



Extraction of high-value added compounds by subcritical water and fractionation by membrane processes : Valorization of vine and wine by-products by eco-innovative processes

Sami Yammine

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THÈSE PRÉSENTÉE
POUR OBTENIR LE GRADE DE
DOCTEUR DE
L'UNIVERSITÉ DE BORDEAUX

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SPÉCIALITÉ : ŒNOLOGIE

Par Sami YAMMINE

EXTRACTION DES MOLECULES A HAUTE VALEUR
AJOUTEE PAR EAU SOUS CRITIQUE ET
FRACTIONNEMENT PAR PROCEDES
MEMBRANAIRES

Valorisation des co-produits de la vigne et du vin par des procédés éco-innovants

Sous la direction de : Martine MIETTON-PEUCHOT

Soutenue le 3 Mai 2016

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À mes parents,
et à Joëlle.

Soyons reconnaissants aux personnes qui nous donnent
du bonheur ; elles sont les charmants jardiniers
par qui nos âmes sont fleuries.

Marcel Proust

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Abstract

This work has dealt with extraction of natural substances from winery by-products using "green" processes such as extraction by subcritical water and purification by membrane processes. These processes are an alternative to solvent extraction traditionally used in the natural products industry. Main part of the work was done on different grape pomace, extraction was optimized and compared in terms of yield, chemical composition, and antioxidant activity of extracts. Dunkelfelder extracts exhibited the strongest antioxidant activity and comparison of chemical compositions of the different extracts indicated. Furthermore this Dunkelfelder grape pomace was used as model in order to optimize the different process parameters such as temperature, pressure and hydraulic retention time. After the subcritical water extraction, extracts produced were found to be rich in several families of molecules. An essential purification step of target compounds prior to industrial use was indispensable. Coupling the subcritical water with membrane processes offers an innovative solution for the purification of these extracts. Thereby, the extract was assayed in a cross-flow apparatus against eleven membranes of ultrafiltration (100 to 2 kDa) and nine membranes of nanofiltration (1000 to 150 Da). The monitoring of the process was carried out by determining performance parameters and retention coefficients of different families of macro and micromolecules. The results obtained have demonstrated that the use of membrane technologies could bring innovative changes in the recovery of bioactive compounds for future industries.

Keywords: Subcritical water extraction, membrane fractionation, phenolic compounds, grape pomace.

Résumé

Ce travail a porté sur l'extraction de substances naturelles de sous-produits de la vigne en mettant en œuvre des procédés "verts" tels que l'extraction par eau sous-critique et la purification par filtration membranaire. Ces procédés représentent une alternative à l'extraction par solvant, traditionnellement utilisée dans la production de substances bio-sourcés.

La majeure partie de cette étude a été menée sur des marcs de raisin de cépages variés, l'extraction a été optimisée et comparée sur la base du rendement, de la composition chimique et de l'activité antioxydante des extraits obtenus. De tous les cépages testés, les extraits de Dunkelfelder ont présenté l'activité antioxydante la plus élevée et la concentration en familles de molécules polyphénoliques la plus importante. En outre, ce marc de raisin de Dunkelfelder a été utilisé comme modèle afin d'optimiser les différents paramètres du procédé tels que la température, la pression et le temps de séjour hydraulique.

Après la phase d'extraction par eau sous-critique, les extraits obtenus se sont révélés riches en de nombreuses familles de molécules. Ainsi, une étape de purification des composés cibles avant usage industriel s'est révélée indispensable. Le couplage de l'extraction par eau sous-critique avec des procédés membranaires représente une solution innovante pour la purification de ces extraits. Des essais de filtration tangentielle de l'extrait ont été menés avec onze membranes d'ultrafiltration (100 kDa à 2 kDa) et neuf membranes de nanofiltration (1000 Da à 150 Da).

Le suivi du procédé s'est appuyé sur une détermination des paramètres opératoires optimisés et sur la détermination des coefficients de rétention des différentes familles des macro et micromolécules.

Les résultats obtenus ont démontré que l'utilisation des technologies membranaires pourrait dans le futur, constituer une innovation technologique pour la purification des composés bioactifs.

Mots clés: Extraction par eau sous-critique, fractionnement par procédés membranaire, composés phénoliques, marc de raisin.

Foreword

A biorefinery is an industrial complex, transforming agricultural biomass, forestry and algae into a variety of bio-based marketable products (ingredients and supplements for human and animal consumption, biomolecules, agro-materials) and/or bioenergy (biofuels, electricity, heat). The biorefinery aims at the complete valorisation of all plant components. In order to do this, biorefinery requires steps of pretreatment, fractionation / purification and conversion of the raw material for the optimized production of high value products. To be economically viable and fit a sustainable development perspective, biorefinery must satisfy a double imperative: the competitiveness of its production costs and use of products and environmentally friendly processes, without the generation of additional waste (minimum environmental impact).

One example of a biorefinery, is a distillery, that acts as a main pathway for the valorization of by-products recovered from the winemaking process.

Grapes (*Vitis vinifera* L.) are one of the most cultivated fruit crops in the world with an annual production of 58 million tons in 2012 (FAOSTAT 2012). Approximately 80% of crops are used for winemaking. Mainly winemaking generates solid residue after pressing: the grape pomace, rich in alcohol. According to European regulations (EC Regulation 555/2008 of the Commission of 27 June 2008), these "by-products" must be disposed of in an environmentally friendly manner. For the French winemakers, the state obliges in either:

- Composting, methanisation or spreading the by-products of all or part of their residues on their own lands
- Or by delivery of grape pomace generated to a methanisation facility, composting, or a distillery (décret n° 2014-903 du 18 août 2014, Art. D. 665-34.-I).

In France, about 50 distilleries collect wine by-products, in an average of a 50 km radius around their site, and allow the recovery of about 850 000 tons of grape pomace each year (Institut français de la vigne et du vin, Novembre 2013).

Until now, the wine distilleries ensure the role of removing the entire load of polluting grape pomace, on national territory, for quality reasons (limitation of over-pressing of grapes, wine quality) and regulations (fight against fraud and guarantee Customs

regulations). However, the Décret n° 2014-903 August 18, 2014 ended obligation to deliver the wine by-products to the distillery, thus threatening the supply of raw material to distilleries. Competitiveness and profitability of the distillery industry is based, therefore, on improving and modernizing processes. The main pedal for improvement is the extraction and purification of high added value compounds from the byproducts. The sector has therefore every interest to move towards an approach of type "biorefinery" maximizing the ways of use of by-products.

At the distilleries, pomace is transformed into various by-products (Figure 1) of more or less high added value (alcohol, grape seed oil, fertilizer, lime tartrate, pulp, etc.).

These by-products are utilized as raw materials in different sectors (agriculture, viticulture, chemical, cosmetic & food industries). This process allows a valorization of the material (compost, feed, chemical ...) and / or energy (bioethanol, biogas...) byproducts.

Due to present industrial equipment, distillation and tartaric acid extraction are currently selected as main method of valorization in the distillery. However, extraction of phenolic compounds can be integrated into the process of valorization.

It would allow a diversification of the distillery activities through the integration of a further step, fractionation of the vegetable biomass to extract high added value compounds. The markets for such products are numerous: the wine, the food (Dyes, natural preservatives), health (food supplements, medicines), cosmetics (natural antioxidants) or the chemical industry (green glue adhesive).

However, to compete in the production of the plant extracts industry (i.e. Naturex, BERKEM, CHR Hansen, DIANA Ingredients, Oenofrance ...), the distillery has to propose extracts with a particular phenolic composition, thus opening up specific markets. Undeniably, the potential application of a plant extract is essentially determined by phytochemical composition, which are particularly dependent on the raw material used and the method of manufacture of the plant extract.

It is in this context overall recovery of bio-compounds and minimizing environmental impacts that is part of the research project VALUXTRACT. The overall objective of VALUXTRACT project is the recovery of high added value compounds from solid waste from winemaking industry with "green" methods. In order produce extracts for oenological applications mainly, but also for the food, cosmetics and pharmaceuticals industries.

This PhD thesis was done within the framework of the European project “Valuxtract”, financed by the French National Research Agency (ANR) under the 1st transnational Call of ECO-INNOVERA (ERA-NET, ANR-12-INOV- 0001-04). It has been conducted at Unité de Recherche Œnologie located in the Institut de Science de la Vigne et du Vin, Villenave d’Ornon in France. It was conducted under the supervision of Professor Martine Mietton-Peuchot.

Part of this work was done in close collaboration with university of Changins - Haute Ecole de viticulture et œnologie, university Hochschule GEISENHEIM - Institut für Oenologie, University of Compiègne - Laboratoire Transformations Intégrées de la Matière Renouvelable and Laboratoire Phenobio - Martillac partners of the project.

The manuscript consists of five publications organized into three chapters, (submitted by the time of writing) that reflect the fruit of the results obtained:

Chapter one presents an overview on extraction and purification of high added value compounds from by-products of the winemaking chain using alternative/non-conventional processes/technologies.

Chapter two is composed of two publications related to the optimization of the extraction of high added value compounds from grape pomace by utilizing subcritical water. The first publication presented the results of the comparative study of the yield of subcritical water extraction of phenolic compounds using multiple raw materials. The second publication describes the optimization of the extraction process grape pomace by subcritical water. The main results of the optimization and the selectivity of this process are described thoroughly.

Chapter three compiles two publications that deal with the fractionation and concentration of high added value compounds from extracts by membrane processes. The chapter will focus on the study of ultrafiltration for the fractionation of the extract obtained in order to separate macromolecules to obtain extract rich in phenolic compounds. The last publication will focus on the utilization of nanofiltration for the fractionation of different families of phenolic compounds.

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1. CHAPTER 1: STATE OF THE ART ALTERNATIVE PROCESS OF EXTRACTION AND PURIFICATION OF HIGH ADDED VALUE COMPOUNDS FROM GRAPE BYPRODUCTS

1.1.INTRODUCTION

Throughout the Valuxtract project a book chapter was written “Yammine, S., Ghidossi, R. & Mietton-Peuchot, M., 2014. Extraction and Purification of Phenolic Compounds from By-Products of the Winemaking Process. In Y. El Rayess, ed. *Wine: Phenolic Composition, Classification and Health Benefits*. NOVA science publishers, pp. 313– 330 ”. In addition with partners of the project a review, which will be presented below, was written to expose all of the publications surrounding this topic. The submitted review displays the main technologies applied or potentially utilizable for the extraction of high added value compounds from wine and vine byproducts on the industrial and laboratory scale. With the aim of giving a general introduction of each utilized technology, to all the process parameters and the limits of the technology. The main approaches such as pressurized liquid extraction, ultrasound-assisted extraction, microwaves assisted solvent extraction, supercritical or subcritical fluid extraction, pulsed-electric fields (PEF) and high voltage electrical discharges (HVED) are the main focus. These technologies are still under development, and so far little or no upscaling industrially has been noticed. Consequently, these technologies have been exploited and are one of the most noticed and published topics.

1.2. EXTRACTION AND PURIFICATION OF HIGH ADDED VALUE COMPOUNDS FROM BY-PRODUCTS OF THE WINEMAKING CHAIN USING ALTERNATIVE/NON-CONVENTIONAL PROCESSES/TECHNOLOGIES

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Abstract

Grape byproducts are today considered as a cheap source of valuable compounds since existent technologies allow the recovery of target compounds and their recycling. The goal of the current article is to explore the different recovery stages used by both conventional and alternative technologies. The intent is to describe the mechanisms involved by these alternative technologies and to summarize the work done on the improvement of the extraction process of phenolic compounds from winery by-products. With a focus on the developmental stage of each technology, highlighting the research need and challenges to be overcome for an industrial implementation of these unitary operations in the overall extraction process. A critical comparison of conventional and alternative techniques is reviewed for the pre-treatment of raw material, the diffusion of polyphenols and the purification of these high added value compounds. This review intends to give the reader some key answers (costs, advantages, drawbacks) to help in the choice of alternative technologies for extraction purposes.

Key words: Extraction, purification, grape by-products, high added value compounds, non-conventional technologies.

1.2.1. Introduction

The valorization of winery waste products is very promising, since grape is one of the largest produced fruit crops with an annual world production of 58 million tons in 2012.¹ About 80 % is used for winemaking and it has been estimated that 13 to 20 % of by-products, which represents about 5-8 million tons of potentially exploitable matter, are generated after the winemaking process.²⁻⁴ Other estimations report higher values up to 14.5 million tons solely in Europe.^{5,6} This represents unquestionably an enormous amount of matter from which high added value components could be extracted. Solid grape wastes are particularly rich in polyphenols, whose use extends to applications in various fields, including cosmetic, nutraceutical, chemical and food industries. Over the last years, polyphenols have attracted a growing interest for their potential health benefits in preventing heart diseases and cancers.⁷⁻¹⁰ Their extraction from winery waste and their following purification are of special interest to produce extracts with high added value.

Phenolic compounds are usually extracted by classical extraction procedure (Figure 1). The natural variability of raw material and the pre-transformation processes (drying, grinding, etc.) could be determinant for the quantity and the composition of extract.¹¹ For instance, high temperatures can lead to denaturation of targeted compounds and grinding leads to a significant increase of undesired components during extraction. Thus, conventional pre-transformation processes decrease the selectivity and/or the efficiency of the extraction process. The selectivity of the extraction processes also depends on the molecular affinity between solvent and solute during the solid-to-liquid diffusion step.¹² However, toxicity, environmental safety, and financial feasibility should also be considered in the selection of a solvent for the extraction of high added value compound. Towards the end of the process, a purification step may be required to obtain extracts with high purity of phenolic compounds. Resin adsorption is commonly used at industrial scale.^{13,14} The major drawback of this technique is the use of a large amount of solvent noticeably during polyphenols desorption, which need to be further evaporated.

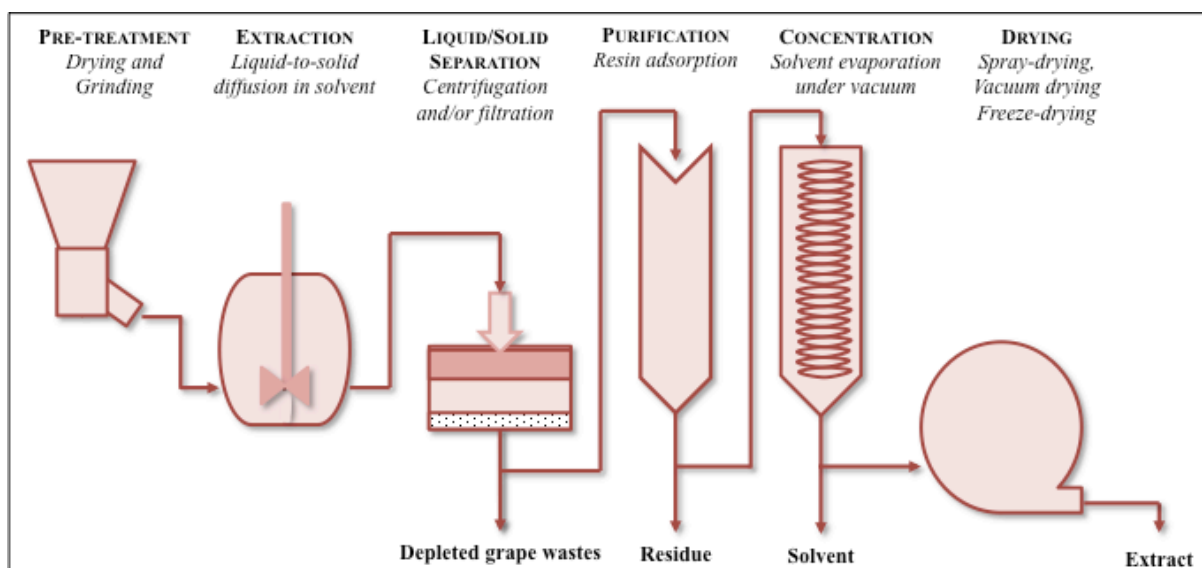


Figure 1: Conventional extraction procedure for the recovery of high added value components from grape wastes

Losses of some compounds, low production efficiency, time- and energy-consuming procedures (prolonged heating and stirring, use of large volumes of solvent...) may be encountered using this conventional extraction procedure. Recent trends in extraction techniques have largely focused on finding solutions that minimize the use of solvent and energy. For these purposes, alternative techniques have been deeply studied to enhance the overall yields in phenolic compounds and to decrease the operational costs of the process. These techniques include:

- Alternative pre-treatments techniques: ultrasounds, pulsed electric fields and high voltage discharges,
- Non-conventional solvent extraction under high pressure: supercritical fluid extraction and subcritical water extraction and,
- Alternative purification technologies, such as membrane processing.

Although lots of experimental studies particularly focused on improving the overall extraction process from solid winery by-products, none of these alternative technologies are currently used at industrial scale for this application. This paper intends to describe the mechanisms involved by these alternative technologies and to summarize the work done on the improvement of the extraction process of phenolic compounds from winery by-products. In this review, the contribution focuses on the developmental stage of each technology, highlighting the research need and

challenges to be overcome for an industrial implementation of these unitary operations in the overall extraction process. A critical comparison of conventional and alternative techniques will be described for the pre-treatment of raw material, the diffusion of polyphenols and the purification of these high added value compounds. This review intends to give the reader some key answers (costs, advantages, drawbacks) to help him in the choice of alternative technologies for extraction purposes.

1.2.2. Pre-treatment of grape by-products for the enhancement of mass transfer phenomena: conventional and alternative techniques

Phenolic compounds exist in plants enclosed in particular structures such as the vacuoles of plant cells and lipoproteins bilayers.¹⁵ In intact cells, the membrane envelope restricts the exchange between the intracellular media and the surrounding solvent. Consequently, conventional solvent extraction techniques such as maceration or diffusion require long extraction time, due to the slow diffusion of solvent and solute through the solid.¹⁶ Thus, the degradation of cell-wall and of intracellular components is a fundamental step to improve the release of these compounds from the grape tissues. Extraction processes can be enhanced by several pre-treatments of the plant materials that are able to physically damage the cells, such as: grinding, pulsed electric field, high voltage electric discharges and ultrasound.

1.2.2.1. Grinding

Grinding is the most conventional pre-treatment technique and is currently used in the extraction industry to shorten the time of diffusion and enhance the yield of targeted bio-compounds. The mechanical action induced by grinding leads to an increase of the exchange surface. However, grinding also leads to the overheating of the plant matrix. Two phenomena are responsible of this released heat:

- Release of energy caused by the fracturing of the matrix,
- Release of energy due to overgrinding of the matrix.¹⁷

Phenolic compounds, and noticeably anthocyanins, are particularly thermosensitive and can be degraded or lose their functionality.¹⁸

On the other hand, the type of plant matrix, and particularly its moisture content, affects the electrical energy requirement, the specific energy consumption and the equipment to be used.¹⁹ Two types of grinding processes can be used in the food industry: dry and wet grinding. A previous study demonstrated that specific energy consumption varied between 420 and 800 kJ/kg of raw rice using dry grinding, while about 14,000 kJ/kg were required in the case of wet grinding (water-to-rice ratio = 2).²⁰ Consequently, a preliminary drying step, which is associated with matrix heating, is often required to facilitate the grinding and reduce its associated cost.^{20,21}

Finally, increased difficulties during the filtration and purification steps due to small particles in suspension in the solvent are another limitation for the use of grinding in the extraction manufactories.

Emerging technologies for the physical alteration of raw material (i.e. pulsed electric fields, high voltage electric discharges and ultrasounds) are based on non-thermal concepts. These three technologies can physically affect the permeability of the cell by different mechanisms.²²⁻²⁵

1.2.2.2. Pulsed electrical field (PEF) assisted extraction

Electroporation phenomena: When subjected to an external electric field, the charge accumulation on the membrane surfaces induces the increase of transmembrane potential of the cell membrane, initiating pore formation.²⁶ Typically, electroporation phenomena requires some threshold value of transmembrane potential around 0.5 - 1.5 V.²⁷ Above the critical value of transmembrane potential, the expansion of pores present in weak areas of the membrane will induce drastic increase of permeability^{28,29} and will facilitate the leakage of intracellular compounds.^{30,31} Thus, Pulsed Electric Field (PEF) treatment increases transmembrane transport of molecules.^{31,32} For cellular tissues of 60-120 μm in diameter, initiation of pore formation can be achieved using electric field strengths of 0.1 - 0.5 kV/cm and treatment times of very short duration (within 10^{-4} - 10^{-2} s)³³ without any significant temperature increase.^{34,35}

Pulsed electric field pre-treatment of winery by-products: PEF treatment prior to conventional extraction allowed a better recovery of phenolic compounds from different winery by-products (**Table 1**). In most of these studies, the raw materials were submerged into water in order to improve electrical contacts between electrodes.

Treatment with liquid-to-solid ratio above 5 required high electric field strength (i.e. $E > 13$ kV/cm) to be effective for the enhancement of polyphenols extraction. As a consequence, specific energy consumptions were relatively high (i.e. $272 < W < 762$ kJ/kg of treated raw material). On the contrary, pre-treatment by PEF combined with an accurate densification of wet pomace or wet skins can be achieved at lower electric field strengths (i.e. $E \approx 1.2$ kV/cm) and lower energy requirements (i.e. $18 < W < 30$ kJ/kg of treated raw material). The treatment of compacted wet winery by-products requires less output current, which can be advantageous for the industrial implementation of PEF.³⁶ Pulse forms used were of different shape (monopolar, bipolar or exponential). However, no comparison of the effect of pulse shape on the extractability of phenolic bio-components is available in the existing literature.

Interestingly, a previous study showed that PEF treatment causes irreversible perforations in the cell wall of the outer hypodermis and distention of the fiber cell wall polysaccharides at the inner hypodermis.³⁷ This electroporation phenomenon may allow the specific recovery of anthocyanins that are particularly located in the upper cell layers of the hypodermis. For instance, High Intensity Pulsed Electrical Field (Hi-PEF) treatment of fermented grape pomace (13.3 kV/cm, $W = 272$ kJ/kg) allowed the selective recovery of anthocyanins and the production of extracts with a high ratio anthocyanins/Total Phenolic Compounds (TPC). This reflects an increase of 40 % that cannot be achieved by conventional extraction procedure, such as grinding combined to diffusion (ratio anthocyanins/TPC < 5 %).³⁸ Moderate PEF treatments ($E < 3.0$ kV/cm, $W < 20$ kJ/kg) were also effective in enhancing anthocyanins extraction from grape skins (+ 17 %)³⁹ and grape pomace (+ 19 %)³⁶. Consequently, PEF can replace conventional pre-treatments of grape by-products (e.g. dehydration and grinding), which have impacts on product quality and are more energy consuming, with the combined objectives of cost reduction and selectivity of extraction.

Scale-up of the technology/Stage of development: Based on existing concepts, and noticeably on sucrose extraction from sugar beets at industrial scale^{25,40,41}, the pre-treatment of grape by-products by PEF should be feasible at larger scale (pilot and industrial scale). Progress in the development of continuous flow treatment chambers for PEF processing have allowed the treatment of material that cannot be pumped

(solid products) using belt or rotating systems.⁴² Moreover, some recent developments of pulsed power systems, which are usable for continuous delivery of high amounts of electrical energy and high electric field strengths and suitable for food industry applications, have allowed the scale-up of the technology.²⁵

Research needs and challenges: While electroporation devices for minimally processed fruits and vegetables are already in operation⁴³, the electroporation devices for extraction of valuable components from grape by-products still require some research and development prior to any reliable operation in an industrial environment.

At the microscopic scale, questions still remain regarding the effect of electric pulses on the cell structure of the plant material and on the targeted bio-components. For instance, it was demonstrated that PEF treatment can modify molecular interactions between intracellular components³⁷ and induce a rupture of polymer chains (decondensation of the tannins).⁴⁴ Further studies might be of importance to evaluate the effect of PEF treatment on the properties of the targeted molecule (bioavailability, functionality, taste...) before using these extracts in food, oenological or nutraceutical applications.

In order to implement the PEF processing step into existing processes in a distillery, a winery or an extracts manufactory, a systemic/integrative approach will be required considering the diversity of raw material to be treated (i.e. pomace, skins, stems, seeds and vine shoots):

- Depending on the grape by-product to be treated, the peak voltage required, the peak current (which depends on product conductivity, on the minimum treatment chamber cross section and on the electrical resistance of the chamber), the average power (dependent on the processing capacity (kg or tons /hour...)) and on the pulse waveform (exponential decay or rectangular pulses) can vary substantially, which renders the design of a power supply for multiple applications challenging.

- The suitability of the treatment chamber may be affected by the raw materials to be treated, most noticeably the materials' pumpability that is critical for a continuous treatment at an industrial scale.

Grape by-product matrix	Operating conditions	Extraction conditions	Targeted bio-compounds (Relative increase)
Red grape pomace (Dornfelder)³⁹	30 exponential pulses in water 3 kV/cm, 10 kJ/kg Total treatment time : 15 sec	1h at 70°C in ethanol/water (50:50, v/v)	Anthocyanins (1.17) * Polyphenols (1.59) *
White grape skins (Chardonnay)⁴⁵	100 bipolar pulses of rectangular shape without addition of conductive liquid 1.3 kV/cm, 30 kJ/kg Effective treatment time: 100 ms at 20°C	3h at 20°C in water	Polyphenols (1.12) *
Grape seeds (Pinot Meunier)⁴⁶	600 exponential pulses in water (L/S ratio: 5) 20 kV/cm, 320 kJ/kg Effective treatment time: 6 ms at 50°C	1h at 50°C in ethanol/water (30:70, v/v)	Polyphenols (1.30) * Reduction of diffusion time by 2
Fermented grape pomace (Dunkelfelder)³⁶	1700 monopolar pulses of rectangular shape without addition of conductive liquid 1.2 kV/cm, 18 kJ/kg Effective treatment time: 170 ms at 20°C	7h at 20°C in ethanol/water (50:50, v/v)	Anthocyanins (1.19) * Polyphenols (1.13) *
Vine shoots (Grenache blanc)⁴⁷	1500 exponential pulses in water (L/S ratio: 20) 13.0 kV/cm, 762 kJ/kg Effective treatment time: 15 ms at 50°C	4h at 50°C in 0.1 M of NaOH in water	Polyphenols (2.09) * Kaempferol: 0.156 mg/g Epicatechin: 1.747 mg/g Resveratrol: 0.032 mg/g
Fermented grape pomace (Dunkelfelder)³⁸	750 exponential pulses in water (L/S ratio: 10) 13.0 kV/cm, 272 kJ/kg Effective treatment time: 7,5 ms at 25°C	Without diffusion	Anthocyanins (5.3)** Polyphenols (0.47)**

Table 1: *Efficiency and operating conditions of PEF-assisted extraction used to extract bioactive compounds from grape by-products*

* *In comparison with control extraction, performed in the same conditions but without PEF pre-treatment*

** *In comparison with control extraction of grinded pomace in water (2h – 20°C under stirring)*

1.2.2.3. High voltage electrical discharges (HVED) assisted extraction

Principles and mechanisms: The first step of HVED is the formation and the propagation of a streamer, which is composed of thin ionized vapor channels, from a needle electrode (pre-breakdown phase). The second phase occurs when the streamer reaches the plate electrode (breakdown phase). These two phases are accompanied by different secondary phenomena such as propagation of pressure shock waves in the surrounding media, emission of UV light, gas bubbles cavitation and chemical reactions generating reactive species.^{22,48,49} At the macroscopic level, the application of electrical discharges on different wine by-products (grape seeds, grape pomace...) results on the fragmentation of the particles.⁵⁰ Depending on the matrix and after effective discharge treatment, the size reduction of the particles treated by electrical discharge is rather similar to that obtained after grinding the product.⁵¹

HVED-assisted extraction of valuable bio-compounds from winery by-products: Electrical discharges have been successfully applied at both laboratory (1 L) and pilot (35 L) scales, in batch, for the enhancement of polyphenols extraction from winemaking by-products (**Table 2**). At the macroscopic level, the treated grape by-products were clearly fragmented after the application of electrical discharges. The increase of the exchange surface promotes the release of non-cell-wall phenolic components and enhances the ethanol transport into cells leading to an increase of phenolic compounds recovery.^{52,53} Moreover, the highly turbulent conditions induced by HVED accelerate the convection of these components from particles to the surrounding medium.

In general, specific energy consumption ranged from 32 kJ/kg and 254 kJ/kg of treated raw material to achieve interesting enhancement of phenolic compounds extraction. At laboratory scale, lignocellulosic biomass (i.e. grape stems, vine shoots) required the highest energy input (> 190 kJ/kg), probably because these biomasses are more resistant to electric discharges than grape skins or seeds.

However, the choice of effective HVED treatment time should be accurately evaluated, as excessively prolonged treatment may deteriorate phenolic compounds. This deterioration of phenolic compounds, and particularly of catechin, epicatechin, quercetin-3-O-glucoside and kaempferol-3-O-glucoside, has been observed above

80 kJ/kg of HVED treatment on grape pomace.⁵⁴ Procyanidin B2 from grape stems showed similar behavior above 122 kJ/kg of energy input.⁵³ Concomitant mechanical and chemical actions induced by the process may be responsible for these observations. Indeed, free radicals can be formed via the thermal dissociation of water during electrical discharge treatment. Under these extreme conditions, antioxidant capacity can be affected.⁴⁸

Stage of development, research needs and challenges: Despite recent research of the effects of HVED for the enhancement of the extraction of phenolic compounds, particularly from winery by-products, this technology is still at its early stage of development. Further research is needed to make this technology feasible at the commercial level.

The detailed mechanism of the establishment of electric discharges in water is still not fully understood. There are two primary principal competing schools, namely, an electron multiplication theory and a phase change mechanism breakdown theory⁴⁹ but until now, there is no consensus on the physical principles involved during this process. Moreover, the evaluation of bioavailability, functionality and/or the taste of the extracts obtained using this process might be of value before oenological or nutraceutical applications.

To intensify the mass transfer phenomena, electric discharges in water have been applied using electrodes with point-to-plane geometry. This geometry is not completely suitable for industrial applications because only restricted volumes can be treated due to low electrical discharges zone. Moreover, identification and application of electrode materials that can provide longer time of operation and lower metal migration would be of value as the lifetime of the needle is rather limited. Another key aspect for the successful application of HVED-assisted extraction is the design uniformity and the processing capacity of the treatment chamber. At this stage of development, HVED treatment would only be dedicated to small batch production of extract.

Grape by-product matrix	Operating conditions	Extraction conditions	Targeted bio-compounds (Relative increase)
Unfermented red grape pomace (Chardonnay) ⁴⁵	80 discharges in water (L/S ratio: 3) 40 kV – 0.5 Hz 32 kJ/kg Effective treatment time: 0.8 ms at 60°C Laboratory scale – in batch	1h at 60°C in water	Polyphenols (2.5) *
White grape skins (Chardonnay) ⁵⁵	60 discharges in water L/S ratio: 6 40 kV – 0.5 Hz 120 kJ/kg 0.6 ms at 20°C Laboratory scale – in batch	3h at 20°C in water	Increase of initial soluble matter extraction by 7.5 Polyphenols (1.2) *
Unfermented red grape pomace (Pinot meunier) ⁵⁴	150 discharges in water L/S ratio: 5 40 kV – 0.5 Hz 80 kJ/kg Effective treatment time: 1.5 ms at 20°C Laboratory scale – in batch	1h at 20°C in ethanol/water (30:70, w/w)	Polyphenols (11.1) *
Unfermented grape skins (Pinot Meunier and Chardonnay) ⁵⁶	750 discharges in water L/S ratio: 5 40 kV 400 kJ/kg Pilot scale – in batch	Without diffusion	Polyphenols (14) **
Grape seeds (Pinot Meunier and Chardonnay) ⁵¹	750 discharges in water L/S ratio: 5 40 kV 400 kJ/kg Pilot scale – in batch	Without diffusion	Polyphenols (50) **
Grape stems (Pinot Meunier and Chardonnay) ⁵⁴	750 discharges in water L/S ratio: 5 40 kV 400 kJ/kg Pilot scale – in batch	Without diffusion	Polyphenols (4) **
Grape seeds (Pinot meunier) ⁵⁶	100 discharges in water (L/S ratio: 5) 40 kV – 0.5 Hz 53 kJ/kg effective treatment time: 1 ms at 50°C Laboratory scale – in batch	1h at 50°C in ethanol/water (30:70, w/w)	Polyphenols (1.5) * Reduction of diffusion time by 4.6
Vine shoots (Grenache blanc) ⁵⁷	500 discharges in water (L/S ratio: 20) 40 kV – 0.5 Hz 254 kJ/kg effective treatment time: 5 ms at 50°C Laboratory scale – in batch	4h at 50°C in 0.1 M of NaOH in water	Polyphenols (3.1) * Kaempferol: 0.213 mg/g (not detected in untreated sample) Epicatechin: 2.459 mg/g (n.d. in untreated sample) Resveratrol: 0.414 mg/g (n.d. in untreated sample)
Fermented grape	120 discharges in water (L/S	Without diffusion	Anthocyanins (3.4) ***

pomace (Dunkelfelder)³⁸	ratio: 10) 40 kV – 0.5 Hz 44 kJ/kg Effective treatment time: 1.2 ms at 25°C Laboratory scale – in batch		Polyphenols (0.88) ***
Grape stems (Cabernet Franc)⁵³	400 discharges in acidified water (pH 2.5, L/S ratio: 7.5) 40 kV – 0.5 Hz 190 kJ/kg Effective treatment time: 4 ms at 20°C Laboratory scale – in batch	2h at 20°C in ethanol/water (50:50, v/v) pH 2.5	Polyphenols (1.4) * Flavan-3-ols (1.4) * Flavonols (1.2) * Stilbenes (1.2) *

Table 2: Efficiency and operating conditions of HVED-assisted extraction used to extract bioactive compounds from grape by-products

* In comparison with control extraction, performed in the same extraction conditions but without HVED pre-treatment

** In comparison with 10 minutes of aqueous diffusion

*** In comparison with control extraction of grinded pomace in water (2h – 20°C under stirring)

1.2.2.4. Ultrasound (US) assisted extraction

Principles and mechanisms: The major effects of ultrasounds (from 20 kHz to 10 MHz) in a liquid medium are attributed to cavitation phenomena. Cavitation is the result of a physical process inducing the creation, the expansion and the implosion of microbubbles, which are formed from gases initially dissolved in the liquid. Held together by attractive forces, molecules are displaced as the ultrasound wave passes through the liquid medium. The sound wave acts as a piston on the surface of the medium, thus inducing cycles of compression phase followed by rarefaction phase. The distance will increase between the molecules of the medium during the rarefaction phase.⁵⁸ If the power is sufficiently high, this distance will reach a critical value, the liquid would breakdown and voids, or cavitation bubbles, can be generated into the liquid.²³ During a compression cycle, upon reaching critical point, bubbles created collapse with temperature and the pressure estimated to be up to 5000 K and 2000 bar. This released temperature and pressure creates microjets pointed towards the solid surface. This leads to destruction of the cell walls of the plant matrix allowing an increase in extraction yields.⁵⁹

US-assisted extraction of valuable bio-compounds from winery by-products:

Ultrasounds can be applied for extraction purposes in two ways: directly to extraction media via an ultrasonic probe, or indirectly through the walls of the extraction media container using a water bath. Both types of apparatus operating at frequencies between 25 and 55 kHz are used at laboratory scale for the enhancement of phenolic compounds extraction from winery by-products (**Table 3**). Optimal US treatment times varied between 25 min and 60 min for phenolic compounds extraction whatever the US device used. This corresponds to specific energy consumption ranging from 187.5 kJ/kg and 4,580 kJ/kg of treated grape by-products. For non-lignocellulosic material (i.e. grape pomace, skins and seeds), US bath is more energy consuming ($W > 4,000$ kJ/kg) than titanium US probe ($W < 1,530$ kJ/kg).

In both cases, ultrasonic systems are composed of a transducer, which converts electrical energy into sound energy by vibrating mechanically at ultrasonic frequencies, generating ultrasounds.⁶⁰ The acoustic intensity (W/cm^2), which is determined as the ratio between the ultrasonic power applied and the emitter surface of the probe system given by common ultrasonic bath at laboratory scale is generally low ($1 - 5 W/cm^2$) in order to avoid cavitation damage to the tank wall. The acoustic intensity provided by a probe is at least up to 100 times greater than the one supplied by the bath, one of the major limitation being the cavitation erosion of the surface of the emitter.⁶¹ This major difference makes each system devoted for a different set of applications, depending noticeably on the operation cost and on the sensibility to degradation of the molecules to be extracted. Thus, US bath should be more adapted for laboratory extraction of phenolic compounds from grape by-products, while US probe can be used at larger scale.

It is generally agreed that ultrasound has weak effects on the stability of extracted compounds. Nevertheless, specific attention should be paid to the stability of extracted component after a long exposure to ultrasonic irradiation. However, long exposure to ultrasonic irradiation, in some cases, may degrade phenolic compounds particularly anthocyanins.⁶²

Scale-up of the technology/Stage of development: Based on the analysis of different advancements in the large scale operation, the development of continuous reactors and use of multiple transducers⁶³ with a possibility of multiple frequency operation⁶⁴ is the key to effective large scale operation and could easily be used for

the treatment of different matrices, including grape by-products⁶⁵. Moreover, some continuous flow systems have been developed for both laboratory and pilot plant scale. Continuous extractors consisting in belt or screw conveyors have been equipped with ultrasound.⁶⁶ US technology for extraction purposes is already in use at industrial scale. The main matrices of ultrasound-assisted extraction are seeds and herbs for food and cosmetic additives application.²³ The high polyvalence of the existing concepts in the ultrasound technologies should allow an easy scale up for the intensification of the extraction of biomolecules from the different matrices of grape by-products.

Research needs and challenges: One of the difficulties reported in the literature is the non-standardized methodologies and control parameters. Thereby, the current challenge is to establish conditions in terms of characterization of sound field, probe types and sample volumes aiming at the techno-economical optimization prior to industrial exploitation.⁶⁷ Another problem hampering the effective operation at commercial scale is from the field of material science and deals with possible erosion of transducer material with continuous use, leading to a decreased transfer of energy and also need for frequent replacements.⁶⁸ Thus, further research needs to be directed in terms of development of high power ultrasonic (HPU) transducers, with higher power capacity, efficiency, radiating surface area and more sophisticated control system.⁶⁹ Theoretical work is indeed required for efficient optimization of the large scale design of the sonochemical reactor.

Grape by-product matrix	Operating/extraction conditions	Targeted bio-compounds (Relative increase*)
Red grape pomace (Dornfelder)³⁹	Ultrasonic bath – 35 kHz V = 90 mL - 140 W 60 min at 70°C ≈ 4860 kJ/kg Ethanol/water (50:50, v/v) (L/S ratio = 4.5 (v/w)) Laboratory scale – in batch	Anthocyanins (0.98) * Polyphenols (1.68) * Antioxidant capacity (1.65) *
Grape seeds⁷⁰	Ultrasonic bath – 40 kHz, V = 100mL - 250 W	Polyphenols: 5.44 mg/100 mL

	<p>29 min at 55-60°C ≈ 4580 kJ/kg Ethanol/water (53:47, v/v) (L/S ratio = 50 (v/w)) Laboratory scale – in batch</p>	
<p>Dried Grape skins (Campbell Early)⁷¹</p>	<p>Ultrasonic bath – 40 kHz V = 100mL - 250 W 25 min at 45°C ≈ 4032 kJ/kg Ethanol/water (52:48, v/v) (L/S ratio = 50 (v/w)) Laboratory scale – in batch</p>	<p>Anthocyanins: 6.26 mg/mL</p>
<p>Vine shoots (Pedro Ximenez)⁷²</p>	<p>Titanium ultrasound probe 14W/mL, (V = 20mL - 280 W) ≈ 6930 kJ/kg 7.5 min extraction at room temperature 80% (v/v) aqueous ethanol at pH 3 (L/S ratio = 20 (v/w)) Laboratory scale – in batch</p>	<p>Polyphenols: 546.4 µg/mL</p>
<p>Red grape pomace Syrah⁷³</p>	<p>Titanium ultrasound probe, 55 kHz 0.44 W/mL (V = 200mL - 87 W) 60 min at 50°C ≈ 1490 kJ/kg Water (L/S ratio = 20 (v/w)) Laboratory scale – in batch</p>	<p>Polyphenols: 770.9±77.5 mg /100 g dry weight (1.62) Antioxidant capacity (ABTS): 705.9±41.7 mg TE/100g</p>
<p>Red grape pomace Syrah⁷⁴</p>	<p>Titanium ultrasound probe, 40 kHz, 0.15 W/mL (V traité = 1 L - 150 W) ≈ 187.5 kJ/kg Water (L/S ratio = 5 (v/w)) 25 min at 17°C Laboratory scale – in batch</p>	<p>Polyphenols: 2.31 mg GA/100 g fresh weight (fw) Flavonols: 2.04 mg quercetin/100 g fw</p>
<p>Vine shoots (Grenache blanc)⁵⁷</p>	<p>Titanium ultrasound probe, 24 kHz 1.3 W/mL (V traité = 300 mL - 400 W) ≈ 3428 kJ/kg 45 min at 50°C in water - (L/S ratio = 20 (v/w)) + Subsequent solid-to-liquid diffusion: 4h at 50°C in 0.1 M of NaOH in water Laboratory scale – in batch</p>	<p>Polyphenols (1.45) Kaempferol: 0.097 mg/g Epicatechin: 0.671 mg/g Resveratrol: 0.024 mg/g</p>

Fermented grape pomace (Dunkelfelder)³⁸	Titanium ultrasound probe, 24 kHz 1 W/mL (V traité = 400 mL - 400 W) ≈ 1527 kJ/kg 28 min at 25°C in water (L/S ratio = 10 (v/w)) - Laboratory scale – in batch	Anthocyanins (4.3)** Polyphenols (0.44)**
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Table 3: Efficiency and operating conditions of US-assisted extraction used to extract bioactive compounds from grape by-products

** In comparison with control extraction, performed in the same extraction conditions but without US treatment*

*** In comparison with control extraction of grinded pomace in water (2h – 20°C under stirring)*

1.2.2.5. Comparison of pre-treatment processes

Several studies performed at laboratory scale have aimed at comparing the pre-treatment techniques described above on the recovery of phenolic compounds from grape by-products. The results evidenced that HVED was the less energy consuming process, followed by PEF and US, and the most efficient for the recovery of total phenolic compounds.^{38,57} However, HVED and US were less selective than PEF regarding the specific recovery of anthocyanins.^{38,39}

Figure 2 compares the specific energy required by each technology for the pre-treatment of the winery by-products. The use of alternative technologies would allow an interesting decrease of the energy consumption, compared to conventional pre-treatment by grinding. The location of targeted compounds with respect to tissue structures seems to be a key issue in the choice of the pre-treatment to be applied. Based on the previous observations, table 4 resumes the advantages and drawbacks of the different pre-treatments.

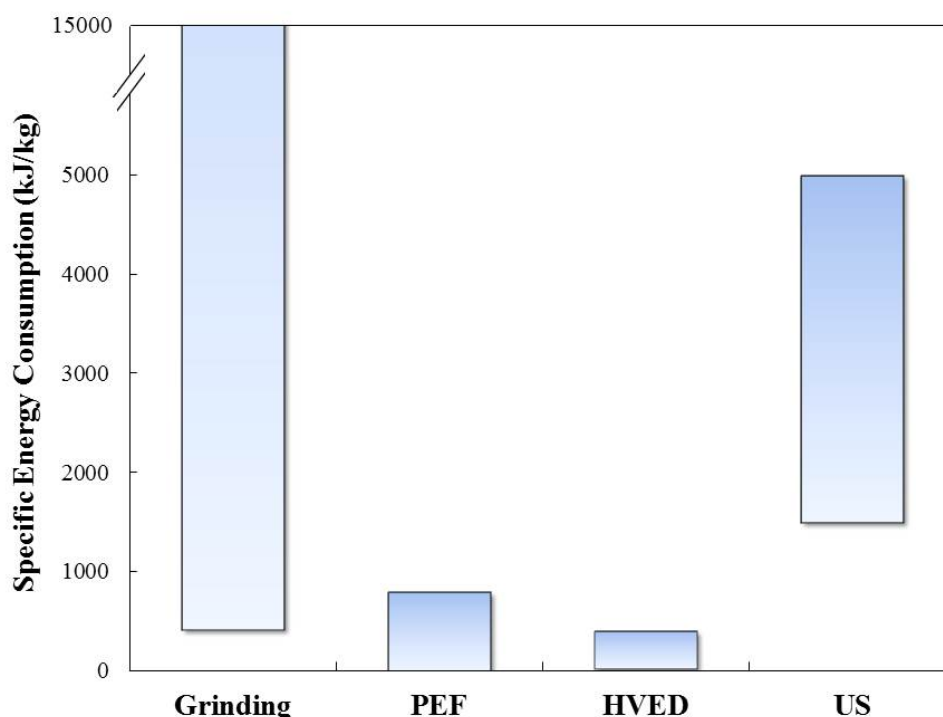


Figure 2: Comparison of specific energy consumption for the different pre-treatments of the raw material

	Drying/Grinding	PEF assisted extraction	HVED assisted extraction	US assisted extraction
Advantages	Easy implementation - High extraction efficiency	- Low energy requirements - Low processing costs - High selectivity, particularly for anthocyanins	- Low energy requirements - High extraction efficiency	Easy implementation Easy-operating High extraction efficiency - High adaptability of the US devices to the different raw material
Drawbacks	- Overheating of the plant matrix - Possible degradation of the bio-components - High energy consumption - Increased difficulties during the filtration and purification steps - Poor selectivity	- Poor adaptability of the PEF apparatuses (generator & treatment cells) to the different raw material - High investment cost	- Possible degradation of bio-compounds - Treatment in batch - Limited lifetime of electrodes - Increased difficulties during the filtration and purification steps - Poor selectivity	- Possible degradation of bio-compounds - Erosion of the transducers

Table 4 Advantages and drawbacks of traditional and alternative pre-treatment techniques

The pre-treatment of the grape by-products is usually followed by a diffusion step that allows the recovery of the solutes in a suitable solvent. New techniques based on high pressure/high temperature promised to reduce diffusion costs by reducing the amount and the type of solvent used, the diffusion time and by enhancing the extraction yields.

1.2.3. Solid-to-Liquid extraction (SLE) of high added value compounds

1.2.3.1. Conventional extraction technique: Low pressure extraction using organic solvents

Principles and mechanisms: A well-established technology, solid-to-liquid extraction is the conventional technique that allows polyphenol components to be removed from the grape by-products matrix using a solvent. Molecular affinity between the solvent and the solute depends on the extracting power of the solvent.¹²

Several interfering parameters are involved for quantitative extraction of phenolic compounds from grape by-products. Work that applied a single-stage solvent extraction is presented in **Table 5**. These literature works aimed at optimization of several extraction process parameters such as: type of solvent, time, temperature of extraction and pH of the extraction medium liquid-to-solid ratio.

Solvent extraction of valuable bio-compounds from winery by-products: Water and ethanol are both accepted as biocompatible solvents, renewable and low-cost resources existent in wineries and distilleries. By increasing the amount of ethanol in water, this has lead to an increased total polyphenol extraction.⁷⁵ The optimum extraction was reached through the use of an ethanol/water solution having a percentage of ethanol between 50 and 70%.^{76,77} Later studies confirmed the results and specified a 66% ethanol solution.⁴⁷

The presence of an acid or a base in the solvent enhances the degradation of the plant material, increasing the extraction capacity from the matrix as well as the solubility of these bioactive compounds. Increasing the acidity of the extraction solvent, lead to the denaturation of the cell membrane, which simultaneously dissolves anthocyanin, and stabilizes them.⁷⁸ To obtain the best yields of anthocyanin extraction, weak organic acid, or low concentration strong acid have been used. An acidic pH, between 1.0 and 2.5, increases both total yields and phenolic contents. For example 0.1% HCl, 1% acetic acid, and tartaric acid have been often used in the solid-liquid extraction process.^{79,80} In addition, sulfured water has also been proposed as an extraction solvent seeking a reduction of the use of organic solvents.⁷⁸ However, excessive acidity of the medium leads to hydroxylation of labile, acyl, and sugar residues during the concentration step.⁸¹

The recovery of phenolic compounds is also influenced by time and temperature. An increase in temperature can cause a higher solubility yet this is accompanied by degradation of polyphenols due to their oxidation. For example several studies have shown an increase of total polyphenol yield at 50°C in comparison to room temperature extraction.^{77,82} However, an increase in the temperature should be coupled by shorter extraction times, lower than 8h, to avoid polyphenols degradation and/or polymerization.

Stage of development, research needs and challenges: Several of these techniques are used for laboratory application and are currently the benchmark for industrial extraction since they are simple, inexpensive and do not require special equipment or qualified personnel. Leading to classifying this extraction as a mature technique, with full industrial practice with little technological risk. Additionally, to cope with the up-scale to industrial processes there is a sufficient variety of large-scale equipment available.

However this mature technique has its own drawbacks. Since large amounts of organic solvents involved during extraction, there is a prominent need for their elimination without traces before commercialization of the extract. Also solvent regeneration e.g., distillation or evaporation increase the utilized energy. Furthermore this technique has low extraction efficiency and a long extraction time. Compared to this technique several selective extractions such as subcritical and supercritical fluid extraction have proven their effectiveness.

Grape by-product matrix	Variables	Targeted bio-compounds
Fresh seeds from white grape pomace (Riesling) ⁸¹	Binary mixtures of ethyl acetate and water (from 3.3 to 20% of water) Extraction time (from 1 to 24h)	Proanthocyanidins
Dried powdered defatted seeds from fresh red grape (Bangalore blue) ⁸³	Solvent: acetone, ethyl acetate, methanol and binary mixtures of ethyl acetate and water (from 10 to 20% of water)	Flavan-3-ols Antioxidant capacity

Red grape pomace (Grenache) ⁸⁴	Solvent: ethanol, methanol, water Liquid-to-solid ratio (from 1 to 5 (v/w)) Extraction time (from 30 to 90 min) Temperature (from 25 to 50°C)	Polyphenols Antioxidant capacity
Red grape pomace (Grenache) ⁸⁵	In a continuous process: Solvent flow rate: (from 2 to 3 ml/min) Sample amount (from 2.5 to 7.5 g) Particle size (from 0.5 to 5.5 mm)	Polyphenols Antioxidant capacity
Fermented grape pomace (Pinot noir) ⁸⁶	Solvent: Water, 70% ethanol in water, 70% methanol in water Extraction time (from 1h to 24h)	Polyphenols Antocyanins Antioxidant capacity
Seeds and skins from white (Chardonnay) and red (Merlot) vinifications ⁷⁶	Binary mixtures of water and ethanol or methanol or acetone	Polyphenols Antioxidant capacity
Grape pomace (Barbera) ⁷⁵	Temperature (from 28°C to 60°C) Maceration times (from 1h to 24h) % of ethanol in water (from 10 to 60%)	Total phenolic compounds, tannins, anthocyanins, Cinnamic acids and flavonols
Dried grape skins from white grape pomace ⁸⁷	Particle size (from 0.16 more than 0.63 mm) Liquid-to-solid ratio (from 10 to 40) Temperature (from 25 to 80°C)	Polyphenols
Fresh seeds and skins from white grape pomace (Roditis) ⁷⁹	Binary mixtures of water and ethanol (from 28.5 to 57%) pH	Polyphenols Flavonoids Flavanols Proanthocyanidins Antioxidant capacity
Dried and milled fermented grape pomaces (Refošk, Merlot and Cabernet) ⁸⁸	Binary mixtures of water and ethanol or ethyl acetate or acetone (from 50 to 100%) pH (from 2 to 6) Temperature (from 20 to 60°C)	Polyphenols Anthocyanins Quercetin Flavanols Resveratrol
Dried, milled and defatted grape stems (Agiorgitiko, Moschofilero, Savatiano) ⁸⁹	pH (from 2 to 6) Binary mixtures of water and ethanol (from 40 to 60%) Extraction time (from 1 to 5h)	Polyphenols Proanthocyanidins Flavones Flavanols
Dried seeds, skin and pomace (Pinot noir and Pinot)	Binary mixtures of water and ethanol or methanol or acetone (50/50, v :v)	Polyphenols Flavonols, Flavanols

meunier) ⁹⁰		Anthocyanins Antioxidant capacity
Dried grape pomace (Tempranillo) ⁹¹	Solvent : Water, Ethanol/water (60/40, w:w) Effect of citric acid (3 g/L) in the solvent	Polyphenols Gallic acid, catechin, epicatechin, resveratrol Antioxidant capacity

Table 5 Comparison of literature works on optimization of solvent extraction of phenolic constituents from grape by-products (adapted from Spigno et al. ⁷⁵)

1.2.3.2. High-pressure extraction

1.2.3.2.1. High temperature and high-pressure extraction/ Subcritical water extraction (SWE)

Principles and mechanisms: Simple experimental equipment, the possibility of online coupling with other techniques, and the ability to use water as a solvent have boosted the number of utilization of Subcritical water extraction (SWE). Established at temperatures between 100°C and 374°C (the critical temperature) under high pressure (usually from 1 to 6 MPa) water stays in the liquid state.

At the higher boundaries, extraction efficiency is obtained due to superior mass transfer properties of subcritical water. The variability of the dielectric constant with temperature is an important variable to consider.⁹³ At room temperature the dielectric constant of water is close to 80. To obtain values of neighboring 27, such as ethanol at ambient temperature, water must be heated to 250°C.

The experimental apparatus required is fairly simple, consisting of a pressure pump, extraction pump, oven where the extraction takes place, valves to maintain a steady pressure in the system and a coolant to swiftly cool the obtained extract.

Grape by-product matrix	Operating/extraction conditions	Targeted bio-compounds (Relative increase*)
Red grape pomace (A-1575) ⁷⁸	10 MPa, 110°C, 100% water, 1400 µg/mL sodium metabisulfite	Total polyphenols: 6.23 g/100g Total anthocyanins: 5.93 mg/100g Anthocyanins, Flavonols, Hydroxycinnamates (1)
Grape seeds (Tempranillo) ⁹⁴	6-7 MPa, 150°C, 100% water	Gallic acid: 232.1 mg/100 g (6.3)
Red grape pomace ¹⁰⁰	8 MPa, 120 °C, 1:1 (v/v) ethanol, 0.8% (v/v) HCl	Total polyphenols: 12.6 mg/100 g (7) Total flavanols: 3.5 mg/100 g (11.6)
Red grape pomace ¹⁰¹	10 MPa, 150°C 100% water	Total polyphenols: 6.070g/100g Total flavonoid: 1.425g /100g
Grape skins (Sunbelt grapes) ⁹⁵	6.8 MPa, 100°C 50% ethanol/water (v/v)	Anthocyanins: 450 mg/100 g (1)
Red grape pomace (Cortina) ¹⁰²	11.6 MPa, 140°C; 100% water	Total polyphenols: 3.08 g/100 g
White grape pomace (Zinfandel) ¹⁰³	10 MPa, 140 ° C, 100% water	Anthocyanins 130 mg/100 g Procyanidin 2077 mg/100 g

Table 6 Efficiency and operating conditions of SWE-assisted extraction used to extract bioactive compounds from grape by-products

Subcritical fluid extraction of valuable bio-compounds from winery by-products:

Several studies were conducted to compare SWE to traditional extraction methods, and numerous extraction parameters such as temperature, pressure, flow rate, sample mass were investigated and taken into consideration (**Table 6**). Optimal conditions for subcritical water extraction are in direct relation with various phenolic acids, anthocyanins and flavonoids. Temperature was the most influential factor in extraction yield and selectivity. With optimum extraction temperature dependent on the by products used and the targeted molecules. For the extraction of anthocyanins from dried red grape pomace, several temperatures ranging from 100 to 160°C were studied, SWE extracts at 110°C had higher levels of anthocyanins values than extracts

obtained using conventional solvent extraction at 60% methanol.⁷⁸ While the extraction recovery of other families of compounds such as catechins and proanthocyanidins from grape seeds showed that selective extractions of compounds can be realized using one-step extraction at 150°C.⁹⁴ The greater the temperature the better the extraction yield of gallic acid, while at lower temperatures thermolabile antocyanins are extracted.

Adding a solvent to water during subcritical extraction leads to the acceleration of extraction and a higher recovery of anthocyanins from grape pomace that decreases the running cost of the extraction process. Optimum ranges for temperature and ethanol concentration are 100–120°C and 50–70% (v/v) respectively.⁹⁵

The main limit of this technology is the degradation of thermolabile compounds during extraction. At high temperature (250°C) during 30 minutes, the majority of flavonoids were degraded.⁹⁶

Stage of development, research needs and challenges:

In general, the use of SWE provides a number of advantages over traditional extraction methods since high diffusion rates promote very efficient extraction of the raw material. Furthermore, those rates vary according to different chemical structures of organic compounds. Therefore, extraction with subcritical water can be both selective and rapid. However SWE extraction in a prolonged period could result in degradation. The development of brown, highly, odiferous compounds with high antioxidant capacity at high extraction temperatures suggested the participation of Maillard reactions in water and in ethanolic extracts.^{97,98}

Most studies have been performed in batch mode on a small scale, but continuous flow equipment has also been tested.⁹⁹ The feasibility of large-scale operations with energy estimation of subcritical water extraction has not been studied until now. A major drawback of this technology is the high operating pressure, which requires high initial investments compared to traditional methods extraction. Nevertheless the high purity of extracts and the efficiency of the process may lead to the future development on the industrial scale. Also, the possibility of fine-tuning the selectivity of polyphenol extraction through changes in water temperature, which directly varies the dielectric constant, is another advantage of subcritical water extraction.⁹⁶

1.2.3.2.2. Supercritical fluid extraction (SFE)

Principles and mechanisms: Above the critical point if a fluid is forced to a pressure and temperature, it becomes a supercritical fluid. Various properties of the fluid change under these conditions. The relatively low viscosity and high diffusivity (gas-like) values provide appreciable penetrating power into the matrix. Its relatively high (liquid-like) density gives good solvent power, generating higher rates of solute mass transfer into a supercritical fluid than into a liquid.

For apolar target molecules the main solvent used in SFE is carbon dioxide (CO₂), which has relatively low critical pressure of 7.4 MPa and a low critical temperature of 31.1°C.¹⁰⁴ In addition CO₂ is safe, foodgrade and widely available with a relatively low cost and high purity.¹⁰⁵ The physicochemical properties of supercritical CO₂ facilitate mass transfer, yield to a solvent-free extract and allow an environmentally friendly operation. However, to use CO₂ as a solvent for polar analytes, a co-solvent such as ethanol is often added as a modifier to CO₂.¹⁰⁶ Depending on the type of sample matrix and the affinity of the targeted compound for the matrix, ethanol may influence the extraction by increasing the solubility of the targeted compound in the supercritical CO₂ (SC-CO₂) as a result of compound–modifier interactions in the fluid phase. This is achieved by inducing the compound desorption from the matrix and/or by favoring the penetration of the SC-CO₂ into the matrix.¹⁰⁷

Supercritical fluid extraction of valuable bio-compounds from winery by-products:

In by-products from wine processing, SC-CO₂ has been interestingly used for the recovery of polar bioactive compounds (**Table 7**). For instance, Bleve et al. developed SC-CO₂ for the purification of anthocyanins from grape skin extracts. The desired fraction containing pure anthocyanins with yields of 80–85%, compared to the initial extract, was obtained under the following optimized process conditions (10–13.10⁶ Pa, 30–40°C; pH 2–4; 25–30% of ethanol in the liquid matrix; CO₂ flow rate 25–50 mL/min).¹⁰⁸

Moreover, Casas et al. reported that resveratrol could be efficiently extracted using SC-CO₂ at high pressure (40 MPa) and low temperature (35°C) using 5% v/v ethanol as a co-solvent. SC-CO₂ extraction enabled resveratrol to be obtained from seeds, which was not possible by conventional methods.¹⁰⁹ However, an increased

ethanol concentration above 7.5% caused a decrease in resveratrol recovery.¹¹⁰ Mixture of ethanol-water was also investigated as co-solvent for biocompounds recovery from grape pomace.^{111,112} The highest extraction yields were obtained at 6 ml/min CO₂ and 10% of ethanol/water (57/43, v/v), which allowed the recoveries of 2.5 g of total polyphenols, 188 mg of monomeric procyanidins, 154 mg of oligomeric procyanidins and 361 mg of polymeric procyanidins /100 g dried material.

Stage of development, research needs and challenges:

Based on existing concepts, and noticeably for food and pharmaceutical products at industrial scale, the extraction of grape by-products by supercritical fluid should be feasible at larger scale (pilot and industrial scale). A study has calculated the economic feasibility of large-scale operations of supercritical fluid extraction (SFE) for the recovery of phenolics using grape by-products.¹¹³ An industrial SFE plant with a capacity of 0.5 m³ for producing an extract with an expected concentration of approximately 23 g of phenolic compounds /kg of dry byproduct, with an estimated manufacturing cost of US\$ 133 /kg.

Several studies stated that one of the biggest drawbacks is the high investment cost needed, qualified manpower in comparison with classical low-pressure equipment, and should be restricted to high added value products. Nevertheless, this far from true the larger the volumes being treated.¹¹⁴

Grape by-product matrix	Operating/extraction conditions	Targeted bio-compounds (Relative increase*)
White grape seeds ¹¹⁵	30 MPa, 55°C, 20 min, 20% v/v methanol/water	Low molecular weight polyphenols (> 0.9)
Grape skins ¹¹⁰	15 MPa, 40°C, 7.5% ethanol/water, time 15 min	Resveratrol (1.0)
Distilled white grape pomace (Grenache blanc) ¹¹⁶	50°C, 90 min, 8% v/v ethanol, L/S 1:1	Gallic acid, catechin and epicatechin
Liquid grape skins extract (Malvasia nera) ¹¹⁷	10–13 MPa, 30–40°C; pH 2–4; 25–30% v/v ethanol/water flow rate 25–50 mL/min	Purification of total anthocyanins (0.85)
White grape pomace (Palomino fino) ¹⁰⁹	40 MPa, 55°C, 5% v/v ethanol/water	Resveratrol: 19.2 mg/100 g (21.3)
Grape seeds (Palomino fino) ¹⁰⁹	40 MPa, 55°C, 5% v/v ethanol/water	Resveratrol: 11.1 mg/100 g (not detectable after conventional extraction in methanol/HCl (0.1%))
Grape skins (Palomino fino) ¹⁰⁹	40 MPa, 35°C, 5% v/v ethanol/water	Resveratrol : 49.1 mg/100 g (15.8)
Grape stems (Palomino fino) ¹⁰⁹	40 MPa, 35°C, 5% v/v ethanol/water	Resveratrol : 0.9 mg/100 g (0.41)
Grape skins (Campbell Early) ¹¹⁸	1.56–1.6 MPa, 45–46°C, 6–7% v/v ethanol/water	Polyphenols: 2.156 mg/100 mL, Anthocyanins: 1.176 mg/mL.
Red grape pomace (Uvina) ¹¹³	20 MPa, 40°C 10% v/v ethanol/water	Gallic acid (11.2), protocatechuic acid (7), vanillic acid (10.7), <i>p</i> -hydroxybenzoic acid (14.6), syringic acid (16.3), <i>p</i> -coumaric acid (8.8), quercetin (3.4)
Grape pomace ¹¹²	20 MPa, 40°C 10% of v/v ethanol/water (57/43, v/v)	Polyphenols (0.92), Monomeric flavan-3-ols (157) Oligomeric flavan-3-ols (38.5) Polymeric flavan-3-ols (2.4)

Table 7 Efficiency and operating conditions of SFE-assisted extraction used to extract bioactive compounds from grape by-products

** In comparison with control extraction*

1.2.3.3. Comparison of extraction processes

Several studies performed at laboratory scale have aimed at comparing the extraction described above on the recovery of phenolic compounds from grape by-products, with no direct comparison between sub and supercritical extractions. The results evidenced that sub and supercritical extraction are more efficient for the recovery of total phenolic compounds. With high selectivity regarding the specific recovery for a family of compounds depending on the process used. Based on the previous observations, **table 8** resumes the advantages and drawbacks of the different extraction techniques.

	Organic solvent extraction	SWE extraction	SFE extraction
Stage of development	Industrial	Laboratory & Pilot	Industrial
Advantages	<ul style="list-style-type: none"> -Easy - Low cost -Doesn't require special equipment and qualified personnel 	<ul style="list-style-type: none"> - Easy implementation - Easy-operating High extraction efficiency - No solvent used - 	<ul style="list-style-type: none"> - Low energy requirements - High extraction efficiency
Drawbacks	<ul style="list-style-type: none"> - Time consuming - Possible degradation of the bio-components - High energy consumption for solvent regeneration - Traces of solvent 	<ul style="list-style-type: none"> - Possible degradation of bio-compounds a high temperatures - Treatment in batch - High cost 	<ul style="list-style-type: none"> - Require low quantities of solvent to be effective - Treatment in batch - Complex expensive -Require specially trained personnel - High Cost
Investment	Low	To be evaluated	High 100–200 k€ (0.05m ³)
Selectivity	Low	High	High

Table 8 Advantages and drawbacks of traditional and alternative extraction techniques

1.2.4. Purification and fractionation of the extract

The large array of utilization of extracts in various fields has lead to further the search for multiple separation techniques of individual compounds. This field has not received much attention thus far, in particular concerning its application to large-scale, industrial purposes.

1.2.4.1. Solid phase extraction

Solid phase extraction (SPE) is a widely used technique in which the sample matrix passes through a column containing sorbent material (solid phase) on which the targeted compound is retained. Subsequently, with a correct choice of wash solvent the chosen compounds can be selectively removed, resulting in a highly purified extract.¹¹⁹ The use of SPE for the separation of nonpolymeric from polymeric phenols was first proposed for red wine.¹²⁰ Recently, SPE was used to specifically extract classes of phenolic compounds and organic acids from white grapes in one step which aimed at simplifying a solid-liquid extraction followed by a solid-phase extraction (SL-SPE) method previously developed by the same group.¹²¹ In that approach, the authors used C18-based sorbents and concluded that matrix solid-phase dispersion (SL-SPE) was good for separation, especially for organic acids.

Additionally rapid solid-phase was proposed as an analysis, extraction and purification technique of resveratrol and other polyphenols in red wine.⁸⁰

The isolation of dimeric to tetrameric procyanidins is achieved after removing the polymeric compounds by solvent precipitation. Purification on polyamide improved the purities of the B-type dimers isolated compounds.¹²²

A complementary study proposed normal phase (NP) HPLC followed by reversed-phase (RP) HPLC as purification technique of B-type dimers. The yield for B1–4 from the grape seed extract using NP/RP-HPLC was about 10 times higher compared to the previously mentioned.¹²³

The uses of these purification methods have so far been restricted to analytical purposes only, due to the fact that preparative scale-up would be too expensive (given the cost of the stationary phases) at the industrial level.

1.2.4.2. Resin adsorption

Similar to solid phase separation, polyphenols in resin adsorption are retained by micro-beads and then specifically eluted using a wash solvent. With its relatively simple design, ease of regeneration, low cost of operation and scale up, resin adsorption appears as the most relevant technique for selective polyphenols recovery from liquid extracts.¹⁴ Polyphenolic compounds are commercially purified and fractionated by resins adsorption, with new developments in the field currently under consideration.^{124–129} The resin used for the adsorption of lipophilic compounds, are lightly hydrophilic acrylic or nonpolar styrene–divinylbenzene resins which can then be washed and eluted with alcohols. Purification of individual compounds or compound classes are done by adjustment of process parameters that included flow rate, concentration of the solute pH value and temperature. Shrikhande *et al.* in their patent have proposed copolymer of trimethylolpropane trimethacrylate as an adsorbent resin to maximize the concentration and purification of the beneficial polyphenolic substances from grape seed extract.¹³⁰ On a laboratory scale using a styrene–divinylbenzene copolymerisate resin for adsorption of anthocyanins from grape pomace for their purification and concentration. Noticably, by varying elution solvent selective results were obtained, with acidified methanol giving the optimum results, recovery rates ranged from 96 to 100% of anthocyanins of the content. Scaling up from laboratory to pilot plant did not affect recovery rates.¹²⁵ This field is fairly new and studies are required to characterize further resin adsorbents with regard to their potential to recover and fractionate phenolic compounds extracted. While this technique has its advantages it usually leads to highly concentrated fractions in organic solvents, and a further step of solvent elimination should be considered.

1.2.4.3. Membrane processes

Employing membranes having different structure and pore sizes it is possible to process and achieve good recovery of polyphenols from grape wastes, while at the same time, fractionating different chemical classes, according to their molecular masses.¹³¹ This process has the clear advantage of being of low environmental impact, as it utilizes little amounts of solvents, mild temperatures and low pressures.

Several studies have used membrane filtration as a concentration step. Accordingly, microfiltration (0.22 and 0.45 μm) of the aqueous EtOH extract from

grape seeds purified the polyphenols in the final recovery: this method turned out to be low-cost, efficient and environmental friendly.¹³² More recently, grape seed extracts obtained with HVED were concentrated using membranes with different molecular weight cut off (0.15 μm , 150 and 50 kDa). Using 150 kDa and 50 kDa membranes, polyphenols were concentrated 2.2 and 2.5 times, respectively, as compared to the initial extract.¹³³ Aqueous extracts from pressed distilled grape pomace were processed using ultrafiltration (1 kDa) and nanofiltration (250 Da, 300 Da, 350 Da) membranes to obtain enriched extracts. The 250 Da membrane allowed the concentration of total phenolic compounds up to 6.3 times the initial extract concentration.¹³⁴ Another aspect is the use of membrane processing to purify and fractionate extracts from winery wastes with the aim of obtaining proanthocyanic fractions with different degrees of polymerization, using different successive cut-off membranes molecules could be separated. Few studies have been conducted on this subject, and literature concerning it is very scarce, making it an advantageous topic for future studies.

1.2.5. Conclusion

The recovery of high added value compounds from winemaking byproducts is rapidly developing. The key to a successful extraction and purification is the identification of effective methods that could lead to extracts that meet consumers' high quality standards. Several of the emerging technologies presented above such as subcritical water extraction and the purification by membranes processes have showed their effectiveness. However as presented in this review, the scale-up of these techniques, is not as simple in practice as it is on paper, in regards to affecting the functional properties of the targeted compounds.

Following the above consideration, restrictions in yield, and energy balance should also be further studied. For the pretreatment process, PEF and HVED technologies have shown their efficiency with low consumption of energy compared to other techniques. Further investigations are required to compare the energy utilized in different extraction and purification technologies.

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CHAPTER CONCLUSION AND OBJECTIVES OF THE PROJECT

To remain competitive, the distilleries have an interest to move towards an integrated biorefinery approach. Through multiplying the number of products from the valorization of grape pomace, particularly the high value-added products such as polyphenols. These products introduce new opportunities in various sectors depending on the phytochemical composition and purity.

The current method for extracting phenolic compounds, presents certain drawbacks such as time-consuming and require a large amount of organic solvents, which are harmful to human health and cause environmental stress. These disadvantages include utilization of organic solvent that needs to be regenerated. That is why many research teams are working actively in the development of alternative technologies extraction and purification of high added value compounds from grape by-products, in a common goal of reducing solvent utilization. To do this, these technologies must:

- **Improve the extraction efficiency of phenolic compounds**
- **Limit the deterioration of thermolabile compounds**
- **Reduce the steps of energy intensive processing (solvent regeneration, drying, concentration, etc.)**
- **Enhance the quality of the extracts by purification**

An alternative method that is the effective, economical, environmentally friendly, safe and fast, is required to alleviate these drawbacks. The most common techniques, which have recently been discussed, include supercritical fluid extraction (e.g., carbon dioxide), pressurized liquid extraction or accelerated solvent extraction, and subcritical water extraction. Of these techniques, subcritical water extraction using water, as the extractant is one of the most interesting methods because water is non-flammable, non-toxic, cheap, and environmentally safe that responds appropriately to the three criteria mentioned above. At present, few comparative studies of these technologies have discussed on the qualitative aspect of extraction and/or do not allow concluding on the selectivity of the process because of the choice of operating parameters.

However, the extracts produced are rich in several families of molecules. A purification step prior to the industrial use of target compounds is essential. Coupling

the subcritical water with membrane processes offers a solution for the purification of these extracts, because of their flexibility. This process has the clear advantage of being of low environmental impact, as it utilizes little amounts of solvents, mild temperatures and low pressures, which appropriately respond to the above criteria.

In this context, the objectives of this PhD project are:

- **A better understanding of the variability byproducts, through the application the subcritical water extraction on different types of byproducts.**
- **Compare the effectiveness of the subcritical water extraction on the phytochemical composition of the extracts obtained, in order to expand knowledge on improving extractability of targeted compounds.**
- **Assess the utilization of ultrafiltration membrane process in order to improve the overall purity of extract of phenolic compounds from grape pomace.**
- **Test Nanofiltration method for the objective of fractionation and/or concentration of the different families of compounds.**

2. CHAPTER 2: SUBCRITICAL WATER EXTRACTION OF HIGH ADDED VALUE COMPOUNDS FROM FERMENTED GRAPE POMACE

2.1.INTRODUCTION

An attractive alternative to conventional extraction methods, is obtaining phenolic compounds is the use of subcritical water (SWE) extraction. This technology uses water at temperatures between 100 and 374°C and enough pressure to maintain water in the liquid state. The critical temperature and pressure of water are 374 °C and 22.1 MPa, respectively (Moran & Shapiro 2006). Under subcritical conditions, the intermolecular hydrogen bonds of water break down and the dielectric constant of water decreases. The dielectric constant of ethanol and of pure water at ambient temperature and pressure are 27 and 79, respectively. As temperature increases to 250 °C, the water dielectric constant is reduced to 27, which is similar to the dielectric constant of ethanol (Ramos, Kristenson, & Brinkman 2002).

On the other hand, grape byproducts are an important and relatively inexpensive source of a wide range of polyphenols including monomeric and oligomeric proanthocyanidins and a diversity of anthocyanins providing important economic advantages. This content varies according to the growth conditions and also undergoes additional changes during different vinification processes. This matter took much of our attention in this chapter due to large variability in the literature. Thus, the aim of the present work was to characterize the phenolic compounds of the grape pomace subcritical water extracts of four different cultivars of *Vitis vinifera* (Chardonnay, Cabernet franc, Merlot, Dunkelfelder), in order to identify their interesting properties to be used as functional ingredients and to compare them at different extraction temperatures by SWE. The varieties selected for this chapter represents a specimen of phenolic diversity. The grape byproduct varieties are: Chardonnay, Cabernet franc, Merlot, Dunkelfelder.

Furthermore different parameters influence the extraction such as temperature, pressure, hydraulic reduction factor, volume of extraction that are correlated directly to the kinetics of extraction and degradation of specific molecules.

Thus, the chapter is composed of two publications dedicated to the presentation of results of extraction of different polyphenol families by subcritical water extraction conditions. When extracting the polyphenols of Dunkelfelder grape variety with subcritical water, a parametric study was implemented to optimize the operating conditions of extraction and compared to extraction using an organic solvent.

2.2.CHARACTERIZATION OF POLYPHENOLS AND ANTIOXIDANT POTENTIAL OF RED AND WHITE POMACE BY-PRODUCT EXTRACTS USING SUBCRITICAL WATER EXTRACTION

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Abstract

A detailed assessment of the high added value compounds content in grape pomace varieties, after the subcritical water extraction, was done. High amounts of anthocyanins and Flavan-3-ols were recovered from fermented grape pomace using differential temperatures with a high variability between by-products. Contrary to anthocyanins, high extraction temperatures (about 200 °C) yielded higher amounts of tannins. Overall, we found that grape pomace antioxidant activity and total polyphenols quantified by Folin Ciocalteu method were not directly related to the main polyphenol content in SWE extracts. The data obtained here using laboratory-scale equipment will be useful to develop an industrial scale SWE process. Finally as observed, grape pomace by-products can be considered as an important source of polyphenols. In this regard, this global characterization may potentially provide the basis for a sustainable process of integrated exploitation of winemaking by-products as potential, inexpensive, and easily available sources of bioactive compounds for the pharmaceutical, cosmetic, and food industries.

Key words: Green process, Subcritical water extraction, Grape pomace, Polyphenols, Valorization.

Abbreviations.

AUC Area under curve

DM Dry matter

DS Dry skin

EtOH Ethanol

GA Gallic Acid

HCl Hydrochloric acid

HPLC High performance liquid chromatography

PTFE Polytetrafluoroethylene

SWE Subcritical water extraction

T Temperature

ϵ Dielectric constant of water

\varnothing Diameter

2.2.1. Introduction

Grapes are one of the most produced worldwide fruit for human consumption but also especially for wine production. About 66.4 million tons of grapes are produced annually in recent years (FAOSTAT 2012). More than 44 % of the production belongs to European countries such as France, Italy, Spain and Germany (OIV 2013). This massive amount of wine production results in commensurate amount of grape by-products. One of the major by-products is grape pomace, which accounts for 17 - 25% of the quantity of grape produced (Pinelo et al. 2005; Arvanitoyannis et al. 2006). Grape pomace is a source of polyphenols, oil, alcohol, and tartaric acid (Jackson 2008). The residual alcohol in grape pomace is generally extracted. The seeds found in the pomace are a main source of grapes seed oil while the grape skin, although contains high level of polyphenols, has not been utilized to its full potential. Due to high polyphenols content, pomace can't be utilized as feedstock or also crop fertilizer (Devesa-rey et al. 2011). For this reason, it is of considerable interest to examine ways to extract polyphenols from the grape by-product.

The most common method for the extraction of polyphenols is by using an organic solvent. This method is simple because the agents and equipment required for the process are easily available (Spigno & De Faveri 2007). However, as a result of the degradation during solvent regeneration the yield is generally low. In addition, organic solvents substantially increase extraction process costs (Galanakis 2012). Furthermore, the remaining solvent needs to be regenerated thoroughly from the product, leading to generation of a large amount of organic solvent wastes (Yammine et al. 2014).

Alternatively, a number of studies demonstrated the use of water at subcritical ($100^{\circ}\text{C} < T < 374.2^{\circ}\text{C}$) conditions (SWE) as an environmentally friendly and effective extraction process (Ju & Howard 2003). At these conditions, the dielectric constant of water (ϵ) changes dramatically with the change in temperature. The value of ϵ at 25 MPa decreases with temperature from 60 at ambient temperature to 21 at 250°C , thus the reaction field changes from ionic reaction to radical reaction. Generally, water at subcritical condition has been demonstrated by several studies to be an effective extraction solvent (Plaza & Turner 2015) and can be applied for the extraction of several bioactive compounds such as phenolic compounds from lemon

balm (Miron et al. 2013), potato peel (Singh & Saldaña 2011), polysaccharides from golden oyster mushroom (Jo et al. 2012).

Table 1: *Efficiency and operating conditions of SWE-assisted extraction used to extract bioactive compounds from grape by-products*

Grape by-product matrix	Operating/extraction conditions	Targeted bio-compounds (Relative increase*)	Reference
Red grape pomace (A-1575)	10 MPa, 110°C, 100% water, 1.4g/L sodium metabisulfite	Total polyphenols: 6.23 g/100g DM*** Total anthocyanins: 5.93 mg/100g DM Anthocyanins, Flavonols, Hydroxycinnamates (1)	(Ju & Howard 2005)
Grape seeds (Tempranillo)	6-7 MPa, 150°C, 100% water	Gallic acid: 232.1 mg/100 g (6.3) DM	(García-Marino et al. 2006)
Red grape pomace (Source n.d.**)	8 MPa, 120 °C, 1:1 (v/v) ethanol, 0.8% (v/v) HCl	Total polyphenols: 1.26 g/100 g (7) DS**** Total flavanols: 3.5 mg/100 g (11.6) DS	(Luque-Rodríguez et al. 2007)
Red grape pomace (Pinot noir)	10 MPa, 150°C 100% water	Total polyphenols: 6.070g/100g DM Total flavonoids: 1.425g /100g DM	(Casazza et al. 2010)
Grape skins (Sunbelt grapes)	6.8 MPa, 100°C 50% ethanol/water (v/v)	Anthocyanins: 450 mg/100 g (1) DM	(Monrad et al. 2010a)
Red grape pomace (Cortina)	11.6 MPa, 140°C; 100% water	Total polyphenols: 3.08 g/100 g DS	(Aliakbarian et al. 2012)
White grape pomace (Zinfandel)	10 MPa, 140 °C, 100% water	Anthocyanins 130 mg/100 g DM Procyanidin 2077 mg/100 g DM	(Monrad et al. 2012)
Red Grape pomace (Cabernet Sauvignon)	10 MPa, 140 °C 70% ethanol/water (v/v)	Total polyphenols: 16.2 g/100 g DM	(Rajha et al. 2014)
Red grape pomace (Cabernet Sauvignon)	10 MPa, 100 °C, 100% water	Total antioxidants 1.06 mg/g Anthocyanins 1.050 g/100g Condensed tannins 0.52 g/100g	(Vergara-Salinas et al. 2015)
Red grape pomace (Pinot Noir)	10 MPa, 120 °C, 100% water	Total polyphenols: 7.76 g/100 g	(Duba et al. 2015a)

* In comparison with control extraction ** not determined (n.d.) ***Dry matter (DM)

**** Dried skins (DS)

As reported in Table 1, almost all the cited works used a subcritical water extraction from grape by-products. The by-products are different and the choice of by-product is not generally motivated, even because many researches were simply aimed to analyze operating parameters in function of the polyphenolic content. Only a few works have tried to optimize some process parameters in function of the different families phenolic compounds and the diversity of the by-product. The most investigated factor is the temperature in extraction yield and selectivity, with optimum extraction temperature dependent on the by-products used and the targeted molecules. For total polyphenol content by Folin-Coicalteau method that extraction at 100°C ranged generally between 1.26 g/100 g DM (Luque-Rodríguez et al. 2007) to 16.2 g/100 g DM for Cabernet Sauvignon by-product (Rajha et al. 2014). Whereas the extraction at 110°C of anthocyanins from red grape pomace was also variable, ranged between 5.93 mg/100g DM (Ju & Howard 2005) to 450 mg/100 g DM (Monrad et al. 2014) for different source of by-products. While the extraction recovery of other families of compounds such as catechins and proanthocyanidins showed that selective extractions of compounds also were greatly variable. This shows a high influence of temperature of extraction, the type of by-product utilized and the manner it was pretreated before extraction.

Thus, the aim of the present work was to characterize the phenolic compounds of the grape pomace by-products of four different cultivars of *Vitis vinifera* (Chardonnay, Cabernet franc, Merlot, Dunkelfelder), in order to identify their interesting properties to be used as functional ingredients and to compare them at different extraction temperatures. This investigation consisted of the determination of the total phenolic and total tannin contents of the grape pomace by-products, the identification and quantification of monomeric and oligomeric (dimer and trimer) flavan-3-ol composition by High Pressure Liquid Chromatography (HPLC) and the estimation of their antioxidant capacity by four different procedures, in particular, ABTS, CUPRAC, FRAP, and ORAC assays. The data may contribute to the selection of suitable grape pomace for the development of antioxidant and polyphenolic rich nutraceuticals.

2.2.2. Material and methods

2.2.2.1. Chemicals

Copper(II) chloride dihydrate, ammonium acetate potassium peroxodisulfate, hydrochloric acid, ethyl alcohol, iron(III) chloride hexahydrate, sodium acetate 3-hydrate, glacial acetic acid, Folin Ciocalteu reagent, and gallic acid were purchased from Scharlau (Barcelona, Spain). TPTZ (2,4,6-tri-(2-pyridyl)-s-triazine) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were from Acros Organics (New Jersey, USA). ABTS (2,2'-azino-bis-(3-ethylbenzothiazoline- 6-sulfonic acid) diammonium salt) was obtained from Biochemica (Darmstadt, Germany). Sodium dihydrogen phosphate dihydrate, disodium hydrogen phosphate dodecahydrate, fluorescein, AAPH (2,2'-azobis-(2-methylpropionamidine) dihydrochloride, Neocuproine (2,9-dimethyl-1,10-phenanthroline), phloroglucinol, (+)-catechin, (-)-epicatechin, (-)-epigallocatechin (EGC), (-)-epicatechin- 3-O-gallate (ECG), procyanidin B1 [(-)-epicatechin-(4 β -8)-(+)-catechin], and procyanidin B2 [(-)-epicatechin-(4 β -8)-(-)-epicatechin] were supplied from Extrasynthèse, Genay, France. Acetonitrile (HPLC grade), formic acid (HPLC grade), methanol (HPLC grade), glacial acetic acid (HPLC grade), L-ascorbic acid, and sodium acetate were purchased from Prolabo-VWR (Fontenay/Bois, France). Trimer C1 [(-)-epicatechin-(4 β -8)-(-)-epicatechin-(4 β -8)-(-)-epicatechin] was obtained from Phenobio SAS (Martillac, France).

2.2.2.2. Raw material

The study was carried out with grape pomace by-products obtained from representative red and white grape varieties (*Vitis vinifera* L.) cultivated in Switzerland: Chardonnay, Cabernet Franc, Merlot, and Dunkelfelder. In particular, Dunkelfelder is a teinturier grape variety known in Changins, respectively, whereas the other three grape varieties considered are well-known and widely cultivated elsewhere. Samples were provided by University of Changins winery (Switzerland) during the 2012 and 2013 harvests. To limit the influence of external factors and to allow a better comparison among results, all samples shared the same geographical area, cultivation system, and viticultural practices. The grapes used were harvested at the optimum technological ripeness, as judged by stabilization of the potential alcohol

content and control of the acidity index, the visual lignification degree of seeds, and the adhesion degree of the skins to the seeds, established by the winery.

While the fourth variety considered, Chardonnay grape pomace was collected the day of grape harvest after destemming and pressing the grapes under identical conditions. A pneumatic press (Bucher-Vaslin– RPS 50, France) was used filled at 75–80% of its capacity. In all cases, the press program applied was as follows: 40 min at a constant pressure of $0.15\text{--}0.20 \times 10^5 \text{ Pa}$ (P_{min}) with cycles consisting of 2 min at the inflated position, followed by a rapid deflation and 3 laps; 40 min at an increasing pressure from P_{min} to $1.75\text{--}1.80 \times 10^5 \text{ Pa}$ (P_{max}), with an inflation cycle of 3 min, followed by deflation and 2 laps; and then 15 min at P_{max} , with cycles of 3 min at the inflated position, a rapid deflation and 3 laps. After pressing, all the grape pomaces were combined and homogenized to ensure a representative sampling of the whole grape pomace. While for the three red varieties pomaces were collected immediately after pressing at $2 \times 10^5 \text{ Pa}$ (RPS 50, Bucher Vaslin SA, France) and were treated with 50 mg of SO_2 per kg of raw material (RM). Samples were stored at -20°C under vacuum until further processing.

In order to avoid complications with repeatability due to the heterogeneity of the raw material, the skins and the seeds were separated with a vibrating sifter (Retsch GmbH, Germany). Afterwards, small fractions (diameter $\varnothing < 2.8 \text{ mm}$) and large fractions ($\varnothing > 5.5 \text{ mm}$) were removed. Then, the two standardized fractions were manually and homogeneously mixed (49% of seeds and 51% of skins fresh weight).

2.2.2.3. Process of extraction and parameters

The schematic diagram of apparatus used for the extraction of polyphenolic compounds using subcritical water is shown on the figure 1.

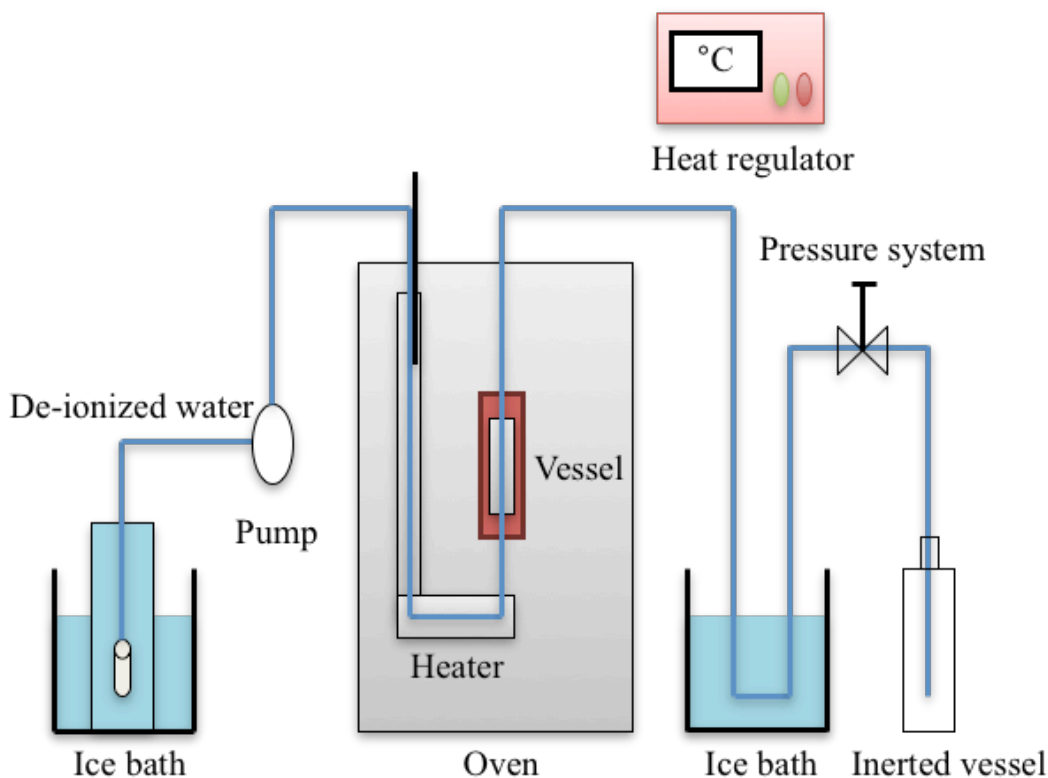


Figure 1. Schematic diagram of the Pressurized liquid extraction process

In the extraction system, a HPLC pump (I.C.S. National 1100) was used for de-ionized water delivery, pressurization and controlling the pressure of system. A pressure transducer (Swagelock NG160) and thermocouple (Eurotherm Automation 90) were installed in the custom-made high-pressure vessel to monitor both pressure and temperature of system. Extract was collected in inerted vessel (65 mL volume) after passing in an ice bath.

In each run pomace (13.00 g) was loaded into the high-pressure vessel, which can contain 26 cm³ of material (Figure 1). The liquid-to-solid ratio was maintained at the value of 5, to obtain 65mL of extract. The vessel was placed in an oven at a several temperatures (tested temperatures: 100°C, 150°C, 200°C). The outlet valve of extraction vessel was then closed and the system was pressurized to a desired pressure of 25 bars at a constant flow rate of 6mL/min. The solution collected in an inerted sampling vessel and pomace were then stored at 4°C for further analysis without preliminary preparation steps.

2.2.2.4. Conventional extraction experiments

Polyphenols extraction from grape pomace (100.0 ± 0.1 g) was carried out in a mixture of ethanol and water (50/50, v/v), maintained at the ambient temperature in a cylindrical extraction cell. The liquid-to-solid ratio was maintained at the value of 5. A gentle agitation at 160 rpm ($16.8 \text{ rad} \cdot \text{s}^{-1}$) was provided using a round incubator of 12.5 mm shaking throw (Infors HT Aerotron, Bottmingen, Switzerland). For untreated samples, the same protocol of extraction was used. Regular sampling was carried out during 420 min of extraction. At the end of extraction, the juice was separated from grape pomace by centrifugation (Model 3-16P, Sigma Laborzentrifugen GmbH, Germany) at 3076 g during 10 min, and stored at -18°C for further analysis.

2.2.2.5. Analysis

2.2.2.5.1. Total polyphenols content

The total phenolic content was spectrophotometrically measured according to a modified Folin Ciocalteu method to be applied in 96-well microplates. Stock solutions (10 mg/mL) of the grape pomace extracts were prepared in EtOH/H₂O (25:75, v/v), and a microplate spectrophotometer (MultiSkan Spectrum, Thermo Scientific) was used for the incubation and measurement. Briefly, each well was filled with 184 μL of distilled water and 24 μL of the sample solution, followed by 12 μL of the Folin Ciocalteu reagent and 30 μL of 20% (w/v) Na₂CO₃ solution. Prior to the measurement of the absorbance at 765 nm, the mixture was incubated for 1h under dark conditions at 25°C. Gallic acid (0–24 mg/L) was used as a standard for calibration. Results, expressed as milligrams of gallic acid per 100 g of grape pomace sample (on a dry matter basis, DM), were a mean of six determinations.

2.2.2.5.2. Antioxidant activity

Polyphenols extracted from grape are well-known for their antioxidant capacity. This antioxidant activity is not a single reaction but comprises a wide range and multiple mechanisms. It usually recommended using several techniques since no single technique is able to take into consideration all antioxidant mechanisms. Therefore, four different antioxidant capacity assays were used: the fluorometric

ORAC assay, which is based on hydrogen transfer and spectrophotometric ABTS, CUPRAC, and FRAP assays, which are based on electron transfer. FLUOstar Optima (BMG LabTech) was used for the first assay and an automated microplate reader (MultiSkan Spectrum (Thermo Scientific) for the other three analyses. As for the total phenolics assessment, for the antioxidant capacity spectrophotometric methods, solutions of the stem extracts (4 mg/10 mL) were prepared in EtOH/H₂O (25:75, v/v). More diluted stock solutions of the sample extracts (20 mg/1L) were prepared in 75 mM phosphate buffer (pH 7.4) for the ORAC measurement. The difference in absorbance between a final reading and the reagent blank reading was correlated with Trolox standard curves in all assays. Because the moisture level of each pomace extract sample was quite different, antioxidant capacity was reported on a dry weight basis to enhance comparison with the literature. Thus, the results were expressed as milligrams of Trolox per gram of grape sample (DM). Each result value was a mean of six determinations

2.2.2.5.2.1. ABTS Assay

In 96-well microplates, the ABTS radical cation (ABTS^{•+}) was prepared by the reaction of equivalent volumes (1:1) of both aqueous solutions of 7 mM ABTS and 2.45 mM potassium persulfate. This stock solution was allowed to react for 12–16 h at room temperature in the dark and used within the two following days stored in the same thermal and light conditions. At the moment of the analysis 8 mL of the ABTS solution was diluted with EtOH/H₂O (25:75, v/v) in a 100 mL volumetric flask to obtain an absorbance of 1.00 ± 0.02 unit at 734 nm. In a 96-well microplate, extract solutions and ABTS reagent (190 μ L in each well) were prewarmed at 25 °C for 20 min. Then, a reagent blank reading was taken at a wavelength of 734 nm. The reaction was carried out by adding 10 μ L of the pomace extract solution to each well. After 3 min of shaking, the mixture was incubated at the same temperature for a 30 min period, and then the absorbance decrease was measured at the same wavelength. Trolox standard solutions were prepared at a concentration ranging from 0 to 0.8 mM ($R^2 = 0.995$), by using EtOH/H₂O (25:75, v/v) as a solvent.

2.2.2.5.2.2. CUPRAC Assay

The cupric reducing antioxidant capacity of the sample extracts was done in 96-well microplates. CUPRAC reagent was prepared just before the analysis by reacting equal volumes (1:1:1) of 10 mM Cu(II) aqueous solution, 7.5 mM neocuproine in EtOH 96% freshly prepared, and ammonium acetate buffer (1 M, pH 7). In a 96-well microplate, pomace extract solution and 190 μ L of CUPRAC reagent for each determination were incubated under the same conditions as the ABTS assay. After the initial absorbance had been read at 450 nm, 10 μ L of the pomace extract solution was added to each well. After 3 min of shaking, the mixture was incubated at 25 °C for 30 min, and then the absorbance increase was measured at the same wavelength. Trolox standard curve was linear between 0 and 1.3 mM ($R^2 = 0.996$).

2.2.2.5.2.3. FRAP Assay

The ferric reducing antioxidant power assay was carried out in 96-well microplates. The fresh working FRAP reagent was prepared by mixing a 0.01 M TPTZ solution in 0.04 M HCl, a 0.02 M $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ aqueous solution, and acetate buffer (pH 3.6, 3.1 g of sodium acetate and 16 mL of acetic acid glacial per liter of buffer solution) at a ratio of 1:1:10. All of these solutions were prepared on the day of analysis, except for the buffer and hydrochloric solutions. For the measurement of the antioxidant activity by the FRAP method, the protocol and experimental conditions were exactly the same as those reported for the ABTS and CUPRAC assays. However, the increase in absorbance was measured at 593 nm and the Trolox calibration curve was obtained using concentrations from 0 to 1.6 mM ($R^2 = 0.996$).

2.2.2.5.2.4. ORAC Assay

The oxygen radical absorbance capacity analysis was applied by using 96-well fluorescence microplates. The reaction was carried out in phosphate buffer (75 mM, pH 7.4). In this order, 30 μ L of the pomace extract solution, 180 μ L of fluorescein (117 nM final concentration), and 90 μ L of AAPH (40 mM) were added to each well. The mixture was shaken and allowed to stand for 1.5 h at 37 °C. Fluorescence was recorded every minute during this period at excitation and emission wavelengths of 485 and 530 nm, respectively. Simultaneously on the same microplate, a blank sample

(phosphate buffer replaced the sample) and Trolox calibration solutions (1–40 μM) were also performed ($R^2 = 0.983$). The area under the curve (AUC) was calculated for each extract sample by integrating their relative fluorescence curves. By subtracting the AUC of the blank, the net AUC of the pomace extracts was calculated and correlated with Trolox concentrations.

2.2.2.5.3. Anthocyanins analyses

Quantitative and qualitative analyses of anthocyanins were performed by high performance liquid chromatography (HPLC) on the extracts after 420 min of extraction. Samples of extracts were diluted (ratio 1/10) in acidified water (0.1% formic acid) and then filtered through Polyamide filters (pores diameter $\varnothing = 0.45 \mu\text{m}$). The system used for anthocyanin analysis was an Agilent 1200 HPLC Series (Agilent Technologies), equipped with a diode array detector. The separation was carried out with a Prontosil C18AQ column ($4.6 \times 250 \text{ mm}$, $5 \mu\text{m}$, Bischoff Chromatography, Germany) operated at 25°C in reverse phase.

UV/VIS spectra were recorded in the range of 200–600 nm. Two mobile phases, (A) water/acetonitrile/formic acid (87/3/10, v/v/v), and (B) water/acetonitrile/formic acid (40/50/10, v/v/v) were used for the separation of phenolic compounds. The elution gradient had the following profile: $t_{0 \text{ min B}}$ (6%), $t_{15 \text{ min B}}$ (30%), $t_{30 \text{ min B}}$ (50%), $t_{35 \text{ min B}}$ (60%), $t_{41 \text{ min B}}$ (6%), $t_{45 \text{ min B}}$ (6%). The injection volume was $30 \mu\text{L}$ and the flow rate was set at $1.0 \text{ mL} \cdot \text{min}^{-1}$. Anthocyanins were detected at 518 nm. Individual anthocyanins were quantified using a calibration curve of the corresponding standard compounds. Results were expressed as weight (g) of individual anthocyanin extracted/100 g DM .

2.2.2.5.4. Flavan-3-ols and gallic acid analyses

The HPLC system used for flavan-3-ols and gallic acid analysis was an Agilent 1200 HPLC Series (Agilent Technologies, Germany) equipped with a diode array detector. The samples were diluted (ratio 1/10) in water and then filtered through PTFE filters ($\varnothing = 0.45 \mu\text{m}$). A volume of $60 \mu\text{L}$ was injected in a Prontosil C18AQ column ($4.6 \times 250 \text{ mm}$, $5 \mu\text{m}$, Bischoff Chromatography, Germany), operated at 25°C in reverse phase. Solvent A, 0.1% trifluoroacetic acid (TFA) in

water and solvent B, 0.1% TFA in acetonitrile, were used for elution at the flow rate of $1 \text{ mL} \cdot \text{min}^{-1}$. The elution gradient had the following profile: $t_{0 \text{ min B}}$ (7%), $t_{2 \text{ min B}}$ (7%), $t_{10 \text{ min B}}$ (16%), $t_{40 \text{ min B}}$ (31%), $t_{45 \text{ min B}}$ (50%), $t_{48 \text{ min B}}$ (100%), $t_{53 \text{ min B}}$ (100%), $t_{54 \text{ min B}}$ (7%), and $t_{59 \text{ min B}}$ (7%). The detection wavelength was 280 nm.

Individual flavan-3-ols and gallic acid were identified using the corresponding standard compounds. Results were expressed as g of catechin equivalent/100 g_{RM} for monomers and g of procyanidin B1 equivalent/100 g_{RM} for oligomers.

2.2.2.6. Statistics

Variance analysis was used for data analysis. The statistical significance of the differences in the data was obtained using the Tukey's test ($\alpha = 5 \%$). Data processing was carried out using XLSTAT (Addinsoft SARL, France) software.

2.2.3. Results and discussion

2.2.3.1. Total Polyphenol Content.

The total phenolic content of subcritical water extracts of grape pomaces of four different grape varieties in function of temperature of extraction is shown in Table 2. The total phenolic content ranged from 0.59 ± 0.05 to 3.66 ± 0.19 g of Gallic acid GA/100g Dry matter (DM) for the extraction Cabernet Franc at 100°C and the extraction Dunkelfelder at 200°C respectively. The Dunkelfelder variety harvested in 2012 yielded the highest values followed by Chardonnay, Dunkelfelder 2013, followed by Merlot and Cabernet Franc, in that order. Since red pomace samples were collected for close vineyards and underwent the vinification and pressing procedure, the differences observed in total phenolics are mainly due to the year of harvest and the inherent characteristics of each grape variety investigated. While the difference between the white Chardonnay and the other red pomace is inherent to the grape variety and vinification practices. As shown in table 2, except between Dunkelfelder 2012 and Dunkelfelder 2013 extracted at 100°C, significant differences ($p < 0.05$) in total phenolic content was found among all varieties.

Temperature had a high influence on the extracted total polyphenols. For example polyphenols extracted from Merlot pomace showed an increase from 1.08 ± 0.2 g to 2.29 ± 0.18 g of GA/100g DM content of polyphenols by increasing the

temperature from 100°C to 200°C and the polyphenol content above conventional solvent extraction at temperatures higher than 150°C.

Another important factor is the influence of year of harvest for Dunkelfleder variety with all three extraction temperatures showing significant differences for total polyphenol content, for example at 200°C extraction of 2012 harvest was $3,66 \pm 0,19$ and 2013 harvest $2,72 \pm 0,09$ g of GA/100g DM.

By comparing to data obtained from other studies from subcritical extraction and conventional solvent extraction, polyphenol content largely varies from 1.06 to 6.23 g of GA/100g depending on the grape cultivar, geographical origin, vintage winemaking process (Ju & Howard 2003; García-Marino et al. 2006; Luque-Rodríguez et al. 2007; Casazza et al. 2012; Vergara-Salinas et al. 2013; Duba et al. 2015a; Vergara-Salinas et al. 2015). The results obtained in this study were in agreement with the aforementioned range. Specifically total phenolic content of weight (g) of GA/100g was presented by Aliakbarian et al. 2012 for grape red Cortina pomace. These results also are comparable which utilized organic solvent for the extraction (González-centeno et al. 2012; Ky et al. 2014).

Table 2 Total Phenolics, Total Proanthocyanidins, and Flavan-3-ol Content of the Grape Pomace Samples (SWE: 100% water, P= 25 bars; Control: 50% ethanol/water 20°C)

By-product	Extraction parameters	Total phenolics ^a		Total pro-Anthocyanin dins ^b		Gallic acid ^c		Flavan-3-ol ^c									
								Catechin		Epicatechin		B1		C1		Total	
Dunkelfelder 2012	SWE 100°C	2.53	± 0.11	62.23	± 3.79	11.85	± 0.41	55.54	± 3.01	25.65	± 1.95	18.83	± 0.44	4.96	± 0.77	104.97	
	SWE 150°C	2.92	± 0.17	68.76	± 2.55	20.46	± 0.96	65.08	± 2.11	35.75	± 1.09	18.65	± 0.31	7.27	± 0.13	126.75	
	SWE 200°C	3.66	± 0.19	72.52	± 2.43	38.83	± 1.39	94.78	± 0.49	65.84	± 2.74	28.49	± 0.70	9.75	± 0.31	198.86	
	Control	3.07	± 0.67	67.25	± 5.29	12.52	± 1.82	47.21	± 0.89	30.21	± 2.06	19.83	± 1.40	4.23	± 0.94	101.47	
Dunkelfelder 2013	SWE 100°C	2.11	± 0.14	44.26	± 1.43	8.58	± 1.01	46.87	± 2.21	19.49	± 2.30	13.12	± 0.53	4.27	± 0.39	83.75	
	SWE 150°C	2.44	± 0.06	52.31	± 0.59	18.37	± 0.73	60.42	± 4.95	31.14	± 1.13	17.55	± 1.21	6.65	± 0.79	115.76	
	SWE 200°C	2.72	± 0.09	67.19	± 1.63	30.86	± 0.33	78.73	± 4.98	59.37	± 2.79	24.69	± 1.24	7.23	± 0.68	170.02	
	Control	2.38	± 0.08	49.11	± 2.93	13.10	± 1.74	49.66	± 3.12	29.50	± 1.92	16.07	± 0.90	4.67	± 0.71	99.89	
Cabernet Franc	SWE 100°C	0.59	± 0.05	11.67	± 1.67	15.08	± 1.58	18.58	± 1.73	15.17	± 1.01	13.87	± 1.06	2.91	± 0.17	50.54	
	SWE 150°C	0.82	± 0.10	20.26	± 0.90	26.20	± 1.19	34.79	± 1.13	18.29	± 1.50	15.13	± 0.47	2.89	± 0.64	71.10	
	SWE 200°C	1.42	± 0.12	34.17	± 2.76	31.41	± 3.36	55.87	± 0.36	21.29	± 0.32	17.54	± 0.90	2.29	± 0.40	97.00	
	Control	0.49	± 0.01	16.07	± 0.42	24.66	± 2.20	23.41	± 1.68	18.28	± 1.40	15.50	± 0.79	2.08	± 0.74	59.27	
Merlot	SWE 100°C	1.08	± 0.20	21.63	± 2.02	0.63	± 0.04	13.29	± 2.53	7.32	± 0.89	5.20	± 0.47	2.08	± 0.18	27.90	
	SWE 150°C	1.56	± 0.06	38.51	± 2.03	1.75	± 0.11	16.78	± 1.02	11.65	± 0.67	6.86	± 1.09	2.82	± 0.33	38.11	
	SWE 200°C	2.29	± 0.18	44.31	± 1.48	1.86	± 0.06	19.69	± 2.09	15.48	± 0.74	3.03	± 1.56	0.49	± 0.35	38.70	
	Control	1.26	± 0.19	36.17	± 3.96	1.52	± 0.07	13.75	± 0.10	11.77	± 0.38	5.11	± 0.70	2.19	± 0.24	32.81	
Chardonnay	SWE 100°C	2.11	± 0.08	54.20	± 1.33	1.78	± 0.33	30.62	± 2.74	26.13	± 2.40	8.24	± 0.80	5.58	± 0.08	70.58	
	SWE 150°C	2.82	± 0.12	63.48	± 2.29	2.11	± 0.37	37.30	± 3.69	28.48	± 1.08	12.03	± 0.17	6.04	± 0.46	83.85	
	SWE 200°C	3.06	± 0.09	68.37	± 4.17	3.59	± 1.85	45.03	± 1.23	31.91	± 0.97	14.53	± 0.48	5.88	± 0.22	97.35	
	Control	2.91	± 0.76	57.17	± 3.69	1.97	± 0.41	38.36	± 0.15	26.78	± 4.06	13.10	± 1.08	5.62	± 0.42	83.86	

^aTotal phenolics expressed as g of GA/100 g DM, ^bTotal proanthocyanidins expressed in mg of tannins/g DM, ^cGallic acid and Flavan-3-ol concentration expressed in mg/100 g DM, C, (+)-catechin; EC, (-)-epicatechin; B1–B2, procyanidin dimers; C1, procyanidin trimer

2.2.3.2. Total Proanthocyanidins Content

The total proanthocyanidins contents of grape pomace by-products, obtained by Bate-Smith reaction, are shown in Table 2. Similar to phenolic content quantification by Folin Ciocalteu method, Dunkelfelder subcritical water extraction at 200°C showed the highest content of 72.52 ± 2.43 mg/g DM, whereas Cabernet Franc pomace presented the lowest value 11.67 ± 1.67 mg/g DM. Significant differences ($p < 0.05$) were observed among the grape varieties investigated, the years of harvest and the temperature of extraction.

Temperature of extraction had a high influence on the extracted total proanthocyanidins. In our case for example polyphenols extracted from Chardonnay pomace showed an increase from 54.20 ± 1.33 to 68.37 ± 4.17 mg/g DM content of total proanthocyanidins by increasing the temperature from 100°C to 200°C and the polyphenol content above conventional solvent extraction at temperatures higher than 100°C.

Additionally the year of harvest had an important influence. Dunkelfelder variety with the three extraction temperatures significant differences were shown for the proanthocyanidin content, for example at 150°C extraction of 2012 harvest was 68.76 ± 2.55 and 2013 harvest 52.31 ± 0.59 g of tannins/100g DM.

Total proanthocyanidin Bate-Smith test is a coloration method to detect the presence of condensed tannins; which is important fraction in the extract that is usually overlooked for quantification subcritical water pomace extracts. For this reason it was difficult to compare results to other subcritical water extracts. Nonetheless results obtained in the present study were similar to values previously reported in the literature for pomace by-products from white and red grape varieties extracted using an organic solvent (Rockenbach et al. 2011; Mandic et al. 2008; Obreque-Slier et al. 2010; Travaglia et al.; González-centeno et al. 2012). Nonetheless, observed total tannins values 68 mg/g DM for Chardonnay pomace 2.2-fold higher than those obtained by González-centeno et al. 2012 using a solvent of MeOH/water (60:40, v/v) extraction. These differences could be attributed to the different vintage and viticulture conditions of the samples.

As previously observed in several studies (Mandic et al. 2008; Lorrain et al. 2013; Ky et al. 2014) a high significant correlation was found between the total

phenolic and total proanthocyanidin contents of the grape pomace extracts ($r = 0.94$, $p < 0.05$).

2.2.3.3. HPLC Analysis of Monomeric and Oligomeric Flavan-3-ols

The monomeric and oligomeric flavan-3-ol composition of the grape pomace by-product from the four grape varieties extracted by subcritical water at different temperatures were investigated and described in Table 2. All the extracts were analyzed by HPLC to identify and quantify the flavan-3-ols procyanidin B1, (+)-catechin, (–)-epicatechin, and the trimer C1, in this order of elution.

The combined amount of the above flavan-3-ols in grape pomace by-products ranged from 27.90 to 198.86 mg/100 g DM, for Merlot (SWE 100°C) and Dunkelfelder (2012, SWE 200°C) varieties, respectively. These results are in accordance to results previously published with the total flavan-3-ol range (29–199 mg/100 g DM) proposed by Luque-Rodríguez et al. 2007 for red grape pomace by-product (8 MPa, 120 °C, 1:1 (v/v) ethanol, 0.8% (v/v) HCl). Significant differences were found among the four varieties considered, the year of harvest, and temperature of extraction ($p < 0.05$), both Dunkelfelder and Chardonnay exhibiting the highest total flavan-3-ol content of 198 mg/100 g DM and 97 mg/100 g DM respectively at 200°C.

Temperature of subcritical water extraction was the most important factor yielding to a varied amount of Flavan-3-ols, for example increasing the temperature from 100°C to 200°C lead an increase of 1.37 to 1.91 folds of extracts Flavan-3-ols. Temperature had a differential influence on individual compounds, (+)-catechin and (–)-epicatechin were optimally extracted at 200°C for all grape pomaces. While Proanthocyanidins B1 and C1 were optimally extracted at 150°C for Cabernet Franc, Merlot and Chardonnay.

The ratio of content of both monomers, (+)-catechin and (–)-epicatechin, accounted for 65–81% of the total flavan-3-ol quantified content of grape pomaces depending on the grape variety considered, temperature extraction, and year harvest. Apart from the Chardonnay variety, in general, the monomeric fraction was greater than the dimeric and trimeric one at high temperatures. This observation agrees with that reported by Monrad et al. 2014 for red grape pomace (*V. labrusca* L.) and, also, with the results described by different authors for red grape pomace (*V. vinifera* L.) (Vergara-Salinas et al. 2015; Duba et al. 2015b) with optimal extraction conditions

shown in Table 1.

A similar ranking of the individual flavan-3-ol compounds was detected throughout all the investigated by-products. (+)-catechin was the major flavan-3-ol component, representing from 46% to 74 % of the monomeric fraction and from 44% to 55% of the quantified flavan-3-ol content. Followed by (-)-epicatechin was the second main component quantified in all extracted by-products except, for Merlot, which had a higher of (-)-epicatechin to (+)-catechin when extracted at 200°C.

These ratios of higher quantity of monomers with respect to dimer and trimer have been previously observed in the literature for skins and/or seeds of different grape pomaces extracted by subcritical water (Srinivas et al. 2011; García-Marino et al. 2006; Vergara-Salinas et al. 2013; Bucić-Kojić et al. 2011; Monrad et al. 2014).

2.2.3.4. HPLC Analysis of Anthocyanins for red grape by-products

The anthocyanin content of skin extracts was analyzed by HPLC and the concentrations obtained compounds were presented in Table 3.

For total anthocyanins, Dunkelfelder 2012 and 2013 extracted at 100°C (47.94 mg/g DM and 40.04 mg/g DM respectively), and Cabernet Franc (12.1 mg/g DM) superior amounts to lower temperatures. Grape variety had an important influence on quantity of total anthocyanins and the ratios of individual anthocyanins.

Undeniably, “teinturier” cultivars as Dunkelfelder had higher anthocyanin content than “non-teinturier” grapes (i.e., Merlot and Cabernet Franc). The table 3 shows the Dunkelfelder pomace contains principally malvidin-3-O-glucoside (72%–78%) of the total anthocyanins found in the extract in comparison to other two cultivars used for the extraction, but also contain unusually high amounts of peonidin-3-O-glucoside (6.7.1 mg/g DM at SWE 100°C) when compared to Merlot and Cabernet Franc at the same condition of extraction. This work is in accordance to several work that has been done on teinturier grape varieties (Hermosín-Gutiérrez & García-Romero 2004; Ky et al. 2014), were peonidin-3-O-glucoside showed higher ratios in comparison to other varieties.

In vintages 2012 and 2013 pomace of Dunkelfelder contained variable levels of anthocyanins the ratio of the anthocyanins stayed the same. For both vintages Dunkelfelder shown an high of malvidin-3-O-monoglucoside was the major anthocyanin and accounted for 72% total anthocyanins while other varieties ranged

between 21 and 65%. In 2013 by-products, less amounts of anthocyanins was observed. Values ranged from 1.4 mg/g DM to 10.6 mg/g DM for glycosylated anthocyanins,

Temperature of subcritical water extraction was the most important factor yielding to a varied amount of for example increasing the temperature from 100°C to 200°C lead a decrease of 1.37 to 1.91 folds of extracted anthocyanins. Temperature had a differential influence on individual compounds, (+)-catechin and (-)-epicatechin were optimally extracted at 200°C for all grape pomaces.

The optimum temperature of extraction depended on the molecules; with an average of optimum temperatures (0.47 mg/100mg) around 100°C. These molecules were optimally extracted at lower temperature than flavonoid and phenolic acids. The presence of a sugar molecule in glycoside anthocyanins intends to make them more soluble in polar substances, and they are subsequently extracted well at a lower temperature than less-polar flavonoid and phenolic acids (Monrad et al. 2010b). Furthermore anthocyanins are highly thermolabile compounds due to the presence of the glucoside function that leads degradation at high temperatures during extraction (Ko et al. 2014). Proanthocyanidins B1 and C1 were optimally extracted at 150°C for Cabernet Franc, Merlot and Chardonnay.

Table 3, Total Anthocyanin Content of the Grape Pomace Samples (SWE: 100% water, P= 25 bars; Control: 50% ethanol/water 20°C)

By-product	Extraction parameters	Anthocyanins (mg/g of DM)					
		Cyanidine-3-O-Glucoside	Delphinidine-3-O-Glucoside	Malvidine-3-O-Glucoside	Petunidine-3-O-Glucoside	Peonidine-3-O-Glucoside	Total
Dunkelfelder 2012	SWE 100°C	0.21 ± 0.01	1.45 ± 0.08	34.79 ± 1.20	4.78 ± 0.55	6.72 ± 1.02	47.94
	SWE 150°C	0.08 ± 0.00	0.30 ± 0.04	19.18 ± 1.75	2.71 ± 0.10	7.80 ± 0.45	30.07
	SWE 200°C	0.05 ± 0.02	0.13 ± 0.02	8.46 ± 0.41	1.08 ± 0.05	3.64 ± 0.17	13.36
	Control	0.15 ± 0.02	0.97 ± 0.08	16.42 ± 1.27	4.83 ± 0.07	4.51 ± 0.07	26.89
Dunkelfelder 2013	SWE 100°C	0.19 ± 0.03	1.33 ± 0.02	31.49 ± 0.15	4.54 ± 0.02	2.49 ± 0.27	40.04
	SWE 150°C	0.08 ± 0.01	0.19 ± 0.08	18.17 ± 1.35	2.60 ± 0.05	7.77 ± 0.97	28.81
	SWE 200°C	0.04 ± 0.00	0.11 ± 0.07	6.96 ± 0.09	0.91 ± 0.07	2.99 ± 0.41	11.02
	Control	0.13 ± 0.02	0.83 ± 0.06	15.14 ± 0.05	0.49 ± 0.04	3.89 ± 0.08	20.48
Cabernet Franc	SWE 100°C	0.51 ± 0.03	2.15 ± 0.06	5.99 ± 0.08	1.87 ± 0.04	1.69 ± 0.02	12.21
	SWE 150°C	0.21 ± 0.02	1.33 ± 0.02	4.31 ± 0.01	0.92 ± 0.02	0.15 ± 0.08	6.93
	SWE 200°C	0.19 ± 0.01	1.47 ± 0.10	1.36 ± 0.04	0.54 ± 0.06	0.12 ± 0.00	3.67
	Control	0.43 ± 0.07	0.17 ± 0.03	0.43 ± 0.03	0.18 ± 0.03	0.12 ± 0.07	1.32
Merlot	SWE 100°C	0.14 ± 0.01	1.59 ± 0.06	2.18 ± 0.08	4.93 ± 0.07	1.33 ± 0.04	10.17
	SWE 150°C	0.10 ± 0.00	1.42 ± 0.05	1.67 ± 0.04	3.59 ± 0.03	1.09 ± 0.14	7.86
	SWE 200°C	0.03 ± 0.00	0.54 ± 0.02	0.97 ± 0.22	1.54 ± 0.02	0.17 ± 0.00	3.25
	Control	0.13 ± 0.03	1.39 ± 0.03	1.89 ± 0.06	3.84 ± 0.05	0.99 ± 0.07	8.23

In units of mg/100 g DM pomace, Data are expressed as the mean of triplicate ± standard deviation,

TPC, total phenol content; total anthocyanins; Dp, delphinidin-3-O-monoglucoside; Cy, Cyanidin-3-O-monoglucoside; Pt, Petunidin-3-O-monoglucoside (eq, Malvidine-3-O-glucoside) ; Pn, Peonidin-3-O-monoglucoside (eq, Malvidine-3-O-glucoside); Mv, Malvidin-3-O-monoglucoside;

2.2.3.5. Antioxidant Capacity

The antioxidant potential of each sample was determined in order to select the most active grape pomace among studied varieties. Antioxidant capacity of each extract cannot be assessed by a single method. Indeed, antioxidant measurements can be related either to the capacity of extracts to directly transfer hydrogen to a radical (ABTS, FRAP) or to act as competitors for the peroxy radicals (ORAC, CUPRAC) (Roginsky & Lissi 2005). Hence, more than one type of antioxidant measurement needs to be performed to take into account the various mode of action of antioxidants (Huang et al. 2005). In that context, the free radical scavenging capacities of seed and skin extracts were evaluated by the four tests, the FRAP, ABTS decolorization, CUPRAC and ORAC assays.

Table 4 presents the antioxidant capacity results, measured by the four aforementioned analytical assays. Similar behavior patterns were observed for the results of ABTS, CUPRAC, FRAP, and ORAC assays, regardless of their action mechanism. Results were obtained for ABTS, CUPRAC, FRAP, and ORAC assays, with the Dunkelfelder variety producing the highest antioxidant capacities extracted at 200°C.

Meanwhile, the Merlot variety showed the lowest values at the same temperature, with an antioxidant potential from 1,6 times lower than that observed for the Dunkelfelder variety. Furthermore, the CUPRAC assay showed significant differences ($p < 0.05$) among the antioxidant capacity values of all four grape pomaces investigated, whereas, in the ORAC assay, both Merlot and Cabernet Franc varieties did not differ significantly as having the lowest antioxidant potential ($p > 0.05$). All the examined grape pomaces extracts showed considerable increase antioxidant activity when increasing the temperature from 100°C to 200°C, in particular, the Dunkelfelder variety, whose total phenolic content was also the greatest among the four varieties considered.

The use of different analytical methods (such as CUPRAC, ABTS, FRAP,...), the utilization of various standards and reference units and also importantly the difference between grape materials of reference, makes a comparison of literature with regard to the antioxidant capacity of winemaking by-products quite challenging. The antioxidant capacity is additionally affected by other factors such as the winemaking procedure, geographical origin of the samples and the extraction methodology.

Nonetheless, the same order of magnitude as previously described in literature for the antioxidant capacity of grape pomace extracts are noted, irrespective of the analytical method applied or of the grape variety used. It was difficult to find antioxidant assay that we could compare with from extracts from grape pomace. One example, Ju & Howard (2005) reported antioxidant capacity ranges measured by ORAC assay were higher in comparison with those of the present research (1105 mg of Trolox/g DM) extracted at 160°C. But a larger scope of comparison to grape extracted solvent showed lower antioxidant values, Sánchez-Alonso et al. (2007) reported the antioxidant capacity of Airén white grape pomace, measured by ABTS (71.1 mg of Trolox/g DM) and FRAP assays (116.6 mg of Trolox/g DM), being similar to the extracts obtained at 100°C.

Table 4. Antioxidant Capacity Determined by ABTS, CUPRAC, FRAP, and ORAC Assays for the Grape Pomace Samples^a

By-product	Extraction parameters	ABTS		CUPRAC		FRAP		ORAC	
Dunkelfelder 2012	100% water/100°C/50 Bar	124.74	± 9.55	163.04	± 3.92	99.90	± 5.81	123.00	± 1.46
	100% water/150°C/50 Bar	239.95	± 15.05	213.64	± 1.20	192.17	± 6.99	236.62	± 5.37
	100% water/200°C/50 Bar	290.39	± 14.02	379.57	± 8.00	232.57	± 5.17	286.36	± 20.03
	50% ethanol/water 20°C	144.08	± 13.82	181.01	± 7.95	162.85	± 6.25	136.93	± 2.47
Dunkelfelder 2013	100% water/100°C/50 Bar	70.60	± 7.01	159.17	± 6.24	73.37	± 2.87	87.00	± 8.79
	100% water/150°C/50 Bar	149.12	± 12.60	336.23	± 2.21	154.99	± 5.73	183.77	± 8.96
	100% water/200°C/50 Bar	219.00	± 13.49	493.78	± 1.34	227.61	± 6.32	269.89	± 5.63
	50% ethanol/water 20°C	87.40	± 9.85	194.34	± 1.23	78.79	± 2.34	123.53	± 4.53
Cabernet Franc	100% water/100°C/50 Bar	60.56	± 4.58	113.63	± 2.28	67.82	± 2.26	66.40	± 6.76
	100% water/150°C/50 Bar	107.97	± 7.90	202.59	± 8.21	120.92	± 3.93	118.40	± 2.37
	100% water/200°C/50 Bar	218.60	± 4.75	410.17	± 7.11	244.81	± 1.21	239.71	± 5.54
	50% ethanol/water 20°C	72.34	± 5.25	132.80	± 9.80	98.88	± 7.02	73.47	± 4.25
Merlot	100% water/100°C/50 Bar	28.76	± 1.16	97.72	± 5.15	47.78	± 5.65	43.44	± 4.14
	100% water/150°C/50 Bar	49.13	± 2.59	166.95	± 6.42	81.62	± 3.81	74.21	± 3.09
	100% water/200°C/50 Bar	97.50	± 3.20	331.31	± 6.34	161.98	± 7.75	147.27	± 8.34
	50% ethanol/water 20°C	32.49	± 1.62	111.77	± 7.29	53.86	± 4.41	55.46	± 5.41
Chardonnay	100% water/100°C/50 Bar	97.90	± 4.17	71.38	± 2.51	55.95	± 6.60	62.30	± 1.51
	100% water/150°C/50 Bar	150.01	± 6.01	109.38	± 1.92	85.73	± 5.86	95.46	± 5.60
	100% water/200°C/50 Bar	271.90	± 7.46	198.25	± 9.59	155.40	± 4.21	173.02	± 8.34
	50% ethanol/water 20°C	123.80	± 3.95	81.15	± 3.07	70.43	± 3.23	82.34	± 2.53

^aAntioxidant capacities expressed as equivalents of mg of Trolox/g DM, Letters following the values in each column show the significant differences among grape varieties (p < 0,05),

Pearson's correlation coefficients were calculated to evaluate the agreement on the expression of the grape pomace antioxidant capacity among the four assays applied. Regardless of the pair of methods considered, a high, significant and positive correlation was observed ($r \geq 0.84$, $p < 0.05$), suggesting that ABTS, CUPRAC, FRAP, and ORAC assays give comparable and interchangeable antioxidant capacity values for grape pomaces. Correlation coefficients among antioxidant capacities based on ORAC and FRAP assays were the highest ($r \geq 0.96$), whereas ABTS data exhibited little to low correlation values ranging from 0.62 to 0.89. The different degree of correlation among these four assays may be due to the different chemical information provided depending on the electron or hydrogen transfer mechanism on which they are based.

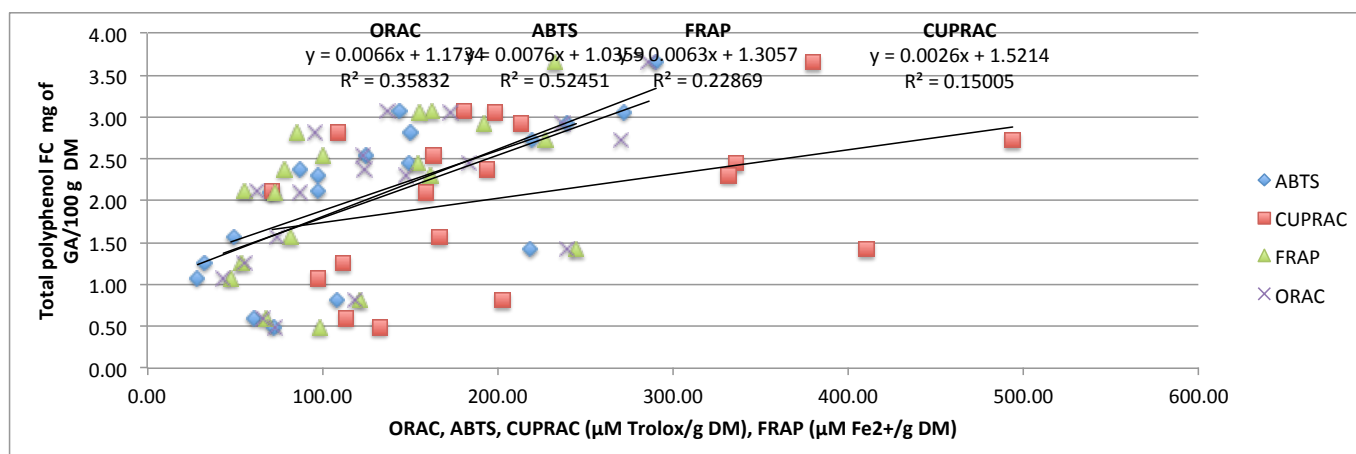


Figure 2. Correlations between radical scavenging capacity assays (ORAC, FRAP, ABTS and CUPRAC) and total flavan-3-ol in grape pomace extract by subcritical water extraction.

Further study of the correlation in the present research revealed that total polyphenols compounds quantified by Folin-Ciocalteu method and antioxidant capacity data (Figure 2) were not significantly correlated. The correlation with total phenolic content was exhibited by the ORAC, ABTS, FRAP and CUPRAC assays ($r = 0.35$ 0.53 , 0.22 and 0.15 respectively at $p < 0.05$), this low correlation was not previously observed in the literature for skins, seeds, and grape pomaces (Ju & Howard 2005; Lafka et al. 2007; Aliakbarian et al. 2012). When comparing the total tannin content and the antioxidant capacity of the grape pomace extracts (Figure 3), a lower correlation was observed ($0.12 \geq r \geq 0.51$, $p < 0.05$). Similar results here obtained when correlating total anthocyanin content and the antioxidant capacity of the grape pomace extracts (Figure 4), a lower correlation was observed ($0.05 \geq r \geq$

0.16, $p < 0.05$). This unexpected because anthocyanins are degraded at temperatures above 100°C, while antioxidant increased with temperature and peaked at 150 to 200 °C.

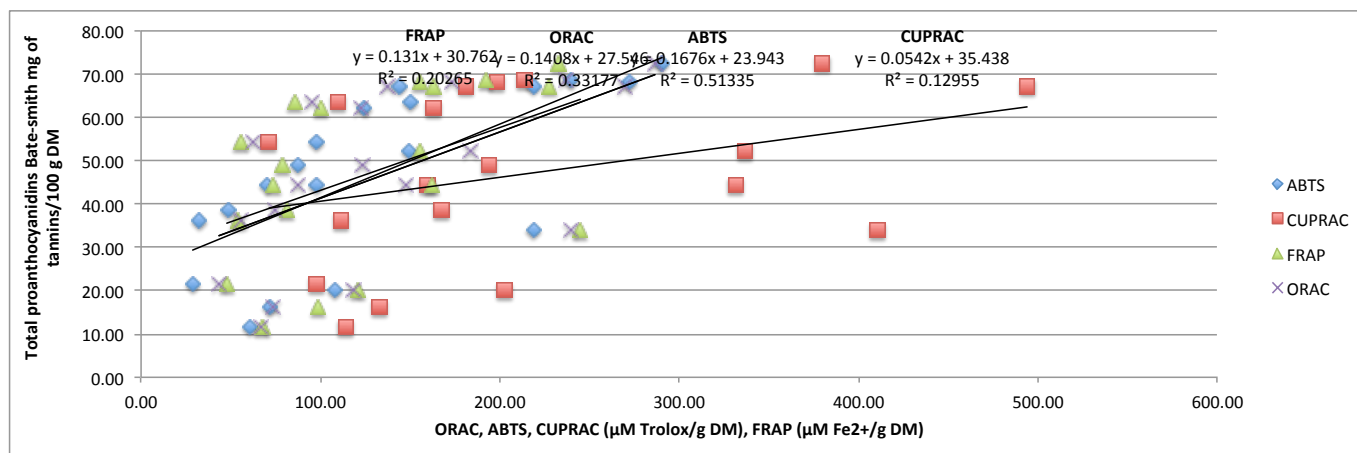


Figure 3. Correlations between radical scavenging capacity assays (ORAC, FRAP, ABTS and CUPRAC) and total proanthocyanidins (Bate - smith) content in grape pomace extract by subcritical water extraction

Increasing extraction temperature above 100°C decreased anthocyanins but increased antioxidant activity and Total polyphenols quantified by Folin Ciocalteu method. It has been reported that high temperatures favor the formation of derived antioxidant compounds from polyphenols (García-Marino et al. 2006; Sadilova et al. 2007) as well as antioxidant Maillard reaction products such as melanoidins (Plaza et al. 2013).

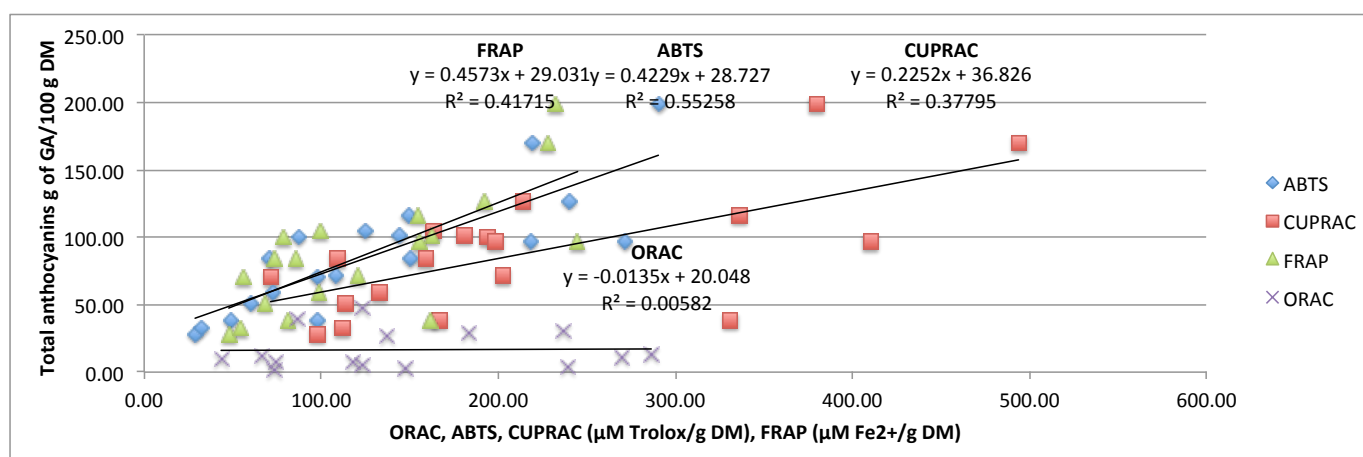


Figure 4. Correlations between radical scavenging capacity assays (ORAC, FRAP, ABTS and CUPRAC) and total anthocyanins in grape pomace extract by subcritical water extraction.

2.2.4. Conclusion

The present research brings a detailed evaluation of the phenolic composition (total phenolic and total proanthocyanidin contents, monomeric and oligomeric flavan-3-ol composition, and proanthocyanidin profile, anthocyanins) and antioxidant potential of white grape pomace by-products derived from the vinification process. To the best of our knowledge, no studies addressing this variability of the by-product such a detailed form for multiple grape varieties have been previously published.

In conclusion, subcritical water extraction of antioxidants from multiple grape pomace, high amounts of anthocyanins and Flavan-3-ols were recovered from fermented grape pomace using differential temperatures with a high variability between by-products. Contrary to anthocyanins, high extraction temperatures (about 200 °C) yielded higher amounts of tannins. Overall, we found that grape pomace antioxidant activity and Total polyphenols quantified by Folin Ciocalteu method were not directly related to the main polyphenol content in SWE extracts, this critical point should be further investigated. The data obtained here in a laboratory-scale equipment will be useful to develop an industrial scale SWE processes.

Finally as observed, grape pomace by-products can be considered as an important source of polyphenols. In this regard, this global characterization may potentially provide the basis for a sustainable process of integrated exploitation of winemaking by-products as potential, inexpensive, and easily available sources of bioactive compounds for the pharmaceutical, cosmetic, and food industries.

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2.3.SUBCRITICAL WATER EXTRACTION AND NEOFORMATION OF ANTIOXIDANT COMPOUNDS FROM DUNKELFELDER GRAPE POMACE

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Abstract

Water, an inexpensive and environmentally friendly solvent is an ideal solvent for industrial extraction of phenolics, but its use is limited due to poor extraction efficiency at low temperatures. In this study, subcritical water (SWE) extractions of grape pomace were studied through independent variables, pressure 25-100 Bar, temperature (100–200 °C), hydraulic retention time and extraction volume. The results were compared with conventional aqueous 50% (v/v) ethanol extractions. The SWE extracts had comparable or higher levels of anthocyanins and ORAC values than extracts obtained using conventional hot water or 50% ethanol. Subcritical water at 100°C appears to be an excellent alternative to organic solvents to extract anthocyanins and from grape pomace and possibly other grape processing byproducts. While the optimized extraction subcritical extraction for flavonols was at 175°C, giving a predicted total yield of flavonols of 190mg/g DM from grape pomace. Furthermore by adjusting the hydraulic retention time of the extraction, degradation effects were minimised, and optimum extraction could be achieved within 6ml/min. Results also obtained suggest that new antioxidant compounds such Hydroxymethylfurfural and Furfural were formed at the higher extraction temperatures.

Key words: Green process, Subcritical water extraction, Grape pomace, Polyphenols, Valorization.

Abbreviations

DS Dry stems

DM Dry matter

GA Gallic acid

HRT Hydraulic Retention Time

n.d. not determined

P Pressure

RM Raw Matter

SWE Subcritical Water Extraction

T Temperature

TE Trolox Equivalent

ε dielectric constant

2.3.1. Introduction

Nowadays, numerous methods of extraction have been developed with the objective of obtaining bioactive compounds from natural sources with higher yields and lower costs. Such is the case of extraction with organic solvents, such as methanol, ethanol and acetone. It is mainly the process of extraction that influences the yield of the extraction. As different compounds have varying levels of solubility in diverse solvents, there is no uniform or complete procedure that is suitable for the extraction of all bioactive compounds. Solubility of the compounds is highly dependent on the polarity of the solvent, with much higher extraction yields possible in less polar solvents, such as methanol and ethanol (Srinivas et al. 2011). The traditional extraction methods have several drawbacks; they are time consuming, laborious, have low selectivity and/or low extraction yields. Also, these processes are not environmentally friendly, because it is difficult to eliminate all solvent traces from the resulting extracts. In addition, organic solvents substantially increase extraction process costs (Yammine et al. 2014). Approaches such as pulsed electric field (Brianceau et al. 2015), supercritical fluid extraction (Díaz-Reinoso et al. 2006) subcritical water extraction (SWE) have been proposed as alternatives to the conventional procedure (Ju & Howard 2005). Subcritical water is obtained at temperatures between 100°C and 374°C (the critical temperature) under high pressure (usually from 10 to 60 10^5 Pa) to maintain the water in the liquid state. At these parameters water polarity declines dramatically with increasing temperature due to hydrogen bond dissolution and reaches values comparable to organic solvent–water mixtures (Plaza & Turner 2015). The lower viscosity and surface tension of hot water also increase mass transfer rates of compounds from the tissue matrix. There is however two concerns to be considered when using subcritical water extraction. The first being is the main degradation of thermolabile compounds during extraction. For instance at high temperature (250°C) during 30 minutes, the majority of flavonoids are degraded (Ko et al. 2014).

The second concern being the extracted compounds undergo chemical reactions such as Maillard and caramelization reactions, forming new compounds with different chemical properties (Liu et al. 2014). The physicochemical parameters of water in a subcritical state has been reviewed extensively (Kronholm et al. 2007; Teo et al. 2010; Wijngaard et al. 2012; Herrero et al. 2013; Saldaña & Valdivieso-

Ramírez 2015; Plaza & Turner 2015). The ability to use water as a solvent, together with the simple experimental equipment required and its potential for on-line coupling with different techniques have boosted the number of applications of the technique. The main physical parameters that influence the extraction are temperature, pressure, flow rate/hydraulic retention time, particle size, and the parameters inherent to the by-product (grape variety, humidity content, pH, stage of fermentation) (Saldaña & Valdivieso-Ramírez 2015).

Polyphenols exhibit wide range of bioactivities as antioxidants, antimicrobials, anti-inflammatory, and anti-cancer (Palma and Taylor, 1999; Casazza et al., 2010; Aliakbarian et al., 2012). The main classes include flavonoids, phenolic acids, tannins and stilbenes (Ignat et al. 2011). Therefore, the isolation of polyphenols can be an additional source of revenue for distilleries besides its use for feedstock, ethanol production and grape seed oil extraction (Monrad et al., 2014).

Several studies have focused on the optimization for the extraction of polyphenols from grape pomace. For example temperature was the most influential factor in extraction yield and selectivity. With optimum extraction temperature varying largely between authors and being dependent on the by-products used and the targeted molecules. For the extraction of anthocyanins from red grape pomace, several temperatures ranging from 90 to 140°C were studied. The optimal temperature extraction conditions using SWE ranged between 75 and 110°C and resulted in higher levels of anthocyanins values than with extracts obtained using conventional solvent extraction at 60% methanol (Zhi et al. 2005). While the extraction recovery of other families of compounds such as catechins and proanthocyanidins showed that selective extractions of compounds can be realized using one-step extraction at 150°C (Garcia-Marino et al. 2006). Pressure is another factor to be considered in the extraction of polyphenols, where some studies have shown a direct effect while others did not (Aliakbarian et al. 2012; Monrad et al. 2014).

Defining flow rates as a variable affecting the extraction of polyphenols is a matter of debate. All studies have mentioned flow rate as a controllable variable in the extraction (Ju & Howard 2003; Monrad et al. 2014; Viganó et al. 2015). This does not account for the amount of the by-product in the reactor, or the geometry of the reactor itself. Instead, hydraulic retention time would best be considered to compare different studies.

In this work, pressurized water extraction of polyphenols from a red grape pomace was performed. An extensive temperature range (100 to 200°C) was investigated in order to further understand the extraction phenomenon. Similarly several extraction pressures, flow rates and volume of water used were considered. The extraction kinetics of the most important polyphenols are discussed taking into consideration the above parameters. Finally, during the SWE of polyphenols from grape pomace, diverse phenomena occur including thermal degradation, selective polyphenol extraction, and formation of neoantioxidant compounds all of which will be presented.

2.3.2. Material and methods

2.3.2.1. Raw material

Dunkelfelder that is a teinturier grape variety was used for red wine processing, including the maceration–fermentation step. The pomace was collected immediately after pressing at 2 Bar Pa (Sutter EPC 50, Bucher Vaslin SA, France) and was treated with 50 mg of sulphur dioxide (SO₂) per kg of raw material (RM). Samples were stored at 4 °C in vacuum bags until further processing. The dry matter was determined by the measurement of the mass of grape pomace before and after drying the samples at 105 °C overnight and was equal to 44.8% having a pH of 3.58. In order to avoid complications with repeatability due to the heterogeneity of the raw material, the skins and the seeds were separated with a vibrating sifter (Retsch GmbH, Germany). Afterwards, small fractions (diameter Ø < 2.8 mm) and large fractions (Ø > 5.5 mm) were removed. Then, the two standardized fractions were manually and homogeneously mixed (49% of seeds and 51% of skins fresh weight).

2.3.2.2. Process of extraction and parameters

The schematic diagram of the apparatus used for the extraction of polyphenolic compounds using subcritical water is shown in Figure 1.

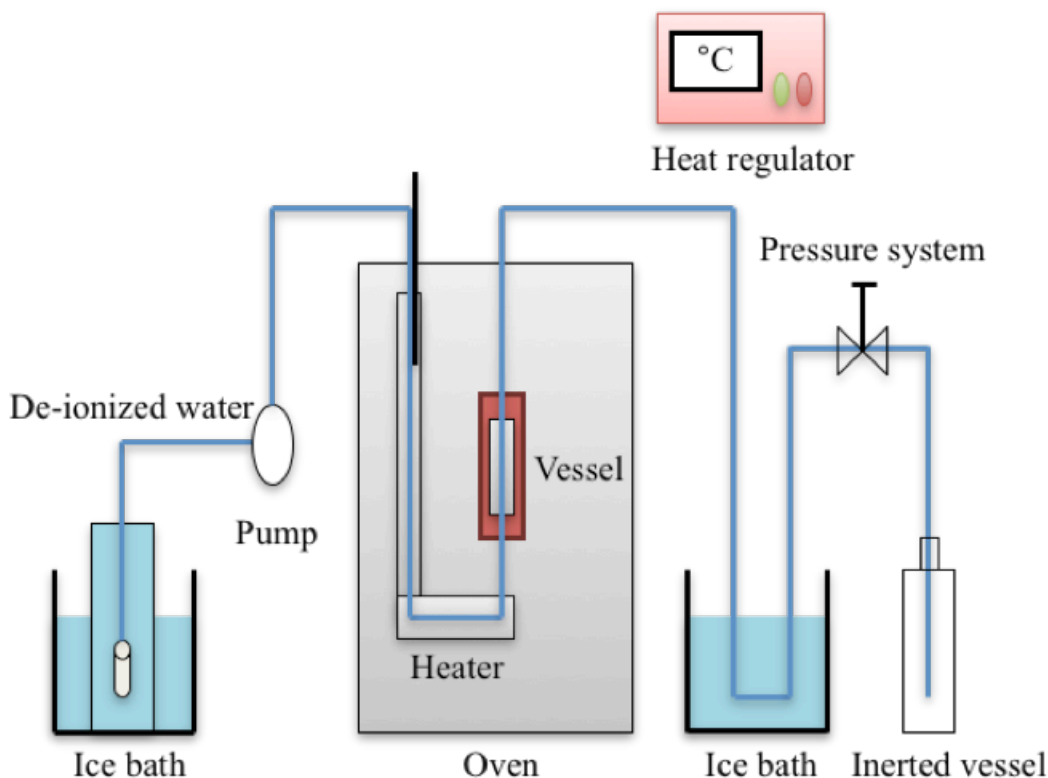


Figure 1. Schematic diagram of the Pressurized liquid extraction process

In the extraction system, an HPLC pump (I.C.S. National 1100) was used for de-ionized water delivery, pressurization and controlling the system pressure. A pressure transducer (Swagelock NG160) and thermocouple (Eurotherm Automation 90) were installed in the custom-made high-pressure vessel to monitor both pressure and temperature of system. Extract was collected in inerted vessels after passing in an ice bath.

In each run, pomace (13.00 g) was loaded into the high-pressure vessel (Internal length of 8.1 cm and diameter of 2.1 cm), which can contain 26 cm³ of material (Figure 1). The vessel was placed in an oven at a predetermined temperature (tested temperatures: 100°C, 125°C, 150°C, 175°C and 200°C). The outlet valve of extraction vessel was then closed and the system was pressurized to a desired pressure (tested pressures: 25, 50, 75 and 100 10⁵Pa) at a constant flow rate. The water flow rate was adjusted between 2-10 mL/min using a metering valve on the HPLC pump. Samples were taken at 65mL, 200mL, 400mL, 600mL. The first fraction of 65 ml was set in order to respect a solid liquid ratio of 1/5. The solution collected in an inerted

sampling vessel and pomace were then stored at 4°C for further analysis without preliminary preparation steps.

For each tested extraction parameter (variation of temperature, pressure, flow rate), experiments were repeated at least three times.

2.3.2.3. Conventional extraction experiments

Polyphenols extraction from grape pomace (100.0 ± 0.1 g) was carried out in a mixture of ethanol and water (50/50, v/v), maintained at the desired temperature (20 °C, 35 °C and 50 °C) in a cylindrical extraction cell. The liquid-to-solid ratio was maintained at the value of 5. A gentle agitation at 160 rpm ($16.8 \text{ rad}\cdot\text{s}^{-1}$) was provided using a round incubator of 12.5 mm (Infors HT Aerotron, Bottmingen, Switzerland). For untreated samples, the same protocol of extraction was used. Regular sampling was carried out during 420 min of extraction. At the end of extraction, the juice was separated from grape pomace by centrifugation (Model 3-16P, Sigma Laborzentrifugen GmbH, Germany) at 3076 g during 10 min, and stored at -18 °C for further analysis.

2.3.2.4. Analysis

2.3.2.4.1. Total polyphenols content

To characterize the subcritical water potential, the extraction rate was quantified by the contents of total polyphenols. The total polyphenols amount was measured spectrophotometrically by the Folin–Ciocalteu (FC) method based on a colorimetric oxidation/reduction reaction of phenols (Singleton, Orthofer, & Lamuela-Raventos, 1999). Note that the Folin–Ciocalteu method is not phenol-specific but it can provide a good estimation of the polyphenols content in the extracts. A volume of 0.2 mL of diluted extract and 1 mL of Folin–Ciocalteu reagent (Sigma-Aldrich, France) (diluted 1:10 with water) were mixed with 0.8 mL of Na_2CO_3 (75 g/L) (VWR, France). The sample was incubated for 10 min at 50 °C and then cooled at room temperature. For the control sample, 0.2 mL of distilled water was taken. The absorbance was measured at 750 nm by the UV/Vis spectrophotometer (Fluorostar OPTIMA BMG Labtech). Gallic acid (Sigma-Aldrich, France) was used for the calibration curve.

Results were expressed as mg gallic acid equivalent per 100 milligram of dry extract (mg GAE/100mg Dry matter (DM)). The analyses were performed in triplicate and average deviation was calculated.

2.3.2.4.2. Antioxidant activity

Oxygen Radical Absorbance Capacity (ORAC) was performed in 96-well microplates. The extracts were diluted in phosphate buffer (75 mM, pH 7) prior to analysis. TroloxTM (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) concentrations were used to build standard curves. Initially, 30 μ L of diluted sample, Trolox standards and blank solution (75 mM, pH 7 phosphate buffer) were added to each well. Then, each well received 180 μ L fluorescein (117 nM) and 90 μ L AAPH (2,2' Azobis (2-methylpropionamidine) dihydrochloride, 40 mM). Fluorescence was detected at 485 nm (excitation) and 520 nm (emission) with a Fluorostar OPTIMA (BMG Labtech) spectrofluorimeter, after the addition of AAPH, and every 60 s thereafter for 60 min to reach a 95% loss of fluorescence. Final fluorescence measurements were expressed relative to the initial reading. Results were calculated based upon differences in areas under the fluorescein decay curve between the blank, samples, and standards. Data were means of four replicates. Activities were expressed as micromoles of Trolox equivalent (TE) per gram dry matter (DM) of extracts.

2.3.2.4.3. Anthocyanins analyses

Quantitative and qualitative analyses of anthocyanins were performed by high performance liquid chromatography (HPLC) on the subcritical water extracts.

Samples of extracts were diluted (ratio 1/10) in acidified water (0.1% formic acid) and then filtered through polyamide filters (pores diameter $\varnothing = 0.45 \mu\text{m}$). The system used for anthocyanin analysis was an Agilent 1200 HPLC Series (Agilent Technologies), equipped with a diode array detector. The separation was carried out with a Prontosil C18AQ column ($4.6 \times 250 \text{ mm}$, $5 \mu\text{m}$, Bischoff Chromatography, Germany) operated at 25°C in reverse phase.

UV/VIS spectra were recorded in the range of 200–600 nm. Two mobile phases, (A) water/acetonitrile/formic acid (87/3/10, v/v/v), and (B) water/acetonitrile/formic acid (40/50/10, v/v/v) were used for the separation of phenolic compounds. The elution

gradient had the following profile: $t_{0 \text{ min B}}$ (6%), $t_{15 \text{ min B}}$ (30%), $t_{30 \text{ min B}}$ (50%), $t_{35 \text{ min B}}$ (60%), $t_{41 \text{ min B}}$ (6%), $t_{45 \text{ min B}}$ (6%). The injection volume was 30 μL and the flow rate was set at $1.0 \text{ mL} \cdot \text{min}^{-1}$. Anthocyanins were detected at 518 nm. Individual anthocyanins were quantified using a calibration curve of the corresponding standard compounds (delphinidin-3-O-glucoside, cyanidin-3-O-glucoside, petunidin-3-O-glucoside, peonidin-3-O-glucoside and malvidin-3-O-glucoside, all purchased from Extrasynthèse, Genay, France). Results were expressed as weight (g) of individual anthocyanins extracted/100 g DM.

2.3.2.4.4. Flavan-3-ols and gallic acid analyses

The HPLC system used for flavan-3-ols and gallic acid analysis was an Agilent 1200 HPLC Series (Agilent Technologies, Germany) equipped with a diode array detector. The samples were diluted (ratio 1/10) in water and then filtered through PTFE filters ($\varnothing = 0.45 \mu\text{m}$). A volume of 60 μL was injected in a Prontosil C18AQ column ($4.6 \times 250 \text{ mm}$, $5 \mu\text{m}$, Bischoff Chromatography, Germany), operated at 25°C in reverse phase. Solvent A, 0.1% trifluoroacetic acid (TFA) in water and solvent B, 0.1% TFA in acetonitrile, were used for elution at the flow rate of $1 \text{ mL} \cdot \text{min}^{-1}$. The elution gradient had the following profile: $t_{0 \text{ min B}}$ (7%), $t_{2 \text{ min B}}$ (7%), $t_{10 \text{ min B}}$ (16%), $t_{40 \text{ min B}}$ (31%), $t_{45 \text{ min B}}$ (50%), $t_{48 \text{ min B}}$ (100%), $t_{53 \text{ min B}}$ (100%), $t_{54 \text{ min B}}$ (7%), and $t_{59 \text{ min B}}$ (7%). The detection wavelength was 280 nm. Individual flavan-3-ols and gallic acid were identified using the corresponding standard compounds (catechin, epicatechin, gallic acid, and procyanidin B1, all purchased from Extrasynthèse, Genay, France). Procyanidin C1 was purified from grape seed extract by Phenobio (Martillac, France). Results were expressed as weight (g) of catechin equivalent/100 g_{RM} for monomers and weight (g) of procyanidin B1 equivalent/100 g DM for oligomers.

2.3.2.4.5. Analysis of Hydroxymethylfurfural (HMF) and Furfural

The formation of HMF and furfural was analyzed with an Agilent 1200 HPLC Series (Agilent Technologies, Germany) equipped with a diode array detector. A Gemini 3 μm C6 phenyl 110 Å (100×2.0 mm i.d.) from Phenomenex (Torrance, CA, USA) was used as an analytical column. The mobile phases consisted of (A) water with 0.5 vol % of formic acid and (B) methanol with 0.5 vol % of formic acid in a gradient elution analysis programmed as follows: 0–5 min, 0% B; 5–15 min, 5% B; 15–16 min, 0% B; with 5 min of post time at a flow rate of 200 $\mu\text{L}/\text{min}$. The column temperature was set at 25 °C, and the injection volume was 5 μL . The detection wavelengths were set at 280 nm.

Quantification of furfural compounds was carried out by an external standard method using a mixture containing furfural and HMF in concentrations from 0.1 to 100 $\mu\text{g}/\text{mL}$ each, corresponding to around 0.8–1042 $\mu\text{mol}/\text{L}$.

2.3.2.5. Statistics

Variance analysis was used for data analysis. The statistical significance of the differences in the data was obtained using the Tukey's test ($\alpha = 0.05$). Data processing was carried out using XLSTAT (Addinsoft SARL, France) software.

2.3.3. Results and discussion

2.3.3.1. Influence of operating parameters

SWE has been considered as a green alternative to obtain functional food ingredients from grape pomace. The objective of this work was to research the principal extraction variables to recover antioxidants from grape by-product from the wine making process. The independent main variables such as temperature (100 to 200°C), flow rate/hydraulic retention time and pressure (25 to 100 Bar) were studied. Several extraction parameters were evaluated: extraction yield of solid solutes; total polyphenols by FC method and antioxidant capacity by ORAC assay; total concentration of polyphenols; formation of brown color as measured at 360 and 420 nm; and concentrations of formed HMF and furfural.

2.3.3.2. Temperature

Taking into consideration an important factor of extraction, Figure 2 shows that the temperature had a significant effect ($P < 0.01$) on the extraction of polyphenols analyzed with Folin Ciocalteu method. Increasing the extraction temperature from 100 to 200°C at 50 bars significantly enhanced the yield from 0.87 mg/100mg to 2.76 mg/g. This result is in agreement with previous studies for the extraction of grape pomace by-products (Ju & Howard 2003; Aliakbarian et al. 2012; Monrad et al. 2014) and other types of by-products (Choi et al. 2003; Deng et al. 2004; Mukhopadhyay et al. 2006; Luthria 2008; Plaza et al. 2013).

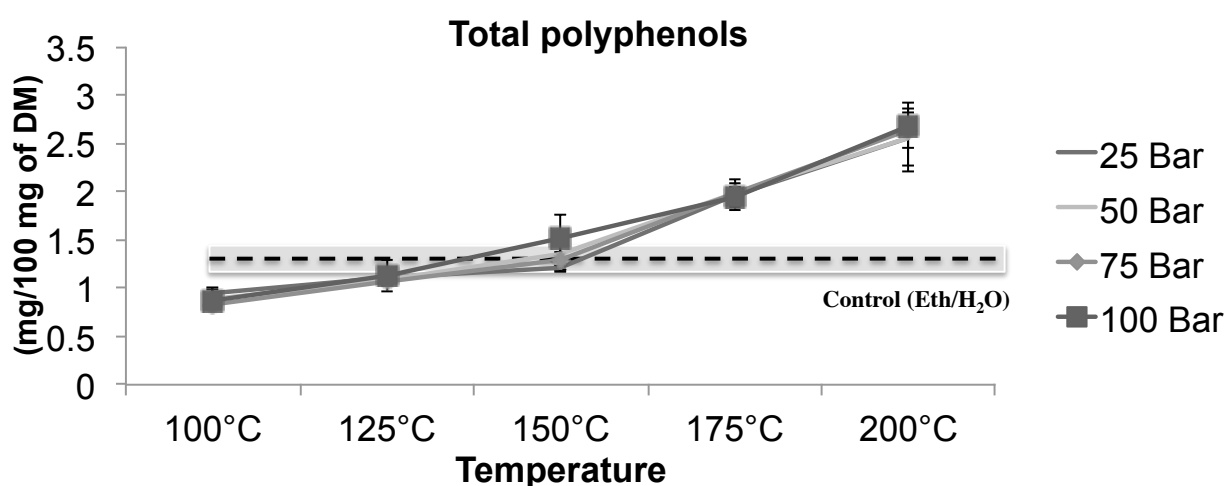


Figure 2. Influence of temperature on the polyphenol concentration for different pressures (25 to 100 bars) and compared with the hydro-alcoholic maceration technique

By increasing the temperature from 100 to 200°C the dielectric constant of water was reduced from 61 to 33 that is comparable to the dielectric constant of methanol at ambient temperature. At high temperatures this leads to the solubility of phenolic compounds comparable to organic solvent. In our case at higher temperatures of 150°C, higher yields were obtained compared to organic solvent. These results were comparable to yields obtained in other studies: 6.23g/100g, 6.070 g/100g, 3.08/100g respectively (Ju & Howard 2003; Casazza et al. 2010; Aliakbarian et al. 2012).

2.3.3.3. Pressure

The extraction pressure in the range of 25 to 100 bars did not have a significant effect ($P > 0.05$) on the yield of polyphenols. This result is in agreement with the following studies (Monrad et al. 2012; Plaza & Turner 2015) and in disagreement with these studies (Aliakbarian et al. 2012). This behavior is due to the small effect of pressure on the dielectric constant at the examined range (Islam et al. 2012).

2.3.3.4. Flow rate/hydraulic retention time

To further optimize the extraction parameters, the flow rate of the water was altered to achieve the best polyphenol yield. An average temperature was set to 150°C for extraction. Figure 3 presents the extraction yield of total polyphenol content (TPC) in function of flow rate/hydraulic retention time and extraction volume. As may be noticed, an increase of the flow rate, decreasing the hydraulic retention time of the solution will accelerate the mass transfer in the SWE system, thus reducing the potential degradation of the extracted molecules and increasing the extraction yield. The hydraulic retention time is calculated by taking the volume of water in the extraction vessel with the 13 grams of pomace added, divided by the linear flow rate. The hydraulic retention time, which is defined by the time it takes for individual compounds to elute from the extraction system, were set between 1 and 5 min depending on the flow rate and presented in Fig. 3. The extraction rate is controlled primarily by a “solubility/elution” step. Denoting that the sub-critical water extraction system as a heterogeneous medium consisting of two immiscible phases, one stationary and the other mobile. If we introduced into this medium, compounds having an affinity to both phases; this will establish, at each point of the column, a balance between the concentration of this compound in the mobile phase and its concentration in the stationary phase. At higher flow rates, the removal of extracted compounds out of the matrix is faster because of a higher concentration gradient between the two phases and an improvement of the hydraulic conditions (decrease of the thickness of the boundary layer). However above a certain value, in our case 6 mL/min ($HRT < 1.6$ min), the hydraulic retention time of water was not sufficient to obtain high concentration due to the too important flow rate which leads to a higher extract volume and subsequently the dilution of the extract.

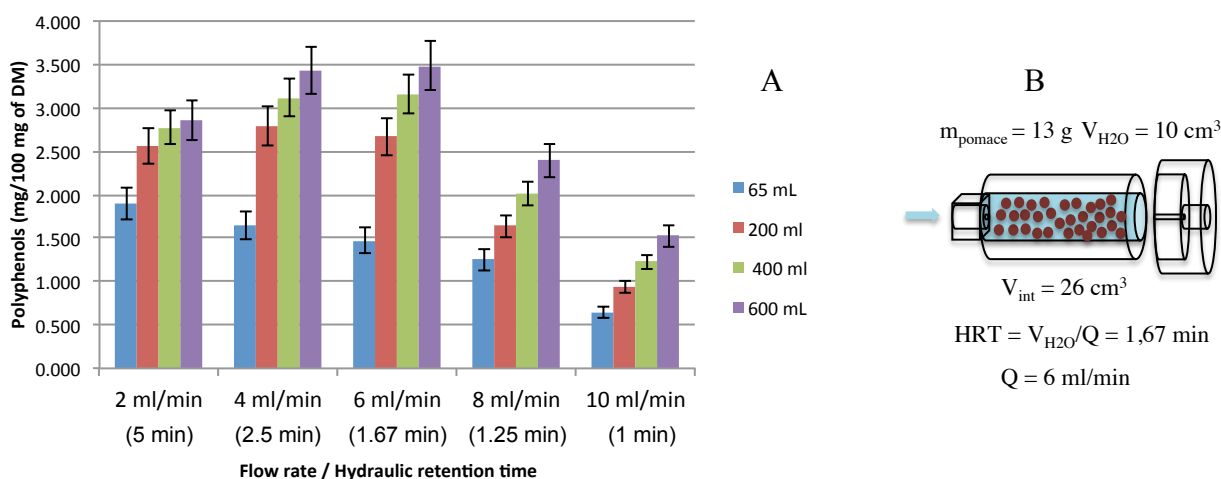


Figure 3. (A) Influence of the hydraulic retention time on the polyphenol concentration for different extraction volumes at 150°C temperature, 50 bars hydraulic retention time calculation.

In addition, the hydraulic retention time (HRT) in the extraction cell is shorter at a higher flow rate, therefore minimizing the effect of the degradation. The volume extracted or the amount of water that passes through the reactor is an important factor. Extracting 65 to 200 ml using a flow rate of 6 mL/min for example (Fig. 3), lead to a 45.1% increase in the extraction yield, from 1.46 to 2.66 mg/100mg DM this may be due to that the original pomace needs to be impregnated in water to initiate extraction. However, extracting 200 to 400 mL using the same flow rate, leads to only a 15.8 % increase in the extraction yield. This signifies that this fraction of total polyphenols is more tightly attached to the skin, and removal of total polyphenols from the system requires desorption/diffusion of the compound from the matrix, which limits the extraction rate. An extraction volume larger than 400 ml was shown to be less significant. In our case an optimum extraction was achieved at 6 mL/min, (HRT time = 1.67 min), which efficiently extracted polyphenols and decreased the risk of degradation. These results were similar to work presented in variation of flow rates on different phenolic compounds. Where they have optimized the flow rate of the extraction solvent, degradation effects were minimized, by selecting a flow rate of 4mL/min and complete extraction was achieved within 60 min (Liu et al. 2014).

2.3.3.5. Temperature influence on the extract composition

A conventional HPLC method was set up, with the aim to separate and identify

the possible antioxidants present in the extracts. The predominant phenolic compound derivative anthocyanins found were Malvidin-3-o-glucoside (0.34mg/100mg), Peonide-3-o-glucoside (0.12 mg/100mg), cyanidin-3-o-glucoside (0.0021 mg/100mg), Petunidin 3-o-glucoside (0.047 mg/100mg) and Delphindin-3-o-glucoside (0.015mg/100mg) (Figure 4). The optimum temperature of extraction depended on the molecules; with a highest cumulative yield of 0.47 mg/100mg was at 125°C. A glycoside anthocyanin is an organic compound that has a monosaccharide sugar portion. The presence of a sugar molecule intends to make them more soluble in polar substances, and they are subsequently extracted well at a lower temperature than less-polar compounds as flavonoid and phenolic acids (Monrad et al. 2010). Therefore, the glucoside flavonoids tended to be extracted well at a lower temperature as compared to the aglycone flavonoids by temperature-dependent dielectric constant (ϵ) of water. Also anthocyanins including hydroxyl group or glucose can exhibit hydrogen bonding interactions with the water solvent due to their large charges. Therefore, anthocyanins glycosides were extracted well using subcritical water at relatively lower temperature (<150°C), whose high dielectric constant facilitates hydrogen-bonding interactions as compared to aglycones (Ko et al. 2014).

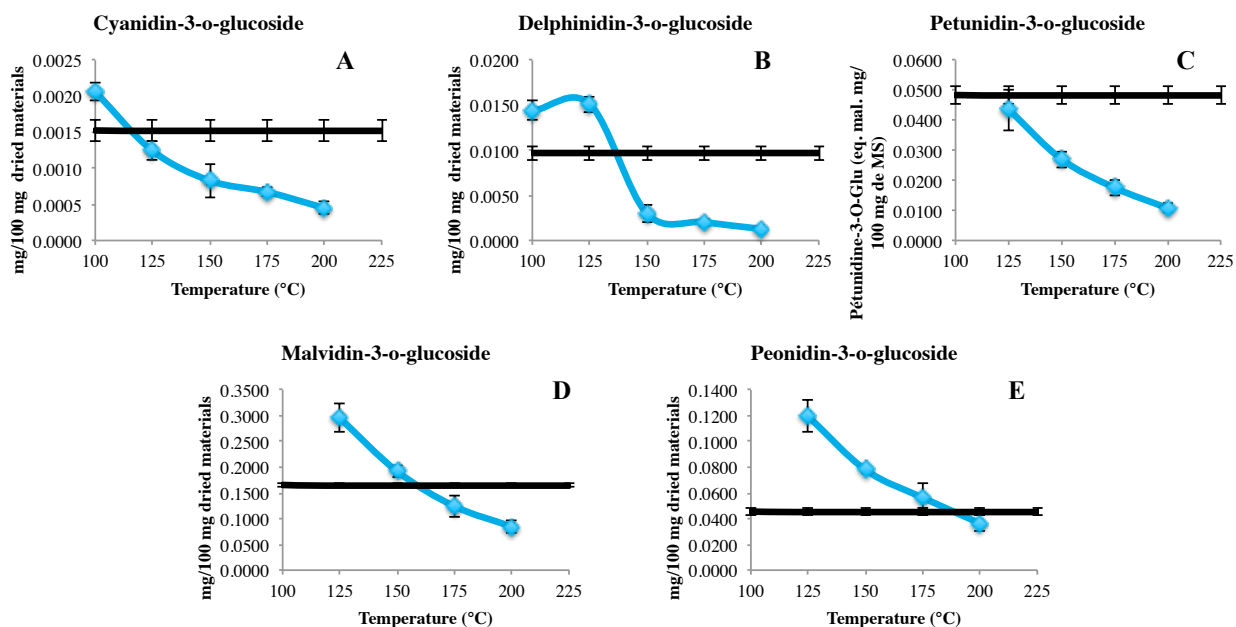


Figure 4. Effect of temperature on the SWE of Anthocyanins for extraction wet pomace; Cyanidin-3-o-glucoside (A); Delphindin-3-o-glucoside (B); Petunidin 3-o glucoside (C); Malvidin-3-o-glucoside (D), Peonide-3-o-glucoside (E). Experiments were conducted in

triplicate. Data points shown as the mean value \pm standard deviations.

Also, due to the fact that Malvidin-3-o-glucoside has a deoxy sugar molecular structure, its structure is unstable and easily degraded. Thus, it should be extracted at a low temperature using SWE (Khanal et al. 2010). The stability of anthocyanins when using the SWE at elevated temperatures varies due to differences in their melting points and molecular weights. The stability of different anthocyanins when using SWE varies. For example, Malvidin-3-glucoside is optimally extracted at 125°C while Cyanidine-3-glucoside is extracted at 100°C. The difference in temperature of extraction is due to the stronger intermolecular forces in more symmetrical anthocyanins having a higher melting point, since the melting points of covalent molecules depends on the identity of the functional group (Smith, 2008, chap. 3).

Optimum extraction conditions have also been determined for another important families of polyphenols such as flavonoids and gallic acid (Figure 5). Catechin (0.11 mg/100mg), followed by Gallic acid (0.0415 mg/100mg), Epicatechin (0.068mg/100mg), Procyanidin B1 (0.03mg/100mg), and Procyanidin C1 (0.008mg/100mg) are extracted at a higher temperature as compared to anthocyanins. The optimum temperature of extraction depended on the molecules; with a highest cumulative yield of 0.248mg/100mg was at 175°C. The results are strongly correlated to their stable structure (Ko et al. 2014) and the direct influence of the solvation. Since these compounds are stable at high temperature of subcritical water (Srinivas et al. 2010), it seemed that a degradation had proceeded above the optimum temperature as shown in figure 3. For example, according to Monrad et al. 2014, the maximum yields of (0.207 mg/100mg) similar extracted molecules were found at the extraction temperature of 140°C.

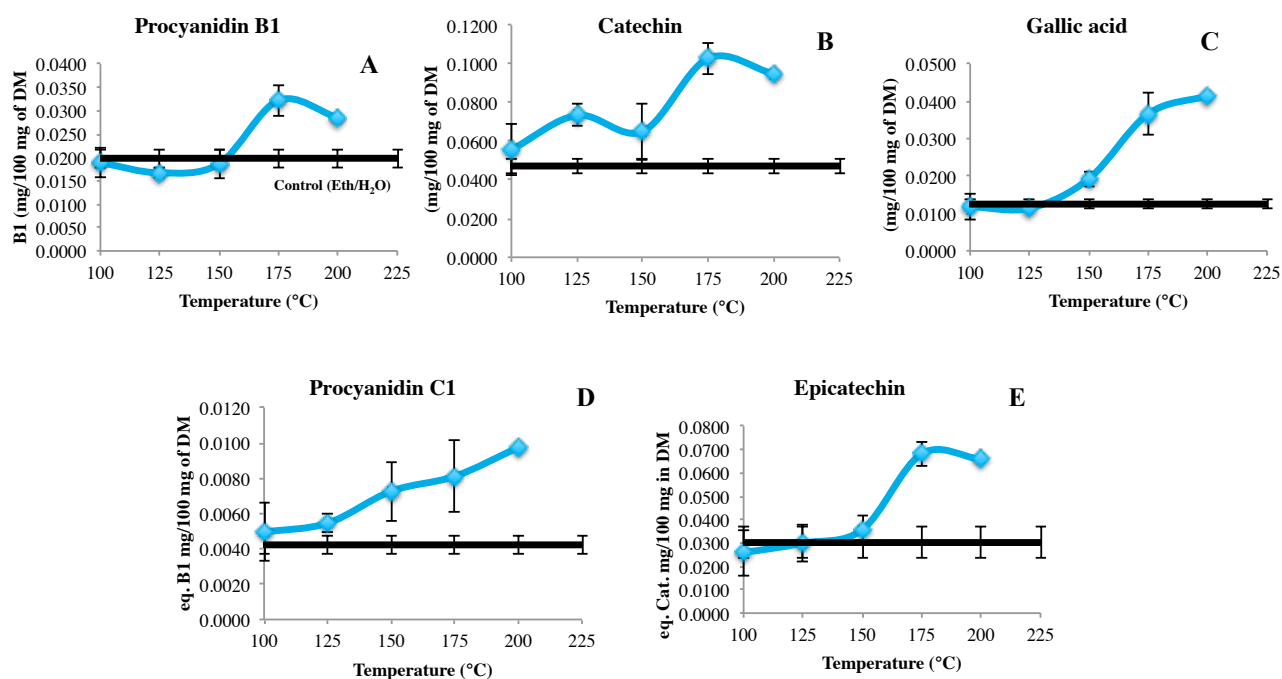


Figure 5. Effect of temperature on the SWE of Flavonol in wet pomace; Procyanidin B1 (A); Catechin (B); Gallic acid (C); Procyanidin C1 (D); and Epicatechin (E). Experiments were conducted in triplicate. Data points shown as the mean value \pm standard deviations.

2.3.3.6. Antioxidant Capacity of the Extracts

All temperatures of extraction above 125°C yielded an extract that showed antioxidant activity. As shown in Figure 6, the temperature of extraction had a strong influence on the antioxidant capacity. Antioxidant capacity increased from 12300 to 28000 $\mu\text{M}/100\text{mg}$ between 100°C to 200°C. As expected, the antioxidant capacity of extracts obtained at 200°C was 2 folds higher when compared to the data obtained at 175°C. The previous observation has also been confirmed by other studies (Srinivas et al. 2011). The best condition considering only antioxidant capacity was found using the highest achievable temperature (200°C). However, the high polyphenol concentration was not necessarily extracted at that high temperature as can be seen in figure 3 and 4.

The optimum extracted amount of polyphenols at these conditions was 0.78 mg/100mg dry grape by-product at 150°C.

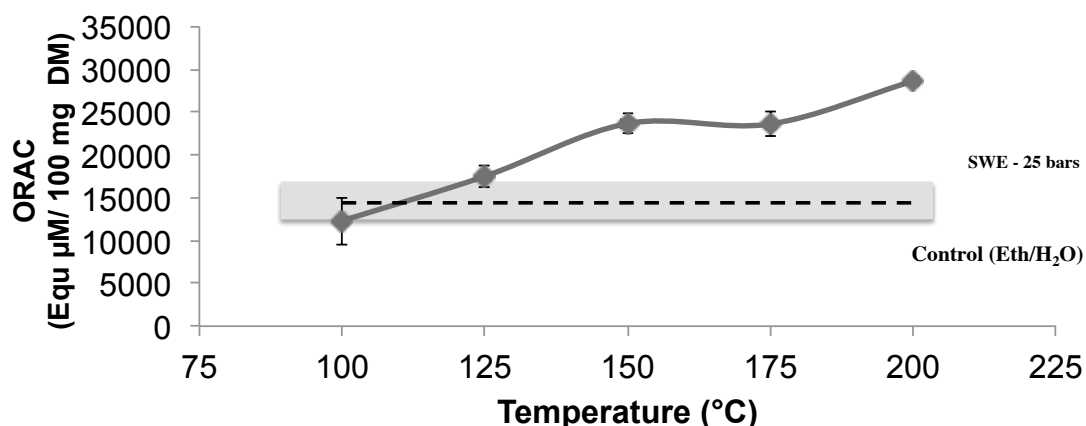


Figure 6. Antioxidant activity measured by the Oxygen Radical Absorbance Capacity (ORAC) assay as a function of SWE process temperature at 25 bars and compared with the hydro-alcoholic maceration technique

At high temperatures between 175 and 200°C the antioxidant activity observed cannot only be explained by the presence of polyphenols. The involvement of Maillard and caramelization reactions have been implicated in the increase of antioxidant activity due to the formation of new compounds in extracts obtained at high temperature from grape pomace by SWE (Monrad et al. 2014; Plaza & Turner 2015). Maillard reactions occur between the amino group of an amino acid and the carbonyl group of an open chain reducing sugar. Therefore, to measure a reduction of sugars as a consequence of Maillard and caramelization reactions Hydroxymethylfurfural (HMF) molecules were determined by HPLC.

2.3.3.7. Maillard and Caramelization Reactions

Furthermore, the formation of neoantioxidants derived from Maillard reaction and caramelization (such as melanoidins) at these extractions conditions, in our knowledge was not previously demonstrated in grape by-products extraction. We demonstrate for the first time an increase in furfural concentrations in SWE extraction from grape pomace, where furfural concentrations increase dramatically between 175 °C and 200°C. We hypothesize that these newly formed compounds could be responsible for the high antioxidant capacity in the extracts obtained at the highest temperatures. Previous studies have shown the formation of HMF and furfural in the apple by-product extracts as determined by HPLC-DAD (Liu et al. 2014).

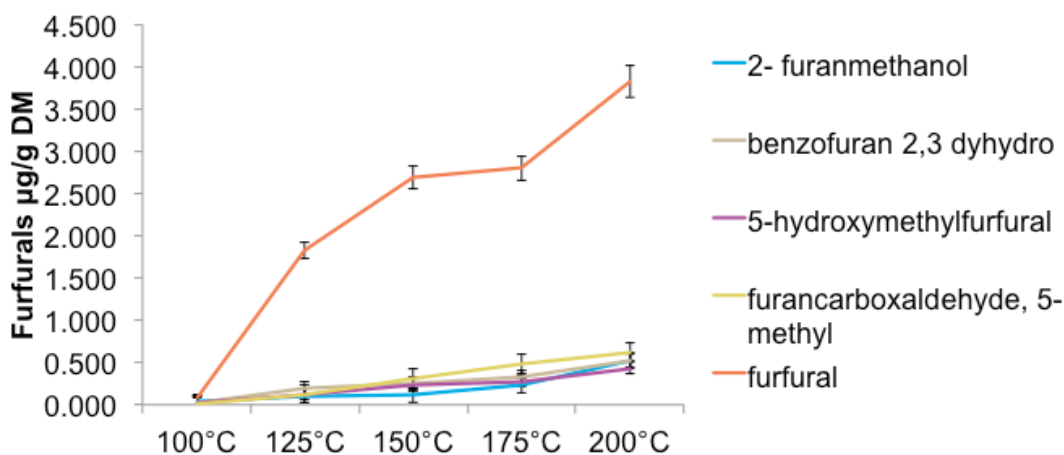


Figure 7. Furfural concentration as a function of SWE temperatures ($\mu\text{g/g}$ of DM) with respect to the temperature at 25 bars

Both compounds were detected at temperatures of 125 °C and substantially increased along with higher temperatures. The formation of brown color (melanoidins), HMF, and furfural suggested that Maillard and caramelization reactions appeared in the final stage between 175-200°C. HMF and furfural are highly reactive compounds that take part in further reactions leading to the formation of melanoidins and other “brown” polymers and aromatic substances (Yilmaz & Toledo 2005).

2.3.4. Conclusions

The advantage of the continuous flow system is that the extraction is accomplished in a dynamic flow. Fresh solvent during the SWE process enhances the mass transfer of the target compounds, and degradation is minimized to achieve higher extraction yield compared to conventional ethanol extraction in a much shorter extraction time. Depending on the yield of extracted compounds, the flow rate was adjusted accordingly to influence the hydraulic retention time in order to the optimal yield. In contrast to the existing work, all the data are available for the extrapolation at another scale. Furthermore, the sample matrix proved to have a protective effect on the thermolabile compounds.

On the other hand the extraction of anthocyanins and flavon-3-ols were directly affected by the extraction condition. The overall results of this study indicate that the efficiency of the SWE depends on the presence of side chains and glucose in the

molecules. The SWE at higher temperature increases the thermal agitation and hence decreases the hydrogen bonding strength, which leads to more efficient extraction of nonpolar hydrophobic compounds than polar hydrophilic compounds.

Results obtained also demonstrate the efficiency of using just water at 150°C extraction temperature, giving the highest content of polyphenols, 0.67 mg/100mg, from dry grape pomace byproducts. However, this extraction condition is not optimal for all polyphenols studied. For instance, the best SWE condition for anthocyanins, is 125° C, giving a predicted anthocyanins content of 0.47 mg/100mg grape byproduct. And the best SWE extraction parameters for Flavon-3-ols and gallic acid are at 175°C with a yield of 0.248 mg/100mg.

Furthermore, the results from this work concerning the concentration of browning and furfural compounds, confirm the occurrence of Maillard and caramelization reactions in the extracts obtained by SWE of grape byproducts at temperatures of 175°C and above. These neoformed compounds present antioxidant capacity, therefore, being able to positively influence the overall antioxidant capacity obtained from grape byproducts under these particular extraction conditions. Therefore the antioxidant capacity and at the same time minimized the formation of undesirable compounds from Maillard and caramelization reactions, giving an optimum SWE conditions of 125 °C.

Finally, our results indicate significant information for the optimization for a rapid extraction different phenolics from grape pomace using SWE, thus avoiding the need for expensive and organic solvents.

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CHAPTER CONCLUSION

Subcritical water extraction is a relatively recent extraction process (early 1995). With considerable success for different analytical applications in developed countries.

The first part of our work screened the phenolic compositions of by-products obtained after vinification of different grape varieties, in order to assess their potential content in high added value compounds after the subcritical water extraction. The comparison of several wine industry by-products with their respective grapes provided evidence that pomaces are very rich sources of antioxidants, flavan-3-ols and anthocyanins. The quantitative and qualitative distribution of polyphenols in grape pomaces showed significant differences through varieties and vintages.

This study of grape pomace characterizations, from Dunkelfelder, Merlot, Cabernet Franc and Chardonnay were evidenced as Dunkelfelder presented the most interesting fractions because of their higher polyphenol contents in term of flavan-3-ols and anthocyanins. These extracts also exerted the highest antioxidant capacities through out four different tests. As a result, these varieties were chosen for further extraction optimization of subcritical water.

The second part of the work was to evaluate the impact of different operating parameters on the overall efficiency of extraction and draw a set of conditions that describe the operative "optimal" in the case of the extraction of natural substances such as grape pomace.

The influence of extraction parameters such as temperature, hydraulic retention time, and pressure were evaluated. The results showed that the temperature and hydraulic retention time of extraction were critical parameters to consider in the process of subcritical water.

The sub-critical water green technology has several advantages over the conventional solvent, in particular the reduction of the extraction time and the amount of required organic solvents. Thus, from this standpoint sub-critical water turns out to be a very good alternative to solvent extraction for natural substances from grape pomace.

3. CHAPTER 3: Fractionation of different phenolic classes from grape pomace extracts by membrane processes

3.1.INTRODUCTION

In the previous chapters we have shown that subcritical water extracts produced are rich in several families of molecules. An essential purification step of target compounds prior to industrial use is indispensable. Coupling the subcritical water with membrane processes offers an innovative solution for the purification of these extracts.

Membrane technologies have been successfully used over the past 30 years in the agro food industries; for example dairy products, fruit juices, wine. Their advantages reside in terms of the absence of phase transition, low energy requirements, high separation efficiency, high productivity, and easy scale-up compared with other conventional methodologies.

Theoretically the separation of target molecules in the extracts using pressure driven membrane technologies seems simple, because it is based on a sieving mechanism and their molecular weight (MW). Nevertheless the membrane molecular weight cutoff (MWCO) is not an absolute barrier. Membrane interactions with the target molecules play an important impact of the MWCO, such as hydrophobicity of the membrane surface and the solubility of the solutes. Another issue is that extract is formed of large and small molecules in the form of clusters and colloids. For instance, polyphenols bind proteins in our extract. This means that small molecules can be recovered in the concentrate, following the structural characteristics of the macromolecules.

Considering these above points, the current chapter explores membrane separation mechanisms and the recovery of target compounds derived from different grape pomace subcritical water extract. The chapter is organized into two parts found in the form of two publications. The first aims to separate the macromolecules from the compounds of interest with ultrafiltration. While the second aims at separating the different families of polyphenols in order to give a higher added value to the extract with nanofiltration.

3.2. SELECTING ULTRAFILTRATION MEMBRANES TO FRACTIONING HIGH ADDED VALUE COMPOUNDS FROM GRAPE POMACE EXTRACTS

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Abstract

The purpose of the current study is to investigate the possibility of utilizing ultrafiltration (UF) for the fractionation of phenolic compounds from subcritical water grape pomace extract (WS) and their separation from other co-extracted components. Thereby, the extract was assayed in a cross-flow apparatus against eleven membranes with molecular weight ranging from 100 to 2 kDa. Monitoring of the process was carried out by determining performance parameters and retention coefficients of proteins, polysaccharides, sugars, phenolic and anthocyanins classes. Results indicated that solutes retention was affected mainly by severe fouling phenomena due to polar solutes adsorption on membrane surface instead of size exclusion. Furthermore, polysulfone membranes were not able to fractionate phenolic classes except for the separation obtained between polymeric and monomeric proanthocyanidins. Membranes starting 20 kDa retained high percentages (i.e. >60%) of polysaccharides and proteins.

Key words: Ultrafiltration, Grape pomace, Proteins, Pectins, Phenolic compounds.

Abbreviations

AUC	Area under the curve
CCD	charge-coupled device
C_{if}	concentrations of compound in feed
C_{ip}	concentrations of compound in permeate
DAD	Diode-Array Detector
DW	Dry weight
HPLC	High performance liquid chromatography
J_p	permeate flux during extract filtration
J_w	pure water flux
MF	Microfiltration
MS	Mass spectrometer
MW	Molecular Weight
MWCO	Molecular weight cut off
NF	Nanofiltration
ORAC	
P	pressure
PEG	Poly Ethylene Glycol
R_a	Fouling resistance
R_i	initial resistance
R_m	membrane resistance
RO	Reverse Osmosis
R_t	total resistance
SE	Standard error
TMP	TransMembrane Pressure
UF	Ultrafiltration
UPLC	Ultra High pressure Liquid Chromatography
UV	Ultra violet
V_0	initial feed volume
V_R	retention volume
VRF	volume reduction factor
μ_p	dynamic viscosity of the extract
μ_w	dynamic viscosity of water

3.2.1. Introduction

Significant amount of grape byproducts produced worldwide as derivative of the wine industry. These byproducts are important source antioxidant molecules such as polyphenols ranging from 2.5 to 7.8 g 100 g⁻¹ dry weight (DW) (Spigno and De Faveri 2007). Polyphenols are divided into two groups, major C₆-C₃-C₆ flavonoids in grapes include conjugates of flavonols, quercetin and myricetin; flavan-3-ols (+)-catechin and (-)-epicatechin; and malvidin-3-O-glucoside and other anthocyanins. Non-flavonoids include C₆-C₁ hydroxy-benzoic acids, and gallic acid, C₆-C₃ hydroxycinnamates caffeic, caftaric, and p-coumaric acids; and C₆-C₃-C₆ stilbenes trans-resveratrol, cis-resveratrol, and trans-resveratrol glucoside (Waterhouse 2002). Thus, grape byproducts contain a large amount of different phenolic compounds, which are important for their physiological roles in plants, and are regarded as significant components of human nutrition. The latter idea is backed by numerous studies reporting high positive antioxidant, cardioprotective, neuroprotective or anticancer effects (for example by Craft et al., 2012; Kähkönen and Heinonen, 2003; Quideau et al., 2011; Stintzing et al., 2002).

After being extracted fractionation the subsequent purification and concentration of polyphenols are a matter of health and, consequently, of economic interest to the food industry. Membrane filtration is a physicochemical separation technique, separating compounds based mainly on their dimension. The pressure driven membrane processes are microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO). The advantages of membrane processes include the low energy consumption compared to other separation methods that involve phase change, high selectivity, no organic solvent usage and low temperatures (crucial when handling thermally unstable compounds like phenolic compounds) (Cissé et al. 2011). While on the hand membrane processes can be saddled with major problems of fouling of the membranes while processing some type of feed streams. This fouling, especially if it is difficult to remove, can greatly restrict the permeation rate through the membranes, modify the retention rate and make them essentially unsuitable for such applications.

Ultrafiltration (UF) have readily been utilized in the food industry, for the clarification of various juices (Rai et al. 2006; Cassano, Donato, and Drioli 2007; Echavarría et al. 2012) and for fractionation (Butylina, Luque, and Nyström 2006; Catarino et al. 2008). In the food and dairy industries, UF is largely used to treat effluents (Kemal Erdem 2005; Cissé et al. 2011; Díaz-Reinoso et al. 2010; Fernández et al. 2010). Applications for the fruit juice and wine industries have recently begun growing in importance (Kalbasi and Cisneros-Zevallos 2007; Versari et al. 2003; Cassano, Donato, and Drioli 2007; Cassano et al. 2007; Echavarría et al. 2012; Rai et al. 2006).

In contrast, the use of UF for plant extracts has been little reported (Tsibranska, Peev, and Tylkowski 2011; Galanakis, Markouli, and Gekas 2013; Díaz-Reinoso et al. 2009). To the best of our knowledge, the membrane studies concerning the separation of phenolic components in different classes are rather scarce. Santamaría et al. (2002) assayed different tubular polymeric membrane sequences in order to fractionate phenolics (gallic acid, catechin, gallates, etc.) recovered from defatted milled grape seeds, using acetone–water mixtures on the basis of molecular weight (MW). While Díaz-Reinoso et al. processed aqueous extracts from pressed distilled grape pomace by nanofiltration (NF) membranes to concentrate. The five tested ceramic nanofiltration membranes were suitable for concentration purposes. The phenolic content in retentates was increased by factors of 3–6 respect to the feed. While Galanakis et al. (2013) suggested that the separation of phenolic compounds recovered from winery sludge is possible using three ultrafiltration organic membranes (100kDa, 20 kDa, 1 kDa). Polysulfone membranes were able to separate phenolic compounds from pectin fractions, but they could not fractionate different phenolic classes and sugars (reducing or not), as they were retained even in rather high percentages at 100 kDa. On the other hand, the application of a non-polar fluoropolymer membrane in the border of UF and nanofiltration (1 kDa), provided a successful methodology to separate different phenolic classes. Recently Zagklis and Paraskeva (2015) proposed purification method for the separation of grape marc phenolic compounds coupling ultrafiltration (100 kDa) and nanofiltration (480 Da) with resin adsorption/desorption. This step apart from the removal of carbohydrates, the concentration of the phenolic compounds was increased. That has lead to an increased concentration of the targeted compounds, as the volume of the final product was only 0.04% of the initial volume.

This study aims to determine the feasibility of using UF to separate and concentrate polyphenolic compounds without utilizing any organic solvent. We have previously optimized the extraction of fermented grape pomace using subcritical water extraction (Yammine, Ghidossi, and Mietton-peuchot 2015). Subsequently to realize the purification of the crude extract, several organic membranes having differential molecular weight cut off 100 kDa to 2 kDa were tested. The performance of the process in terms of retention, permeate flux and transmembrane pressures (TMP) in a UF apparatus was evaluated. Conditions consisted of constant temperature and circulation flow rate. Retention percentages of phenolic acids, stilbenes, anthocyanins, monomeric flavan-3-ols and polymeric flavan-3-ols were compared with different molecular weight cutoffs and the different types of membranes, with the determination of the fouling agents such as pectins and polysaccharides in respect to our application. pomace.

3.2.2. Materials and methods

3.2.2.1. Subcritical water extraction

In the extraction system, a HPLC pump (Shimadzu LD-AC10) was used for deionized water delivery, pressurization and controlling the pressure of system. A pressure transducer (Davidson, Druck) and thermocouple (Caveland Electric) were installed in the custom-made high-pressure vessel to monitor both pressure and temperature of system. Extract was collected in an inerted vessel after passing in an ice bath.

In each run red Dunkelfelder pomace supplied by the university of Changins (70 g) was loaded into the high-pressure vessel, which can contain 325 cm³ of material. The vessel was placed in an oven at a predetermined temperature of 150°C. The outlet valve of extraction vessel was then closed and the system was pressurized to a desired pressure of 25 bars at a constant flow rate. The water flow rate was adjusted at 20 mL/min using a metering valve on the HPLC pump. After 3L of extraction, the solution collected in an inerted sampling vessel and pomace were then stored at 4°C for further analysis and membrane separation.

3.2.2.2. Experimental analysis and membranes

UF experiments were performed in a pilot unit (Fig.1), equipped with a Sepa® CF II Membrane Cell System (GE Osmonics, Minnetonka, MN, USA) featuring an effective membrane area of 0.0153 m^2 . The temperature was maintained at $20 \pm 0.5^\circ\text{C}$, by a thermal bath. Permeate flux was determined at a 2 m s^{-1} of crossflow velocity, by weighing the amount of permeate with a balance connected to a computer. Weight and pressure values were recorded every 1 s by an electronic system. The eleven commercial UF flat-sheet membranes were acquired from the manufacturers. Table 1 lists their characteristics. Only new membranes were used throughout the experiment.

3.2.2.3. Membrane performance

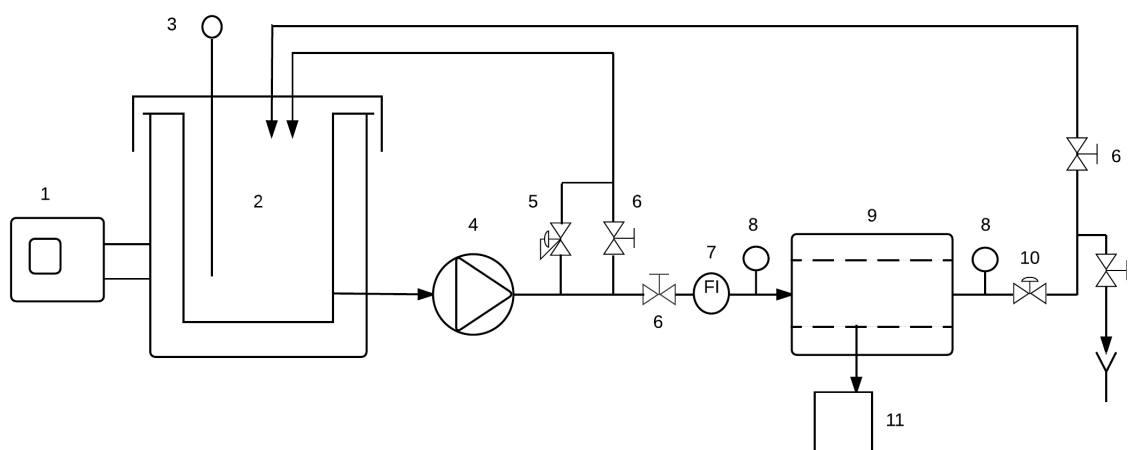


Figure 1. Flow sheet of the experimental apparatus: 1. thermal bath, 2. feed tank, 3. temperature probe, 4. high-pressure pump, 5. security valve, 6. valves, 8. pressure probes, 9. membrane cell system, 11. pressure control valve, 12. balance

The membranes were preconditioned with deionized water for 60 min at 20°C using transmembrane pressure 5.10^5 Pa and 2 m.s^{-1} of crossflow velocity. Water permeability was determined for four pressures between 10^5 Pa and 5.10^5 Pa during the last 20 min of preconditioning, using the slope of the plot of permeate weight recovered against time. Immediately after preconditioning, a trial with grape pomace extracts was filtered. The filtration experiments were conducted at the natural pH of the extract (3.7) in tangential crossflow mode, with the feed stream flowing tangential to the membrane surface. The operating method was batch concentration mode: that is, the retentate or concentrate stream was flowed back to the feed tank, while the

permeate stream was collected separately and not recirculated to the storage vessel. The initial volume of extract treated was 2L in all cases, and the flow rate was fixed at $v=2 \text{ m s}^{-1}$, corresponding to a flow rate of 29.70 mL s^{-1} . The temperature was set at a constant 20°C . The transmembrane pressures tested for each membrane were 1, 2, 3, 4 and $5 \cdot 10^5 \text{ Pa}$.

Table 1 Characteristics of the tested ultrafiltration (UF) membranes (manufacturer data)

Designation	Manufacturer	Polymer Type	Nominal Molecular Weight Cutoff (MWCO)	% Rejection	Maximum Pressure (10^5 Pa)	Recommended pH range	Maximum Temp. ($^\circ\text{C}$)
FS40PP	Alfa laval	Fluoro polymer	100000		10	1-11	60
GR40PP	Alfa laval	Polysulfone	100000		10	1-13	75
MW	GE Osmoncis	Polyacrylonitrile	50 000	50K-Protein	7	1-11	50
GR51PP	Alfa laval	Polysulfone	50000		10	1-13	75
GR61PP	Alfa laval	Polysulfone	20000		10	1-13	75
PW	GE Osmonics	Polyethersulfone	10000	10K-Dextran	13	1-11	50
GR81PP	Alfa laval	Polyethersulphone	10000		10	1-13	75
PT	GE Osmonics	Polyethersulfone	5000	5K-Dextran	3,4	1-11	50
GK	GE Osmonics	Thin Film*	3500	3K-PEG	5,2	1-11	50
GH	GE Osmonics	Thin Film*	2000	2K-PEG	10	1-11	50
GR95PP	Alfa laval	Polyethersulphone	2000		10	1-11	65

The duration of each experiment varied according to the desired value of the volume reduction factor (VRF) to be reached. This parameter is defined by:

$$VRF = \frac{V_0}{V_R}$$

where V_0 is the initial feed volume and V_R is the retention volume, that is the extract volume remaining in the storage vessel ($V_R = V_0 - V_P$).

For the MWCO membranes ranging from 50 kDa to 100 kDa the permeate flux was expressed at a $VRF = 10$, which implies 1.8 L of permeate was obtained. While for lower MWCO membranes of 20 to 2kDa, due to lower filtration fluxes, permeate flux was expressed at a $VRF = 2$, which implies that a retentate 1L was obtained.

The samples of raw material and permeate collected were immediately frozen and kept at -20°C until analyzed.

To measure membrane selectivity for a solute, the observed retention was calculated, as shown in the following expression:

$$R_i = \left(1 - \frac{C_{ip}}{C_{if}} \right) \cdot 100$$

where R_i is the observed retention of compound i (%), and C_{ip} and C_{if} are the concentrations of compound in permeate and feed (mg L^{-1}), respectively.

3.2.2.4. Hydraulic resistance, using Darcy's law

According to Darcy's law, total hydraulic resistance (R_t) during UF of grape pomace extract was calculated as follows:

$$R_t = \frac{\Delta P}{J_p \mu_p}$$

Where J_p is the permeate flux, ΔP is the transmembrane pressure applied and μ_p is the dynamic viscosity of the product.

To assess resistance due to fouling and/or polarization, the intrinsic membrane resistance (R_m) was measured during filtration of pure water, using clean membranes (hydraulic permeability). Intrinsic membrane resistance was calculated as follows:

$$R_m = \frac{\Delta P}{J_w \mu_w}$$

Where J_w ($\text{m}^3 \text{s}^{-1} \text{m}^{-2}$) is the pure water flux, ΔP (Pa) is the transmembrane pressure applied and μ_w (Pa s^{-1}) is the dynamic viscosity of water.

Then, resistance created by fouling and/or concentration polarization (R_a) during grape pomace extract filtration was calculated as the difference between total resistance (R_t) obtained during the filtration experiment and membrane resistance (R_m):

$$R_a = R_t - R_m$$

3.2.2.5. Contact angle

Surface hydrophobicity and wettability of the membranes were evaluated by contact angle measurements using a contact angle meter (Digidrop, GBX, France), equipped with a diffuse light source, a CCD camera (25 frames per second) and a closed chamber with controlled temperature (20 ± 1 °C). A drop of distilled water (≈ 5 μ L) was deposited on the film surface and a magnified image of the drop profile was conveyed to a computer via a CDD camera thanks to a system of video acquisition. Changes in the drop shape over time were recorded as a sequence of images that was then analyzed frame by frame with the GBX software (Windrop, GBX, France). For each membrane, the hydrophobicity was deduced from the initial contact angle values (averaged value of contact angles measured on both sides of the drop). The evolution of the drop form as a function of time allowed evaluating the contribution of absorption phenomenon involved in membrane wettability. At least five measurements per membrane were performed.

3.2.2.6. Chemical analysis

3.2.2.6.1. pH, Total sugars, Polysaccharides

pH values of feed samples were measured with a digital pH-meter (Thermo Scientific™ Orion™ Star A324)

The total sugar content was determined by the anthrone method (Trevelyan et al., 1952), and expressed as glucose equivalents (GE).

Total polysaccharides were determined using the modified Usseglio Tomasset method based on the precipitation of the polysaccharides with ethanol.

3.2.2.6.2. Proteins

Protein content was determined by EZQ[®] protein quantitation kit (Invitrogen) following the manufacturer's instructions. The calibration curve was built using serial dilution from 0 to 250 mg/L of thaumatin from *Thaumatococcus daniellii* (SigmaAldrich, France). Fluorescence measurements were taken using excitation/emission settings of 485/590 nm with a FLUOstar Omega microplate

reader (BMG LABTECH, France).

3.2.2.6.3. Total polyphenols content

The total phenolic content was spectrophotometrically measured according to a modified Folin Ciocalteu method to be applied in 96-well microplates. Stock solutions (10 mg/mL) of the grape pomace extracts were prepared in EtOH/H₂O (25:75, v/v), and a microplate spectrophotometer (MultiSkan Spectrum, Thermo Scientific) was used for the incubation and measurement. Briefly, each well was filled with 184 µL of distilled water and 24 µL of the sample solution, followed by 12 µL of the Folin Ciocalteu reagent and 30 µL of 20% (w/v) Na₂CO₃ solution. Prior to the measurement of the absorbance at 765 nm, the mixture was incubated for 1h under dark conditions at 25°C. Gallic acid was used as a standard for calibration. Results, expressed as milligrams of gallic acid per 100 g of grape pomace sample (on a dry matter basis, dm), were a mean of six determinations.

3.2.2.6.4. Antioxidant activity – ORAC

The oxygen radical absorbance capacity analysis was applied by using 96-well fluorescence microplates. The reaction was carried out in phosphate buffer (75 mM, pH 7.4). In this order, 30 µL of the pomace extract solution, 180 µL of fluorescein (117 nM final concentration), and 90 µL of AAPH (40 mM) were added to each well. The mixture was shaken and allowed to stand for 1.5 h at 37 °C. Fluorescence was recorded every minute during this period at excitation and emission wavelengths of 485 and 530 nm, respectively. Simultaneously on the same microplate, a blank sample (phosphate buffer replaced the sample) and Trolox calibration solutions (1–40 µM) were also performed ($R^2 = 0.983$). The area under the curve (AUC) was calculated for each extract sample by integrating their relative fluorescence curves. By subtracting the AUC of the blank, the net AUC of the pomace extracts was calculated and correlated with Trolox concentrations.

3.2.2.6.5. Phenolic classes

UPLC analyses were performed in an Agilent 1260 apparatus consisting in an autosampler module, a degasser, a binary pump, a column heater/selector and a UV–

visible DAD detector (Agilent Technologies, USA). Chromatographic separation was performed on a Agilent C18 (2.1 mm x 100 mm, 1.8 μ m). Anthocyanins were eluted with a flow rate of 0.4 mL/min and a gradient of water/acetonitrile/formic acid (87/3/10; solvent A) and water/acetonitrile/formic acid (40/50/10; solvent B) according to the following gradient program (v/v): 0 min 94% A 6% B, 15 min 70% A 30% B, 30 min 50% A 50% B, 35 min 40% A 60% B, 40 min 35% A 65% B, 41 min 100% B isocratic for 5 min. Detection was performed at 518 nm. Other polyphenols were eluted with a flow rate of 0.4 mL/min and a gradient of water/formic acid (99.9/0.1; solvent A) and acetonitrile/formic acid (99.9/0.1; solvent B) according to the following gradient program (v/v): 0 min 93% A 7% B, 15 min 86% A 14% B, 40 min 65% A 35% B, 44 min 50% A 50% B, 54 min 30% A 70% B, 55 min 100% B isocratic for 5 min. Detection was performed at 280 nm for flavanols, 306 nm for stilbenes, 310 nm for coumaric acid derivatives and 370 nm for flavonols. Phenolic compounds were eluted with a flow rate of 1 mL/min and a gradient of water/formic acid (99.9/0.1; solvent A) and acetonitrile/formic acid (99.9/0.1; solvent B) according to the following gradient program (v/v): 0 min 70% A 30% B, 18 min 65% A 35% B, 46 min 20% A 80% B, 47 min 100% B isocratic for 5 min.

This HPLC was coupled to an Esquire 3000+ ion trap mass spectrometer using an ESI source from Bruker – Daltonics (USA). Nitrogen was used as drying gas. ESI-MS parameters: positive mode, nitrogen flow rate 10L/min, nebulizer pressure 0.275 10^5 Pa, drying gas temperature 365 °C, HV capillary -3700 V, end plate offset -500 V, capillary exit 111.2 V, skimmer 40 V and trap drive 45.9; negative mode, nitrogen flow rate 10 L/min, nebulizer pressure 0.172 10^5 Pa, drying gas temperature 350 °C, HV capillary +3400 V, end plate offset -500 V, capillary exit -115.3 V, skimmer -40 V and trap drive 42.9.

Identification of phenolic compounds was achieved using their UV/vis spectra, ion mass and MS/MS fragments using available standards. The results were expressed as mg of specific compound per L of extract, and the data represent the means of three replicates \pm SE.

3.2.3. Results and discussion

3.2.3.1. Grape subcritical extract composition

The main components and antioxidant capacity of the grape pomace extract

used as raw material for the UF experiments are presented in Table 2. The extract presents relatively low contents of proteins and high levels of acidity, polysaccharides, polyphenols and anthocyanins. These results agree with those obtained by (Valiente et al. 1995; Llobera and Canellas 2007). Polyphenols content is slightly higher than that for dried apple pomace (Schieber et al. 2003), almond hulls (Takeoka and Dao 2003) or artichoke byproducts (Llorach et al. 2002). The antioxidant capacity of grape pomace extracts, as assessed by the ORAC method, is relatively high ($198 \mu\text{mol Trolox g}^{-1}$), compared with roselle extract ($182 \text{ Trolox g}^{-1}$), grape juice ($88 \mu\text{mol Trolox g}^{-1}$), grape ($87 \mu\text{mol Trolox g}^{-1}$), blackberry ($72 \mu\text{mol Trolox g}^{-1}$), tomato juices ($67 \mu\text{mol Trolox g}^{-1}$), orange ($24 \mu\text{mol Trolox g}^{-1}$) or strawberry fruit pulp ($5.3 \mu\text{mol Trolox g}^{-1}$) (Besco et al. 2007; Lam et al. 2006; Xu, Yuan, and Chang 2007).

Table 2 Characteristics of the winery pomace extracts used as feed liquids.

Values represent mean \pm standard deviation (n = 6).

Molecules	Unit	Extract
Proteins	mg/L	$238,0 \pm 34,7$
Pectins	mg/L	$864,2 \pm 32,4$
Sugars	mg/L	$4096,4 \pm 216,9$
Total polyphenols	mg/L	$3309,1 \pm 366,4$
Phenolic acids	mg/L	$243,7 \pm 90,3$
Anthocyanins	mg/L	$153,6 \pm 12,4$
Stilbenes	mg/L	$21,3 \pm 2,3$
Monomeric Flavan-3-ols	mg/L	$76,3 \pm 3,8$
Polymeric Flavan-3-ols	mg/L	$153,0 \pm 8,5$
ORAC	$\mu\text{mol Trolox g}^{-1}$	$192,2 \pm 14,0$

3.2.3.2. Membrane performance

3.2.3.2.1.1. Water permeability determination

The hydraulic permeability L_p is an intrinsic feature of a non-fouled membrane that must be determined. Therefore and prior to the general filtration experiments of

the grape pomace extracts, several filtration experiments of pure water were carried out with each one of the filtration membranes selected, and with the aim to measure the evolution of the water permeate flux (J_w) with the variation of TMP. The results obtained showed the permeability that is the slope of J_w in function of TMP.

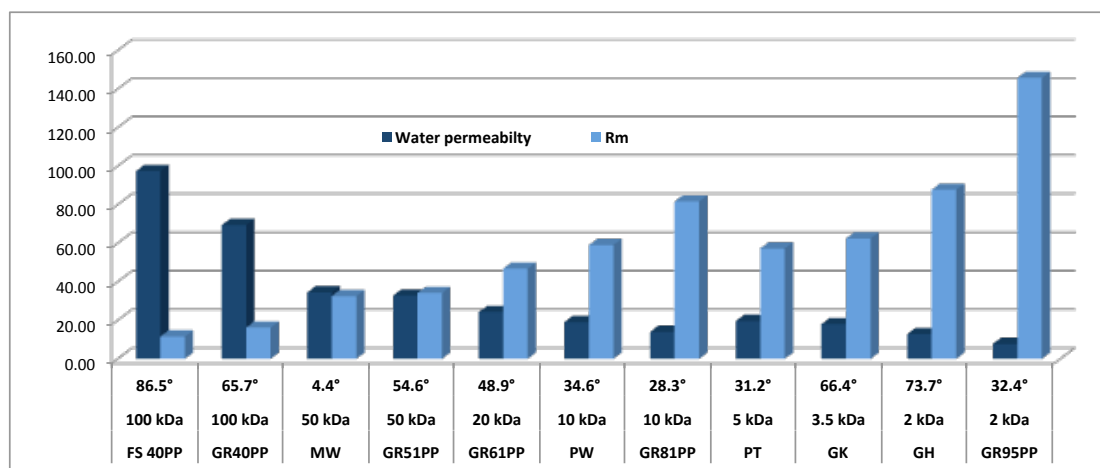


Figure 2. Hydraulic permeability ($Lm^{-2}h^{-1} \cdot 10^{-5}Pa$) and membrane resistance (Rm) $10 m^{-1}$ to water and contact angle for the ultrafiltration membranes ($T = 20\text{ }^{\circ}C$).

Thus, after regression analysis, the following values were deduced, with correlation coefficients higher than 0.99: ranging from 7.79 to 97.38 $L h^{-1} m^{-2} 10^5 Pa^{-1}$, for the GR95PP (2 kDa) to FS40PP (100kDa) membranes, respectively, at 20 °C. For the utilized UF membranes, the increase in the hydraulic permeability occurred as could be expected: among membranes of the same nature, larger pore sizes or MWCO lead to higher pure water flux.

Contact Angle was used for the differentiation between a hydrophilic and a hydrophobic membrane, which influences the membrane affinity to the molecules in the filtration extract. The mean value water contact angle and its standard deviation were calculated for each sample. The values obtained are shown in figure 2.

These values indicate that the MW membranes present a highly hydrophilic surface, FS40PP membranes present a hydrophobic surface, while remaining membranes are relatively hydrophilic.

3.2.3.2.1.2. Influence of operating conditions on the permeate flux

Figure 3 presents the permeate fluxes against transmembrane pressure (1 to 5.10^5 Pa), obtained for eleven UF membranes when filtering the grape pomace subcritical water extract. These show different trends: some UF membranes (MW, FS40PP, GR40PP and GR51PP) are characterized by a steady increase of permeate flux when transmembrane pressure increases (VRF=10). These membranes have a nominal MWCO equal to 50 kDa and 100kDa. For a same cutoff the permeability depends on the membrane and vary from 9 to $70 \text{ Lm}^{-2}\text{h}^{-1}.10^5\text{Pa}^{-1}$. Another group of membranes, with a nominal MWCO equal to or less than 20 kDa (GR95PP, GH, PT, GR81PP, PW, GR61PP and GK), behave in different manner, that is, when the transmembrane pressure is increased (VRF=2), at about 5.10^5 Pa, the permeate flux does not correlate with the increase in pressure. This behavior is well known for UF membranes due to the fouling (cake and/or concentration polarisation) at higher pressures (Bohonak and Zydney 2005; Kallioinen et al. 2007).

Membranes from GE Osmonics (i.e., GH, GK, PT and PW) presented high permeate fluxes, considering their relatively low nominal MWCOs, compared with the Alfa Laval membranes (GR95PP, GR81PP, GR61PP), made of polysulfone or polyethersulfone which have much similar nominal MWCOs. For example, a thin-film membrane (PW) from GE Osmonics, with a 10-kDa MWCO, presented a high permeation flux at 5.10^5 Pa ($11 \text{ Lh}^{-1}\text{m}^{-2}$), whereas a polyethersulfone Alfa Laval membrane with a 10kDa MWCO (GR81PP) presented one of the lowest of all permeation fluxes ($8 \text{ L h}^{-1} \text{ m}^{-2}$) under the same conditions. This result shows that, not only does transmembrane pressure affect permeate flux, but it also may be affected by the membrane material and structure, and the different interactions between solutes and membrane.

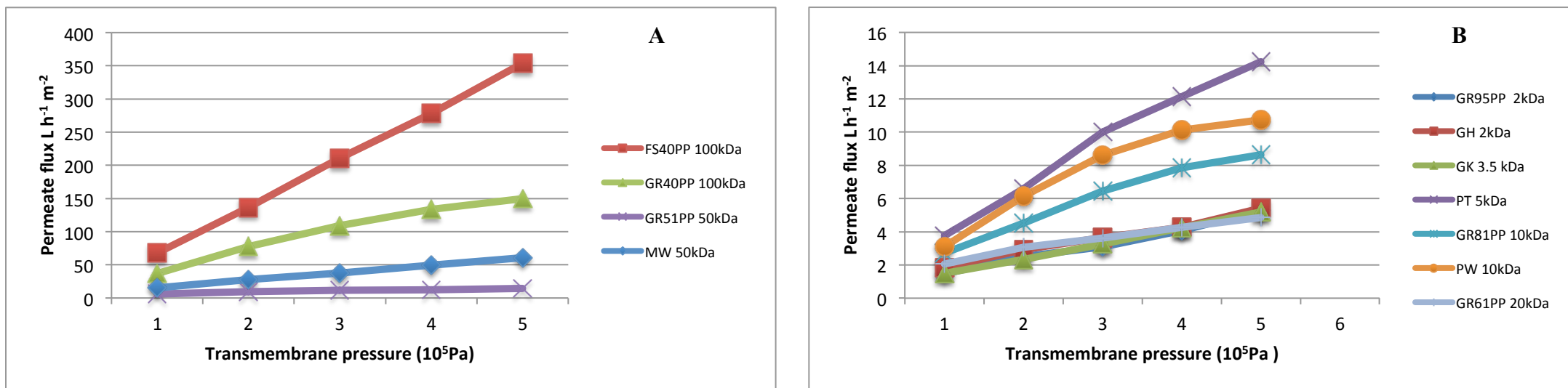


Figure3. Permeate flux during ultrafiltration of grape pomace extract with respect to transmembrane pressure for 11 different membranes; **A** (VRF=10); **B** (VRF=2).

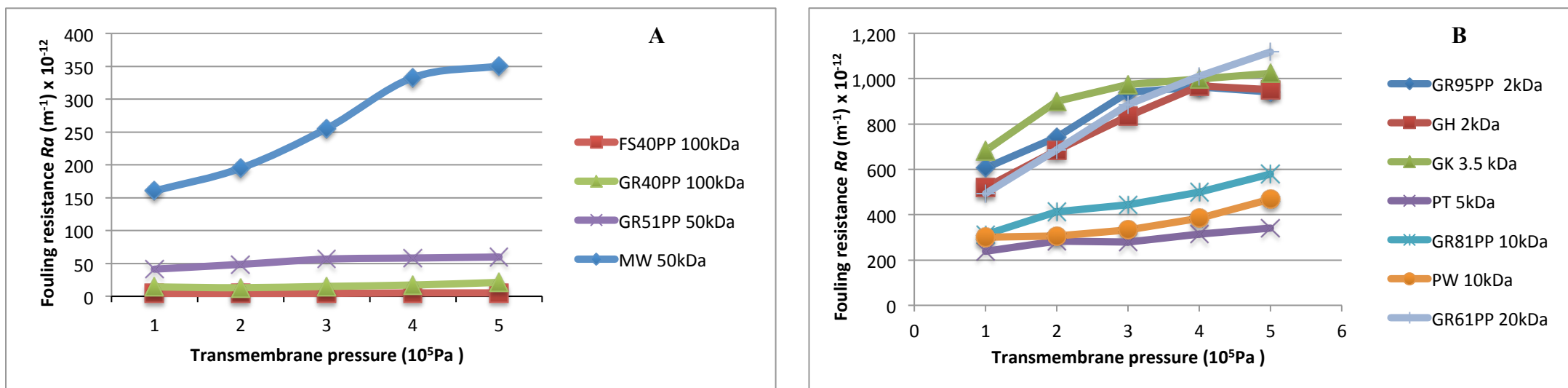


Figure 4. Evolution of fouling resistance for ultrafiltration membranes tested: GE Osmonics and Alfa Laval membranes. ; **A** (VRF=10); **B** (VRF=2).

The fouling resistance (R_f) calculated for all UF membranes at different transmembrane pressures tested is shown in Figure 4. Fouling resistance (R_f) includes intrinsic membrane fouling resistance, fouling layer resistance and resistance due to concentration polarization phenomena and/or gel layer formation (Bernat et al. 2009; Butylina, Luque, and Nyström 2006). As the transmembrane pressure increases, fouling resistance increases for all UF membranes tested. When transmembrane pressure varies from 1 to $5 \cdot 10^5$ Pa, R_f increases up to 2 times, depending on the membranes.

In most cases, R_f is higher for the Alfa Laval UF membranes than for the GE Osmonics membranes. This may explain the decrease of permeate flux observed previously with membranes GR95PP, GR81PP, GR61PP for transmembrane pressures for up to $5 \cdot 10^5$ Pa. Similar fluxes are observed with GR95PP (2kDa), GH (2kDa), GK (3,5kDa) and GR61PP (20kDa).

The resistance due to fouling and/or polarization was calculated, using Eq. (4); it represented at least 80% of total resistance in all cases. Nonetheless, for the higher MWCO membranes (>50 kDa), with an exception to the MW membrane, no correlations were observed between fouling resistance (R_f), the nominal MWCO and the contact angle. The structure of the membrane, nature of materials and the different interactions most likely explain the differences observed.

3.2.3.2.2. Retention of compounds

Table 3 presents the retention values of total protein, pectin, sugars, total polyphenols, and different families of polyphenols for UF membranes extracted at $3 \cdot 10^5$ Pa of pressure. The different compounds showed various retention percentages that were dependent on the MWCO and the type of membranes. Taking into consideration the two 50kDa membranes proteins showed a lesser retention with the MW membrane (21%) than the GR51PP (41%) . For all the others compounds, the retention is higher for the MW membrane. The contact angle shown in table 1 shows that Ultrafilic MW membrane is more hydrophilic than polysulfone. The more hydrophilic membrane leads to the higher retention of proteins and the lower retention of polysacharrides. The two membranes have similar water fluxes (figure 2). The

membrane fouling is more important for the MW membrane (figure 4 A); this may explain the higher retention of molecules, even retention of anthocyanins that have a molecular weight of 300Da. It would be probable that the macromolecules are responsible for membrane fouling since they interact with the membrane such MW that is highly hydrophylic. The membranes MWCO also influence retention of different families of molecules. Pectins values showed highest retention values on 20 kDa membranes (72%) compared to high MWCO membranes (\approx 40% with 50KDa). With the exception of the membrane MW whose behavior is different from other membranes, retention increases as the MWCO decreases for the three families of compounds: proteins, pectins and polymeric flavan-3-ols. Thus, the retention is a function of MWCO when it comes to large MW molecules compared to other molecules whose MW are smaller. For phenolic acids and anthocyanins, retention becomes dependent MWCO from 10 kDa and up 2KDa. Above 10 kDa, retentions are generally more important. It should be noted that the membranes of which the MWCO is larger than 10 kDa are mainly polysulfone as membrane MWCO less than or equal to 10 kDa are polyethersulfone or thin film. The physico-chemical interactions between the compounds and polysulfone may explain the observed retention. The stilbenes and monomeric flavan-3-ol are retained similarly on different ultrafiltration membranes tested. Sugars, although corresponding differences can be found they were not significant showed variable retention between 12 and 51 %. Total phenols showed retentions similar to those of sugars, especially in the case of more hydrophilic polysulfone membranes 54 %. These results are in accordance with Díaz-Reinoso (2009) et al. Galanakis (2013), Markouli, and Gekas, Zagklis and Paraskeva(2015), where both studies have shown that for these families of molecules separation, initial fouling and membrane resistance due to solutes polarity seem to play a more important role in the separation process examined compared to MWCO.

Table 3. Retention coefficients (%) obtained for several parameters of subcritical grape pomace extracts as a function of different ultrafiltration membranes.

Membrane	FS40PP 100kDa	GR40PP 100kDa	MW 50kDa	GR51PP 50kDa	GR61PP 20kDa	PW 10kDa	GR81PP 10kDa	PT 5kDa	GK 3.5kDa	GH 2kDa	GR95PP 2kDa
Proteins	64,7 ± 4	41,2 ± 6	21,4 ± 2	57,7 ± 0	62,3 ± 8	79,4 ± 4	84,2 ± 0	89,2 ± 9	90 ± 9	94,3 ± 7	94,8 ± 8
Pectins	21,3 ± 1	29,4 ± 4	43,3 ± 3	37,5 ± 4	72,2 ± 5	77,1 ± 0	79,3 ± 1	80,7 ± 4	79,4 ± 6	93 ± 8	91,3 ± 4
Sugars	17 ± 8	12,1 ± 7	34,1 ± 8	29,2 ± 3	26,7 ± 7	21,9 ± 2	30 ± 2	26,6 ± 0	17 ± 7	48,3 ± 6	36,5 ± 3
Total polyphenols	10,1 ± 1	54 ± 6	68,8 ± 5	62,3 ± 1	64,7 ± 5	48,3 ± 8	39,9 ± 3	29 ± 7	48,5 ± 1	56,8 ± 0	45,5 ± 3
Phenolic acids	34,4 ± 6	50,4 ± 6	74,2 ± 5	55,9 ± 9	54,7 ± 5	34,4 ± 10	46 ± 4	49,8 ± 3	62,7 ± 7	64,2 ± 6	69,4 ± 9
Stilbenes	52 ± 9	61,4 ± 5	75,5 ± 5	69,1 ± 2	59,7 ± 9	72,6 ± 1	72,1 ± 9	70,9 ± 4	67,5 ± 2	74,4 ± 9	74,1 ± 7
Monomeric Flavan-3-ols	44,3 ± 3	63,3 ± 4	71,3 ± 1	67,4 ± 1	60,6 ± 7	10 ± 6	63,4 ± 1	43 ± 3	41,3 ± 9	57,7 ± 7	54,8 ± 8
Polymeric Flavan-3-ols	21,5 ± 1	44,4 ± 7	56,2 ± 1	42 ± 1	51,8 ± 8	64 ± 1	69,7 ± 0	76 ± 6	77,9 ± 3	85,4 ± 8	88,1 ± 1
Anthocyanins	12 ± 4	43,4 ± 9	71 ± 6	57,6 ± 2	64,2 ± 6	27,4 ± 7	26,9 ± 5	38,4 ± 8	38,4 ± 10	56,7 ± 4	61,6 ± 8

3.2.4. Discussion

As it is well known, UF process is typically governed by the MWCO of the membranes and the so-called “sieving mechanism”. However, when the solubility of the components and the hydrophobicity of the membranes are incorporated, sieving mechanism attenuates and other phenomena such as charge exclusion are enhanced (Pinelo, Jonsson, and Meyer 2009; Reddy et al. 2003). At the current study, eleven membranes with different MWCO ranged from 100 to 2 kDa were assayed for the UF of grape pomace subcritical water extracts. Four of them possessing the highest MWCO (100 and 50 kDa) were made of polysulfone, polyacrylonitrile and fluoro polymer while seven with the narrower pores were made of polyethersulfone and Thin film. Despite the lacking literature concerning the interactions among phenols, pectins, sugars and membrane materials, it is expected that the numerous hydroxyl and carboxylic groups of the solutes in combination with the acidic pH of the medium would lead to molecules’ negative polarity and the so-called “polarity resistance” (El Rayess et al. 2012).

3.2.4.1. Retention of macromolecules

3.2.4.1.1. Retention of polysaccharides

While the valorization of these polysaccharides is little interest, they are mainly responsible of membrane fouling. The presence of these polysaccharides in extract is due to the contribution of the cell walls of either microorganism during alcoholic fermentation or grape berries after hydrolysis of pectic chains by pectolytic enzymes. Grape pomace polysaccharides include pectins, which are chains formed almost exclusively of galacturonic acid units linked with α -(1,4) bonds (homogalacturonane), and pectic substances such as arabinanes, arabinogalactanes, arabinogalactan-proteins (AGP) and rhamnogalacturonans (RG-I, RG-II) with a molecular weight ranging between 40 and 250 kDa (Pellerin et al. 1996; Vidal 2001; Vidal et al. 2003).

The polarity of these molecules depends on the size and the methylation degree of polysaccharides, i.e. low methoxy pectin possesses numerous negatively charged carboxylic groups. Similar pectinolytic fragments have been reported to pass optimally through a 10 kDa membrane reactor (Rodriguez-Nogales et al. 2008), denoting that their size was eventually smaller than this MWCO.

The retention of the polysaccharides and initial fouling and membrane resistance due to these molecules is due to a combination of the solutes polarity and the molecular weight cut off of the membrane. The results of the current study confirm this hypothesis, depending on the material used coupled with high retention percentage retention of polar solutes having MW < 2 kDa. The fouling tendency of polysaccharides is less strong than proteins, but can still lead to significant flux reductions; the hydrophilic macromolecular solutes can bind to less hydrophilic membrane surfaces (Susanto and Ulbricht 2005, 2007).

Pectins showed higher rejection values for the narrower membranes irrelevant of their material used. While at higher MWCO (100-50 kDa), the hydrophilic polyacrylonitrile membrane showed higher rejection rates of pectins compared to the less hydrophilic polysulfone and fluoropolymer membranes. This also led to rather high *Ra* values for the polysulfone and polyacrylonitrile membranes. Figure 4 confirmed the presence of membrane resistance via entrapment and adsorption of the polar solutes on membrane surface. Moreover, although the increase of the solutes concentration in the feed is known to increase rejection percentages via solutes adsorption, precipitation and gel layer formation (Patsioura, Galanakis, and Gekas 2011; Galanakis, Tornberg, and Gekas 2010). This result could be correlated to the solubility of specific molecules, as less protection of the non-polar molecular sides could lead to enhanced repulsion from the hydrophobic membrane surfaces. All of the above factors play a direct role in membrane fouling and retention of this family compounds. Finally the impact of pectins on the flow is dependent on their concentration. This effect results from their natural abundance and their high molecular weight (El Rayess et al. 2012).

Several studies (Mould and Synge 1954; Pritchard, Howell, and Field 1995; Alvarez et al. 1998; Hilz et al. 2005; Galanakis, Tornberg, and Gekas 2010) have reported the incidence of grape polysaccharides on the performance of ultrafiltration membranes. They have demonstrated their negative effect on the permeation flux.

It has been reported that depectinization led directly to the enhancement of permeate flux when ultrafiltration was applied in apple (Alvarez et al. 1998), pineapple juice (ros Sueli et al. 2004) processing and black currant Juice processing (Pap et al. 2010). The researchers noticed that the membrane fouling by a given juice is not directly related to its total polysaccharides content but rather to the composition, structure of these polysaccharides and the balance between different

groups of polysaccharides. While studying the effects of membrane surface properties on grape polysaccharides adsorption, it was shown that polysaccharides adsorption was negligible under static conditions and shown to be governed by membrane polarity. It decreased as surface polarity increased due to hydrophilic repulsion between surface and the hydrophilic macromolecules (Sharma, Patel, and Sugandha 2016).

A recent study (Galanakis, Markouli, and Gekas 2013) had provided evidence that different membrane materials polysulfone, polyethersulfone and fluoropolymer exhibit different levels of adsorption of typical foulants in grape extracts such as polysaccharides. In contradiction with (Vernhet et al. 1997), it was shown that larger amounts of polysaccharides were adsorbed to PES than to PP membrane. To notice that, PES membrane presents hydrophilic character while PP membrane has hydrophobic character.

3.2.4.1.2. Retention of proteins

Together with amino acids and peptides, proteins constitute the main components of nitrogenous fraction of pomace, and they are essential for fermentation (Marangon et al. 2011, 2012). While in our case we have focalized on this group, due to their negative effect on extract filtration (El Rayess et al. 2012). In the literature, some studies show that wine proteins are a mixture of grape proteins and proteins from autolyzed yeasts (Ferreira et al. 2001), or these macromolecules come only from grapes (Ferreira et al. 2000). Wines may have variable proteins concentrations of up to a few hundred (10-500) mg/l, mainly originating from grapes (Ribéreau-Gayon et al. 2006). A similar range found in our extracts. Among these proteins, the most abundant are chitinases and thaumatin-like proteins with low molecular masses ranging between 20 and 30 kDa (Marangon et al. 2012).

The polarity of these molecules is highly variable and to our knowledge no work has been done to in regards to characterizing their polarity of the grape proteins and membrane interaction. The current results have shown that retention of the proteins is due to the molecular weight cut off of the membrane. The results of the current study confirm this hypothesis, independent of the material used coupled with high retention percentage retention of polar solutes having $MW < 1\text{kDa}$.

El Rayess et al. (2012) showed the negative effect of wine proteins on permeate

fluxes on microfiltration membranes. They observed a strong decrease of the fluxes after the addition 300 mg/g proteins extract to the wine like solution. Fouling was more important when the quantity of extract is doubled. To point out this suspected “protein effect” on solution filterability, proteins were removed by fining with bentonite. Permeate fluxes obtained with the fined solutions were 25% higher than those containing 0.5 g/l of extract.

While alternative studies on the diverse types of byproducts containing proteins retention showed with respect to membrane types. They have shown Polyethersulphone exhibits protein and polysaccharides repellency (Ma et al. 2007; Peng et al. 2011) and is also susceptible to concentration polarization caused by whey proteins. Separation of soy proteins from sugars has been reported to be very efficient (90% against negligible retention) using a 18 kDa polyvinylidene difluoride membrane (Kumar, Yea, and Cheryan 2003).

3.2.4.1.3. Retention and fractioning of polyphenols

The structure of grape phenolic compounds is based on structures of multiple aromatic rings with multiple of hydroxyl and carboxylic groups, which alters the respective MW and polarity of the molecule. Phenolic acid derivatives, such as p-coumaric and ferulic acids, own a similar MW to the aforementioned monosaccharides (164 and 194, respectively). Stilbenes are generally larger (MW= 186) and more polar molecules than the rest phenolic acid compounds (Spigno and De Faveri 2007). On the other hand, flavon-3-ols (i.e., quercetin and kaempferol) are generally larger molecules (MW = 302). Whereas polymerized flavon-3-ols such as procyanidin B1, B2 and C1 have higher molecular weights (MW between 600 to 900) due to the existence of 4 aromatic rings surrounded by hydroxyl groups and polymerization. While simple anthocyanins (i.e. malvidin, MW = 331) possess a MW close to saccharose.

It is worth to note that polyphenols are amphipathic molecules with hydrophobic aromatic rings and hydrophilic phenolic hydroxyl groups. So their adsorption involves both hydrophobic effects and the formation of hydrogen bonds. The preferential adsorption of phenolic compounds with low polarity suggests the predominance of hydrophobic interactions (El Rayess et al. 2011).

Phenolic compounds have a much more important affinity for membranes than the polysaccharides and there are both quantitative and qualitative differences between the different materials tested. To better understand the impact of phenolic compounds on types of membranes, some studies were investigating the relationship between the polarity of surfaces and adsorption of flavan-3-ols, also known proanthocyanidins monomers and polymers (Ulbricht et al. 2009). The overall results show that flavan-3-ols and proanthocyanidins mainly react as acidic compounds (acceptor of electron pair or donor of H⁺) due to their hydroxyl (OH) groups of phenolic nuclei and highlight the importance of the formation of H bonds in their physico-chemical reactivity. They also note that once the number of nuclei phenols is greater than two, the affinity of compounds to surfaces is greatly increased regardless of the polarity of the latter.

The separation of specific phenolic classes was not so distinct using the different types membranes at different MWCO, as differences between retention coefficients were below 20%. The highest retention values were observed for the higher molecular weight polyphenols, such as Procyanidin B1 and C1. These results are in contradiction was previously proven on polysulfone membranes (Galanakis, Markouli, and Gekas 2013). The fact that these components were recovered quantitative (80%) verifies the presence of severe initial fouling phenomena upon saturation of adsorption sites from the various phenolic compounds depending on the membrane used. Indeed, these compounds could eventually have the exact molecular size and sequence of polar and non-polar sides to absorb and “lock” like a key on the polyethersulfone surface. The importance of matching phenols’ size, polar and non-polar sites with membrane characteristics (polarity and MWCO) is confirmed by the lower rejection of phenols obtained in other cases, i.e. during treatment of kiwifruit juice with a more polar, cellulose acetate 30 kDa-membrane (Cassano et al. 2008) or polysulfone 10 kDa- membrane grape juice (Kalbasi and Cisneros-Zevallos 2007) .

Sugars, which are more polar but have similar MW to phenolic acids, showed lower rejection coefficients, probably due to their repulsion from the non-polar sides of the polyethersulfone membrane. The retention of the phenolic acids could be attributed to saturation of adsorption on the membrane, as a very high retention percentage difference, 26-79 % respectively was observed between the compounds.

The above results are in accordance with Santamaría et al. (2002) who fractionated dimeric and trimeric proanthocyanidins from the monomeric ones in grape seeds extract (>70% and 30%, respectively) with a 20 kDa polysulfone membrane. Nevertheless, the fact that the difference between polymeric and monomeric proanthocyanidins herein decreased and retention percentages of both proanthocyanidins classes increased implies the potential appearance of concentration polarization phenomena, too. Besides, adsorption of proanthocyanidins on the membrane surface cannot be excluded, too. Polyethersulfone is known to have an asymmetric pore size distribution (Cheryan 1998) that could lead to retention percentages variations, depending on the size of the “gaps” found on the membrane surface.

At this case, retention is expected to be governed by the molecular size and the specific structural characteristics of each solute. Perhaps this could explain the observed much broader variation of retention percentages. For instance, the larger and less polar components (i.e. pectin, proteins) would be able to come close to membrane surface and pass occasionally through the pores resulting in moderate retention percentages at MWCO >50kDa. On the other hand, the smaller and more polar molecules (i.e. Phenolic acids, stilbenes and sugars) could come even closer to membranes surface, leading to local concentration polarization and increased rejection percentages of 60%. Besides, a partial adsorption of the more polar molecules onto membrane surface cannot be excluded. In every case, it is important to state that the more hydrophilic polyethersulfone showed a selective separation concerning the polarity of particular compounds and their concentration in the feed. These characteristics could lead to an effective enrichment of phenolic classes in different streams, i.e. phenolic acids against polymeric Flavan-3-ols or stilbenes against anthocyanins, as the retention of the first class was almost 2-fold. Enriched concentrates in polymeric proanthocyanidins derivatives could be utilized as antioxidants in foodstuff, while lower permeates rich in proanthocyanidins and anthocyanins could be used as flavorings and colorants, respectively.

3.2.5. Conclusion

The current study suggests that the purification of phenolic compounds recovered from grape extract is possible using UF. The multiple membranes were able to separate phenolic compounds from pectin and protein fractions, but they could not fractionate different phenolic classes and sugars, as they were not retained at variable MWCO from 100 to 2 kDa. Using several membrane materials, separation was mainly affected by severe fouling phenomena due to polar solutes adsorption on membrane surface and less by sieving mechanism. On the other hand, the application of a thin film membrane in the border of UF and nanofiltration (2 kDa), provided a successful methodology to separate different phenolic classes like monomeric, polymeric proanthocyanidins, and anthocyanins on the basis of polarity, and MWCO. Another proposal could be the sequential application of a fluoropolymer, polyethersulfone and thin film membranes (100, 50 and 2 kDa, respectively), aiming at clarifying of protein and pectins in the first step and the separation of polymeric and monomeric polyphenols in the second. The further purification of macromolecules and micromolecules may be done by diafiltration.

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3.3. THE USE OF NANOFILTRATION MEMBRANES FOR THE FRACTIONATION OF POLYPHENOLS FROM GRAPE POMACE EXTRACT

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Abstract

Filtration experiments in batch concentration mode (with recycling of the retentate stream) of the grape pomace extract were performed in laboratory filtration membrane equipment, by using nine commercial membranes: NF membranes with an approximate MWCO of 1000-150Da. The filtration experiments of the selected pomace extract was performed by modifying the most important operating variables: transmembrane pressure, tangential velocity, temperature, and the nature and MWCO of the membranes. The evolution of the cumulative permeate volumes and permeate fluxes with processing time were analyzed till a VRF of 10 was reached. The effect of the mentioned operating conditions was discussed. The effectiveness of the filtration treatments was determined by the evaluation of the rejection coefficients for several families of polyphenols. With membranes possessing MWCO between 1000 to 500 Da were able to quantitatively recover polymeric proanthocyanidins in concentrate stream and separate it from phenols that pass into the permeate stream. On the other hand, the 600 to 300 Da membranes could also be utilized for the fractionation of monomeric phenolic families. The membrane was able to partially remove the anthocyanins fragments of phenolic acids derivatives and flavonols in the concentrate stream and at the same time.

Key words: Nanofiltration, Grape pomace extract, Anthocyanins, Proanthocyanidins, Phenolic compounds.

Abbreviations

AUC Area under the curve

CCD charge-coupled device

C_{if} concentrations of compound in feed

C_{ip} concentrations of compound in permeate

DAD Diode-Array Detector

DW Dry weight

HPLC High performance liquid chromatography

J_p permeate flux during extract filtration

J_w pure water flux

MF Microfiltration

MS Mass spectrometer

MW Molecular Weight

MWCO Molecular weight cut off

NF Nanofiltration

NF Nanofiltration

ORAC

P pressure

PEG Poly Ethylene Glycol

R_a Fouling resistance

R_i initial resistance

R_m membrane resistance

RO Reverse Osmosis

R_t total resistance

SE Standard error

TMP TransMembrane Pressure

UPLC Ultra High pressure Liquid Chromatography

UV Ultra violet

V_0 initial feed volume

V_R retention volume

VRF volume reduction factor

μ_p dynamic viscosity of the extract

μ_w dynamic viscosity of water

3.3.1. Introduction

Grape pomace is a by-product in wine production, representing around 30% of the original fruit, consisting of skin, seed, and stems tissue. A large quantity of grape pomace is produced worldwide every year and its disposal has caused a serious environmental problem. Researchers have proposed the use of grape pomace for the production of different value-added products including enzymes, organic acids, ethanol, aroma compounds, and natural antioxidants (Arvanitoyannis, Ladas, & Mavromatis, 2006).

As is well known, grapes represent an important source of bioavailable polyphenolic compounds such as flavonols, monomeric and oligomeric flavanols, and anthocyanidins (Spigno, Tramelli, & De Faveri, 2007). Conventional wine production results in a wine rich in phenolic compounds but only 10-40% of the phenolic compounds of the fruit are transferred to the wine (Fragoso, Guasch, Aceña, Mestres, & Busto, 2011), most of the compounds remaining in the grape pomace. As a result of its abundance, and owing to the increasing interests in new natural sources of antioxidant products, grape pomace has been investigated as a potential source of bioactive polyphenols during recent years Ayaprakasha, 2002, Kammerer et al., 2005, Vergara-Salinas et al., 2013 and Vergara-Salinas et al., 2015, which can be used for various purposes in the food, pharmaceutical and cosmetic industry for their effective antioxidant and free radical scavenger activities.

In recent years, more environmentally friendly techniques have been investigated and used for the separation, purification and concentration of bioactive compounds allowing to reduce extraction time and solvent consumption as well as to increase bioactive compounds yield (Galanakis, 2012).

Membrane operations are recognized as powerful tools for the purification and concentration of various solutions (e.g., juices, extracts, whey) and the separation of valuable compounds from by-products of the agro-food industry (Li & Chase, 2010).

The basic properties of membrane operations make them competitive with conventional methodologies: they do not involve phase changes, chemical additives and heat treatment, they are modular and easy to scale-up, and are characterized by unlimited selectivity of separation, thereby enabling a more rational utilization of raw materials and recovery and reuse of by-products. In addition, they respond efficiently to the requirements of so-called “process intensification”, allowing drastic

improvements in manufacturing and processing, substantially decreasing the equipment-size/production-capacity ratio, energy consumption, and/or waste production (Akin, et al., 2012; Drioli & Romano, 2001).

Pressure-driven membrane operations such as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) are based on the principle of selective permeation of solutes through polymeric or inorganic semi-permeable membranes: the driving force for mass transfer of solutes across the membrane is the transmembrane pressure. NF is a unit operation which separation characteristics between UF and RO whose molecular weight cut-off (MWCO) ranges from 100 to 1000 Da ($\text{g}\cdot\text{mol}^{-1}$). The complex separation mechanisms that occur in nanofiltration (physical, chemical and electrical interaction between the solvent, solutes and membrane) make the number of the operating parameters that control separation efficiency long and give different results for the same feed and the same membrane. The specific performance of nanofiltration membranes and the large choice of the membranes should facilitate their application (Massot, et. al, 2008). It appears to have great potential in the production of high quality food, including water softening, wastewater treatment, beverage industry, dairy industry and sugar industry (Salehi, 2014). The recovery of biologically active compounds from agro-food by-products, also in combination with other membrane operations (*i.e.*, UF and RO), is another research area of growing interest. For example, a composite a composite fluoro polymer membrane (1 kDa), was able to separate hydroxycinnamic acids satisfactorily from anthocyanins and flavonols in winery sludge extracts and diluted wine samples (Galanakis et al., 2013b, 2015). The same membrane has been reported to recapture low-MW polyphenols (ie, hydroxytyrosol, protocatechuic acid, catechol, tyrosol, caffeic acid, p-coumaric acid, and rutin) from pretreated olive mill wastewater (Cassano et al., 2013) before the permeate stream is processed with NF to concentrate the valuable compounds (17% polyphenol rejection). In another application reported by Diaz et al. (2009), two different 1-kDa membranes (Inside Céram and GE 2540) were used to recover total phenols from fermented grape pomace, and showed at least 80% rejection of these components. In addition, Diaz et al. (2010) recovered antioxidant and phenolic compounds from liquors obtained by pressing distilled grape pomace, using a 1-kDa membrane (Inside Céram). This application showed a higher rejection of total phenolics (up to 72%). Finally, the separation and concentration of phenolic compounds from press liquors obtained from

pigmented orange peels was carried out by Conidi et al. (2012). High rejection of anthocyanins (89%) and flavonoids (70%) was observed using a 1-kDa membrane (NP010).

In a previous work, a conceptual process design for recovering and concentration phenolic compounds from grape pomace was proposed on the basis of a NF treatment of multiple grape extracts with different NF membranes in selected operating conditions (Díaz-Reinoso et al., 2009; Galanakis et al., 2013; Santamaría et al., 2002; Zagklis & Paraskeva, 2015). In a different context of these precedent researches, NF fractionation experiments (with recycling of the retentate stream) of the grape pomace extracts were performed in the present work with several objectives: the study of the evolution of the permeate flux with filtration time and volume retention factor; the establishment of the effect of operating parameters transmembrane pressure (TMP), crossflow velocity (v), temperature and molecular weight cut-off (MWCO) of the membranes used on the permeate flux; fractionated streams were characterized in terms of total antioxidant activity (TAA), sugars and phenolic compounds. The performance of selected membranes in terms of productivity and selectivity towards compounds of interest is evaluated and discussed.

3.3.2. Materials and methods

3.3.2.1. Experimental equipment and membranes

Nanofiltration experiments were conducted in a laboratory cross-flow mode filtration apparatus, Sepa® CF II Membrane Cell System (GE Osmonics, Minnetonka, MN, USA). The equipment was constituted by a 2 L pressurized and inerted storage vessel and a M-03S Hydracell feed flow pump which fed the solution to the flat-sheet membrane module at the desired flow rates. The whole equipment is temperature controlled by means of a water stream at the desired temperature that circulated through an external jacket that surrounded the storage vessel. A pressure control valve controlled the transmembrane pressure (TMP) in the experiments after the filtration apparatus. The cumulative permeate volume was measured with a Mettler balance.

The 9 flat-sheet membranes used were provided by GE Osmonics and Alfa laval. The majority of the membranes of NF membranes were made of polyamide, while the ENTA01P and BQ01 membranes were made from Fluoropolymer and

Polysulfone respectively; with MWCO ranging from 1000 to 125 Da. Table 1 lists their characteristics.

Table 1 Characteristics of tested Nanofiltration (NF) membranes (manufacturer data)

Designation	Manufacturer	Polymer Type	Nominal Molecular Weight Cutoff (MWCO) Range	% NaCl Rejection	Recommended Pressure (MPa)	Maximum Pressure (MPa)	Recommended pH range	Maximum Temp. (°C)
MS19	GE Osmoncis	Polyamide	125-200	≥99	1,4	6,8	2-12	80
DL	GE Osmoncis	TF (Thin Film)	150-300	≥96	0,70	4,10	2-10	50
DK	GE Osmoncis	TF (Thin Film)	150-300	≥98	0,70	4,10	2-10	50
NF	Alfa laval	Polyamide	200-400	≥98	2,2	5,5	3-10	50
MX07	GE Osmoncis	Polyamide	300-600	50-70	0,7	6,8	2-12	80
BQ01	GE Osmoncis	Polysulfone	500-1000	20-30	0,7	6,8	0.5-11	100
GE	GE Osmonics	Polyamide	1000	1K-PEG	2,76	6,8	1-11	50
ETNA01PP	Alfa laval	Fluoro polymer	1000		1	5,5	1-11	60

All the membranes had an effective area of 0.014 m² and an experimentally measured flow section of 14.9 mm² (4.5 mm × 3.3 mm). A new membrane was used in each experiment, rinsed with ultrapure water, and compacted by filtering ultrapure water for 1 hour before starting the next filtration experiment. The water contact angles of the membranes were measured by the sessile drop technique.

3.3.2.2. Subcritical water extraction

In the extraction system, a HPLC pump (Shimadzu LD-AC10) was used for deionized water delivery, pressurization and controlling the pressure of system. A pressure transducer (Davidson, Druck) and thermocouple (Cavland Electric) were installed in the custom-made high-pressure vessel to monitor both pressure and temperature of system. Extract was collected in an inerted vessel after passing in an ice bath.

In each run red Dunkelfelder pomace supplied by the university of Changins (70 g) was loaded into the high-pressure vessel, which can contain 325 cm³ of material. The vessel was placed in an oven at a predetermined temperature of 150°C. The outlet valve of extraction vessel was then closed and the system was pressurized to a desired

pressure of 25×10^5 Pa at a constant flow rate. The water flow rate was adjusted at 20 mL/min using a metering valve on the HPLC pump. After 3L of extraction, the solution collected in an inerted sampling vessel and pomace were then stored at 4°C for further analysis and membrane separation.

3.3.2.3. Filtration experiments

The filtration experiments were conducted at the natural pH of the extract (3.7) in cross-flow mode, with the feed stream flowing tangential to the membrane surface. The extract was prefiltered at 0.3 μ m (GE Osmonics JX) to microbial stabilize the extract. The operating method was batch concentration mode: that is, the retentate or concentrate stream was flowed back to the feed tank, while the permeate stream was collected separately and not recirculated to the storage vessel. The initial volume of extract treated was 2L in all cases, and the flow rate was dependent on the tangential velocity selected, $v=1, 2$ or 3 m s^{-1} . During each experiment, the temperature, tangential velocity and TMP remained constant, but they were varied among the different experiments. The duration of each experiment varied according to the desired value of volumic reduction factor (VRF) to be reached.

A standard protocol for NF experiments included three steps. At first, the new membrane was rinsed with ultrapure water (from a Milli-Q system) and the membrane hydraulic permeability was determined by measuring the water permeates flux (J_w) at different TMP. Secondly, the storage vessel was emptied and filled with grape pomace extract to perform the filtration experiment. During these experiments, the cumulative permeate volume (V_p) was measured at regular time intervals. In addition, several parameters frequently used in grape pomace to evaluate the content of the extract were analysed in the permeate stream: sugar content, absorbance at 280nm, total polyphenol content, antioxidant activity by ORAC. The concentration of different families of phenolic acids was also measured: these compounds were specifically selected for their high added value, and the interest in their purification. With the values obtained for these parameters, their respective rejection coefficients were determined.

3.3.2.4. Analytical methods

3.3.2.4.1. Contact angle

The water contact angles of the membranes were measured by the sessile drop technique. Prior to the experiments, the membranes were fixed to a smooth support surface by using a double side sticky tape. Ultrapure water droplets with a volume of 5 μL were automatically deposited on the membrane surface by using a Digidrop, GBX, France equipped with needles of 0.5 mm of external diameter. Once the drop was placed on the surface, the Drop Shape Analysis System of the GBX software (Windrop, GBX, France) allowed the direct measurement of the water contact angle by averaging the water contact angles measured on the left and right sides of the sessile drop. At least 10 drops were deposited on different zones of the membrane at room temperature. The mean value water contact angle and its standard deviation were calculated for each sample. The values obtained are shown in table 2.

Table 2 contact angles of tested Nanofiltration (NF) membranes

Designation	Contact angles
MS19	37,3
DL	27
HL	32,7
DK	45,1
NF	48,7
MX07	33,2
BQ01	57,1
GE	51,2
ETNA01PP	65,3

These values indicate that the ETNA01PP, GE and BQ01 membranes present a hydrophobic surface, while the MX07, NF, DK, HL, DL, MS19 membranes are relatively hydrophilic.

3.3.2.4.2. pH and Total sugars

pH values of feed samples were measured with a digital pH-meter (Thermo Scientific™ Orion™ Star A324)

The total sugar content was determined by the anthrone method (Trevelyan et al., 1952), and expressed as glucose equivalents (GE).

3.3.2.4.3. Total polyphenols content

The total phenolic content was spectrophotometrically measured according to a modified Folin Ciocalteu method to be applied in 96-well microplates. Stock solutions (10 mg/mL) of the grape pomace extracts were prepared in EtOH/H₂O (25:75, v/v), and a microplate spectrophotometer (MultiSkan Spectrum, Thermo Scientific) was used for the incubation and measurement. Briefly, each well was filled with 184 µL of distilled water and 24 µL of the sample solution, followed by 12 µL of the Folin Ciocalteu reagent and 30 µL of 20% (w/v) Na₂CO₃ solution. Prior to the measurement of the absorbance at 765 nm, the mixture was incubated for 1h under dark conditions at 25°C. Gallic acid was used as a standard for calibration. Results, expressed as milligrams of gallic acid per 100 g of grape pomace sample (on a dry matter basis, dm), were a mean of six determinations.

3.3.2.4.4. Antioxidant activity – ORAC

The oxygen radical absorbance capacity analysis was applied by using 96-well fluorescence microplates. The reaction was carried out in phosphate buffer (75 mM, pH 7.4). In this order, 30 µL of the pomace extract solution, 180 µL of fluorescein (117 nM final concentration), and 90 µL of AAPH (40 mM) were added to each well. The mixture was shaken and allowed to stand for 1.5 h at 37 °C. Fluorescence was recorded every minute during this period at excitation and emission wavelengths of 485 and 530 nm, respectively. Simultaneously on the same microplate, a blank sample (phosphate buffer replaced the sample) and Trolox calibration solutions (1–40 µM) were also performed ($R^2 = 0.983$). The area under the curve (AUC) was calculated for each extract sample by integrating their relative fluorescence curves. By subtracting the AUC of the blank, the net AUC of the pomace extracts was calculated and correlated with Trolox concentrations.

3.3.2.4.5. Phenolic classes:

UPLC analyses were performed in an Agilent 1260 apparatus consisting in an autosampler module, a degasser, a binary pump, a column heater/selector and a UV–visible DAD detector (Agilent Technologies, USA). Chromatographic separation was performed on a Agilent C18 (2.1 mm x 100 mm, 1.8 μ m). Anthocyanins were eluted with a flow rate of 0.4 mL/min and a gradient of water/acetonitrile/formic acid (87/3/10; solvent A) and water/acetonitrile/formic acid (40/50/10; solvent B) according to the following gradient program (v/v): 0 min 94% A 6% B, 15 min 70% A 30% B, 30 min 50% A 50% B, 35 min 40% A 60% B, 40 min 35% A 65% B, 41 min 100% B isocratic for 5 min. Detection was performed at 518 nm. Other polyphenols were eluted with a flow rate of 0.4 mL/min and a gradient of water/formic acid (99.9/0.1; solvent A) and acetonitrile/formic acid (99.9/0.1; solvent B) according to the following gradient program (v/v): 0 min 93% A 7% B, 15 min 86% A 14% B, 40 min 65% A 35% B, 44 min 50% A 50% B, 54 min 30% A 70% B, 55 min 100% B isocratic for 5 min. Detection was performed at 280 nm for flavanols, 306 nm for stilbenes, 310 nm for coumaric acid derivatives and 370 nm for flavonols.

Phenolic compounds were eluted with a flow rate of 1 mL/min and a gradient of water/formic acid (99.9/0.1; solvent A) and acetonitrile/formic acid (99.9/0.1; solvent B) according to the following gradient program (v/v): 0 min 70% A 30% B, 18 min 65% A 35% B, 46 min 20% A 80% B, 47 min 100% B isocratic for 5 min.

This HPLC was coupled to an Esquire 3000+ ion trap mass spectrometer using an ESI source from Bruker – Daltonics (USA). Nitrogen was used as drying gas. ESI-MS parameters: positive mode, nitrogen flow rate 10 l/min, nebulizer pressure 0.275 10^5 Pa, drying gas temperature 365 °C, HV capillary –3700 V, end plate offset –500 V, capillary exit 111.2 V, skimmer 40 V and trap drive 45.9; negative mode, nitrogen flow rate 10 l/min, nebulizer pressure 0.172 10^5 Pa, drying gas temperature 350 °C, HV capillary +3400 V, end plate offset –500 V, capillary exit –115.3 V, skimmer –40 V and trap drive 42.9.

Identification of phenolic compounds was achieved using their UV/vis spectra, ion mass and MS/MS fragments using available standards. The results were expressed as mg of specific compound per L of extract, and the data represent the means of three replicates \pm SE.

3.3.3. Results and discussion

3.3.3.1. Water permeability determination

The hydraulic permeability L_p is an intrinsic feature of a non-fouled membrane that must be determined. Therefore and prior to the general filtration experiments of the grape pomace extracts, several filtration experiments of pure water were carried out with each one of the filtration membranes selected, and with the aim to measure the evolution of the water permeate flux (J_w) with the variation of TMP. The applied pressures during this process ranges from 10 to 30 10^5 Pa for the NF membranes. The results obtained showed that J_w increased linearly with TMP, as can be seen in Fig. 1 for the nine types of membranes tested ($T = 20^\circ\text{C}$).

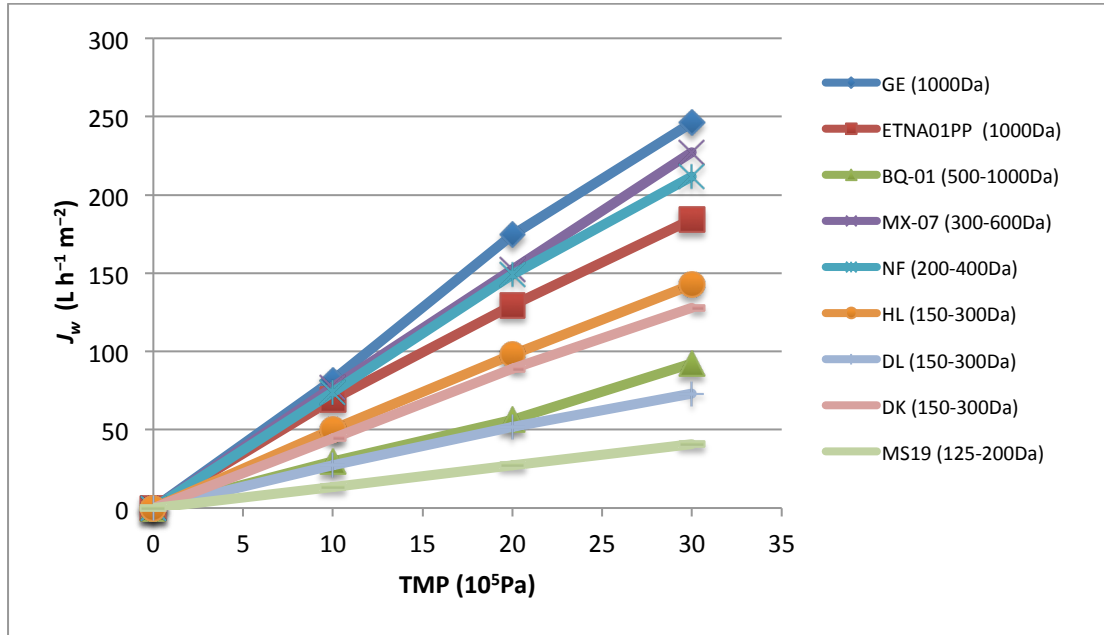


Figure 1. Hydraulic permeability L_p for the nanofiltration membranes ($T = 20^\circ\text{C}$).

The hydraulic permeability L_p was obtained from the slopes of the straight lines. Thus, after regression analysis, the following values were deduced, with correlation coefficients higher than 0.99: ranging from 1.35 to 8.4 $\text{L h}^{-1} \text{m}^{-2} 10^5 \text{Pa}^{-1}$, for the MS19 (150-200 Da) to GE membranes (1000 Da), respectively, at 20°C . In the previous work UF membranes showed that the increase in the hydraulic permeability occurred as could be expected: among membranes of the same nature, larger pore sizes or MWCO lead to higher pure water fluxes. The different L_p in the NF

membranes can be attributed to their internal structure and not only to the MWCO. The L_p value is also an inherent characteristic related to the composition, morphology, porosity and hydrophobicity/hydrophilicity of the membranes, and is not indicative of process flux. In the present case, the ENTA01PP membrane (1000 Da) in fluoro polymer exhibited relatively hydrophobic surface and lesser pure water permeability compared to the GE membrane (1000 Da) in polyamide.

Since the temperature effect on the filtration process was investigated with the GE membrane, its hydraulic permeability was also measured at several temperatures. The values of L_p obtained at 30 and 40 °C were 5.7 and 6.5 L h⁻¹ m⁻² 10⁵Pa⁻¹, respectively. Therefore, a temperature increase leads to higher pure water permeate flux due to a decrease of the viscosity.

3.3.3.2. Influence of operating conditions on the permeate flux

Filtration experiments of the grape pomace extract were performed with the 9 selected membranes in batch concentration mode, by modifying the most important operating variables: TMP, crossflow velocity, temperature, and the nature and MWCO of the membranes. These conditions were varied according to the values depicted in Table 3, which summarizes the experiments carried out with the Nanofiltration membranes.

The cumulative permeate volume V_p obtained as a function of time for the GE membrane (1000 KDa) at different TMP and temperatures, and with a constant $v=2\text{ms}^{-1}$, are represented in Fig. 2. As it can be observed, these volumes increased with processing time, but simultaneously, a decrease occurred in the permeate rate. Additionally, for a given time, the volumes increased with the increasing TMP; and increased with the increasing temperature, in the investigated range of operating conditions. Similar effect of the TMP was obtained for all of the used membranes.

Table 3. Experimental conditions applied in the NF experiments performed and results obtained at VRF=10.

Experiment	TMP (10 ⁵ Pa)	v (m s ⁻¹)	T (°C)	J_v (L h ⁻¹ m ⁻²)
ENTA01PP-1	10	2	20	34,0
ENTA01PP-2	20	2	20	53,4
ENTA01PP-3	30	2	20	67,5
GE-1	10	2	20	15,4
GE-2	20	2	20	25,8
GE-3	30	2	20	29,7
GE-4	30	1	20	23,1
GE-6	30	3	20	35,8
GE-7	30	2	30	34,2
GE-8	30	2	40	37,4
BQ01-1	10	1	20	7,8
BQ01-2	20	2	20	12,8
BQ01-3	30	3	20	17,1
MX-07-1	10	2	20	14,7
MX-07-2	20	2	20	29,0
MX-07-3	30	2	20	37,5
NF-1	10	2	20	18,2
NF-2	20	2	20	21,8
NF-3	30	2	20	25,0
HL-1	10	2	20	10,8
HL-2	20	2	20	23,6
HL-3	30	2	20	36,2
DK-1	10	2	20	7,5
DK-2	20	2	20	15,8
DK-3	30	2	20	23,6
DK-4	30	1	20	19,5
DK-5	30	3	20	26,9
DL-1	10	2	20	2,2
DL-2	20	2	20	3,5
DL-3	30	2	20	4,7

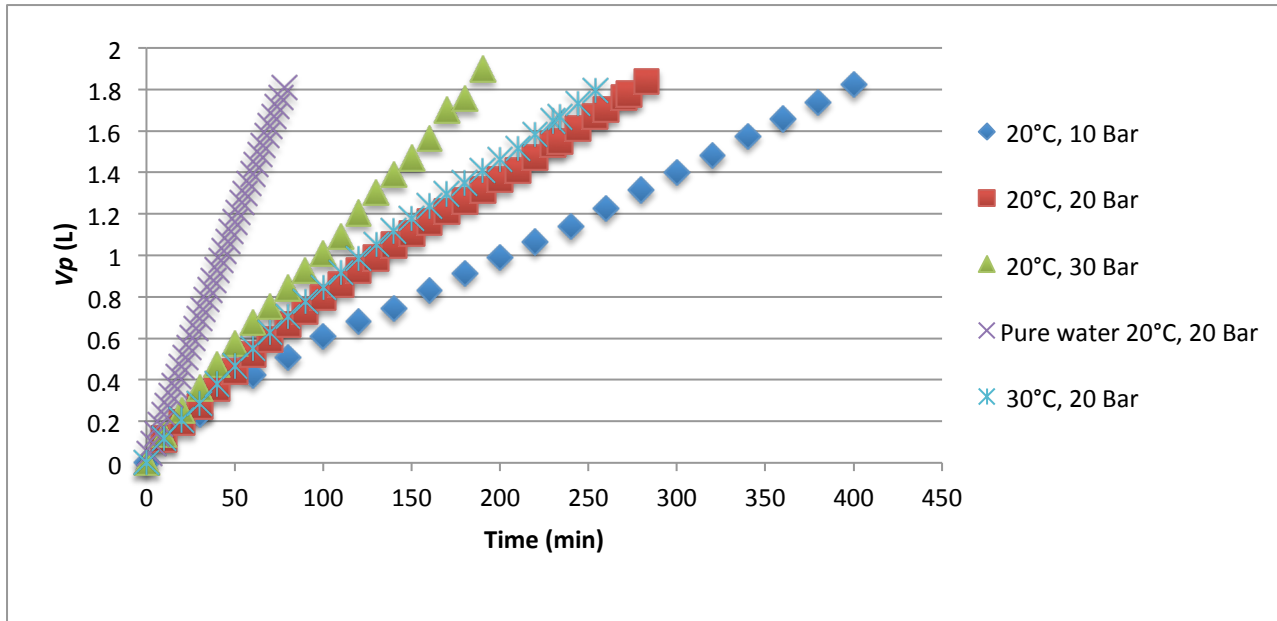


Figure 2. Evolution of the cumulative permeate volume with processing time for the grape pomace filtration experiments performed with the GE membrane at a tangential velocity $v = 2 \text{ m s}^{-1}$.

Fig. 2 also includes the cumulative permeate volume obtained in the previous experiments for the filtration of pure water with the new membrane. The lower values of V_p obtained for the grape pomace extract in comparison to those of pure water were due to the fouling of the membrane (Cissé et al., 2011). The membrane resistance is defined by the following equation for the water permeate flux:

$$R_m = \frac{\Delta P}{J_w \mu_w}$$

where μ_w is the viscosity of the pure water permeate and R_m is the hydraulic resistance to pure water.

The decline of the grape pomace extract permeate flux J_w with filtration time is represented in Fig. 3 for some experiments performed with the GE membrane, where the TMP was varied at 2 m.s^{-1} and 20°C . The results show that J_w decreased gradually with the operating time, which is due to fouling caused by the compounds found in grape pomace extract (Díaz-Reinoso et al., 2009) and (Zagklis & Paraskeva, 2015).

At the same time, Fig. 3 also depicts the volume reduction factor (VRF) evolution with filtration time with the GE membrane which is defined by:

$$VRF = \frac{V_0}{V_R}$$

where V_0 is the initial feed volume and V_R is the retention volume, that is the extract volume remaining in the storage vessel ($V_R = V_0 - V_P$). For the experiment with GE at $\text{TMP} = 3 \cdot 10^5 \text{ Pa}$, the initial permeate flux was $60.4 \text{ L h}^{-1} \text{ m}^{-2}$ and decreased up to $30 \text{ L h}^{-1} \text{ m}^{-2}$ after 2.25 h of operation, which corresponded to a final value of $\text{VRF} = 10$.

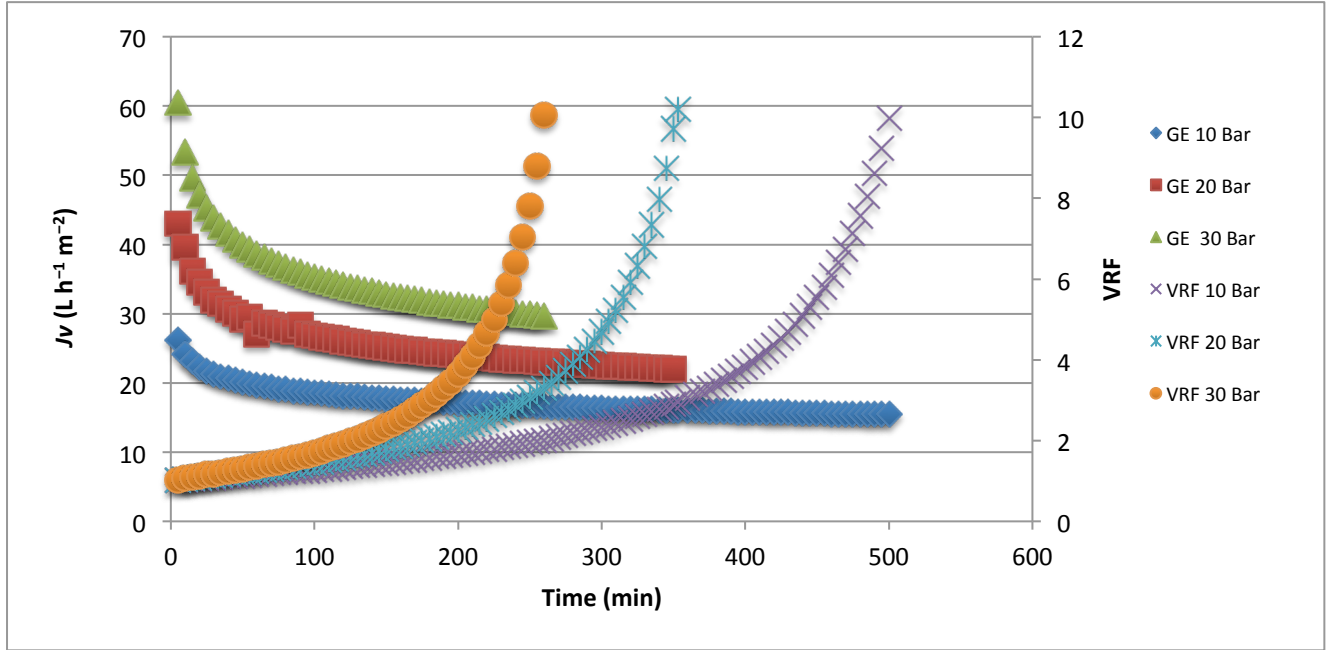


Figure 3. Evolution of the permeate flux and volume retention factor with processing time for the grape pomace extract filtration experiments performed with the GE membrane at $v = 2 \text{ m s}^{-1}$ and $T = 20 \text{ }^{\circ}\text{C}$.

Fig. 4 depicts the evolution of J_v with VRF for the selected experiment GE-2 taken as example: a clear decline of J_v occurs with the increase in VRF, due to the increase of the concentration in the retentate, increasing fouling effect on the membrane. Moreover, this curve could be divided into three periods: an initial stage with a rapid decrease of the permeate flux; a second stage with a smaller decrease of the permeate flux that takes place around $\text{VRF} = 1.25$, and a final stage with a very slight decrease in J_v up to near steady-state conditions, that occurred after $\text{VRF} = 4$. Similar trends have been observed in previous studies for the filtration of grape juice (Cancino-Madariaga et al., 2012) and kiwifruit juice (Cassano et al., 2008). As similar curves were obtained for the remaining experiments, it was adopted $\text{VRF} = 10$ as the standard value that provided the VRF for steady-state conditions in the filtration of grape pomace extract; Table 1 only depicts the permeate fluxes J_v specifically obtained at $\text{VRF} = 10$.

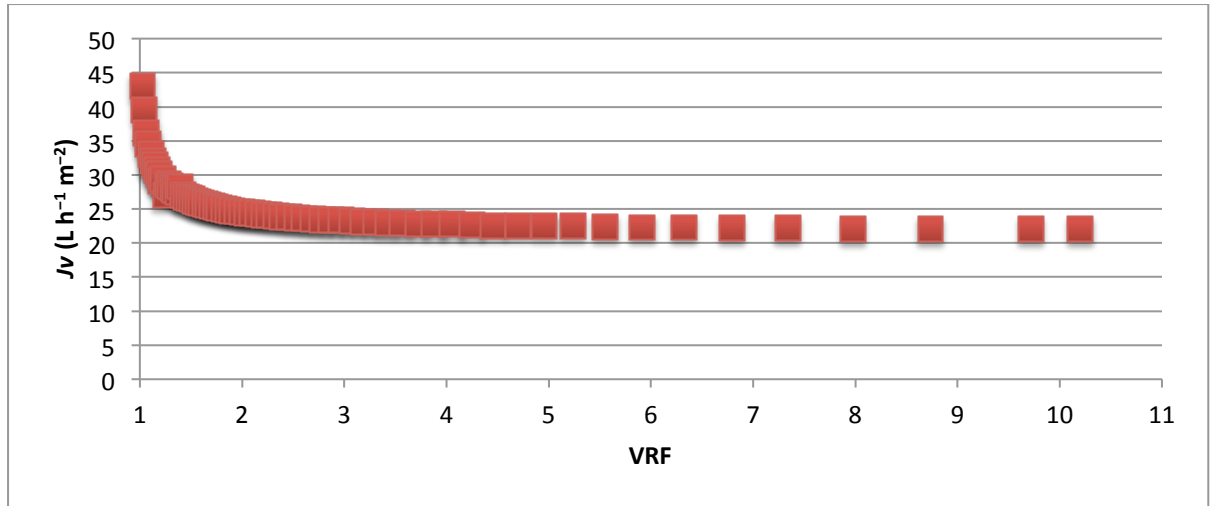


Figure 4. Evolution of the permeate flux with the volume reduction factor for the experiment GE at $20 \cdot 10^5$ Pa.

Those calculated J_v values are affected by the main operating parameters already mentioned: tangential velocity, TMP, temperature and MWCO and nature of the membranes. Thus, the effect of the tangential velocity on the steady-state permeate flux can be observed for the GE (1000 KDa) and DK membrane (150-300 Da) in Fig. 5. As it is seen for $\text{TMP} = 30 \cdot 10^5$ Pa, J_v increases when the tangential velocity is increased, due to an increase of the shear stress at the membrane surface, which prevent the accumulation of the components in the laminar sublayer and decrease the thickness of the concentration polarisation layer (Wei et al.,2007).

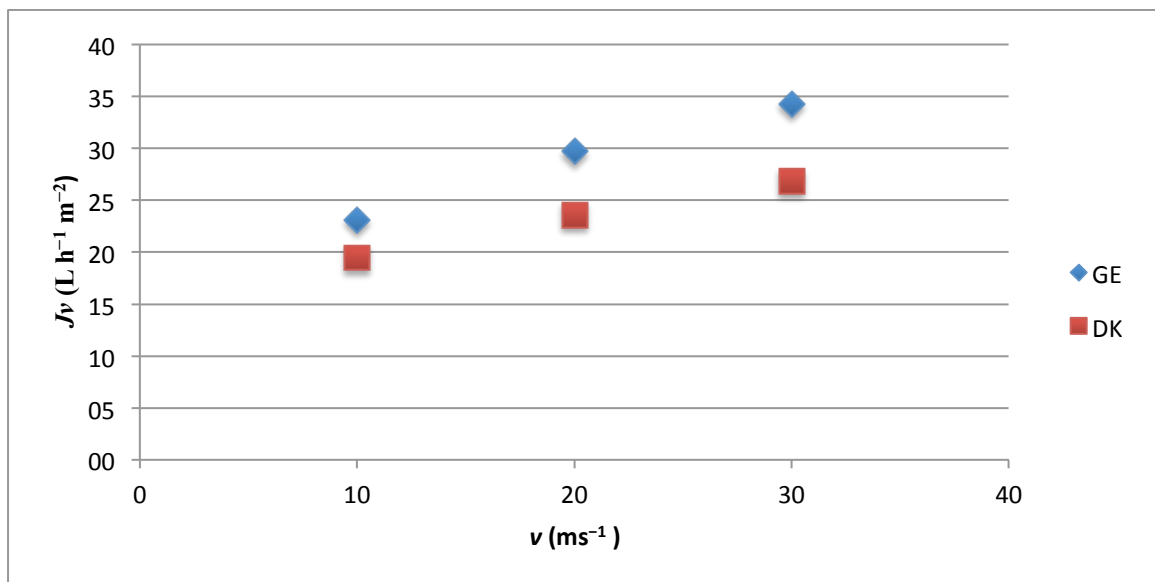


Figure 5 Effect of the crossflow velocity on the permeate flux for $\text{VRF} = 10$ with the GE and DK membranes at 20°C and $3 \cdot 10^5$ Pa.

In a similar way, Fig. 6 shows the evolution of the steady-state permeate flux with the TMP in the experiments carried out with NF membranes and with a crossflow velocity of 2 m s^{-1} . It is observed that J_V increased linearly with increasing pressure in the range of TMP used, as it has been reported by other authors in similar studies performed with different extracts filtrations (Santamaría et al., 2002) and (Díaz-Reinoso et al., 2009).

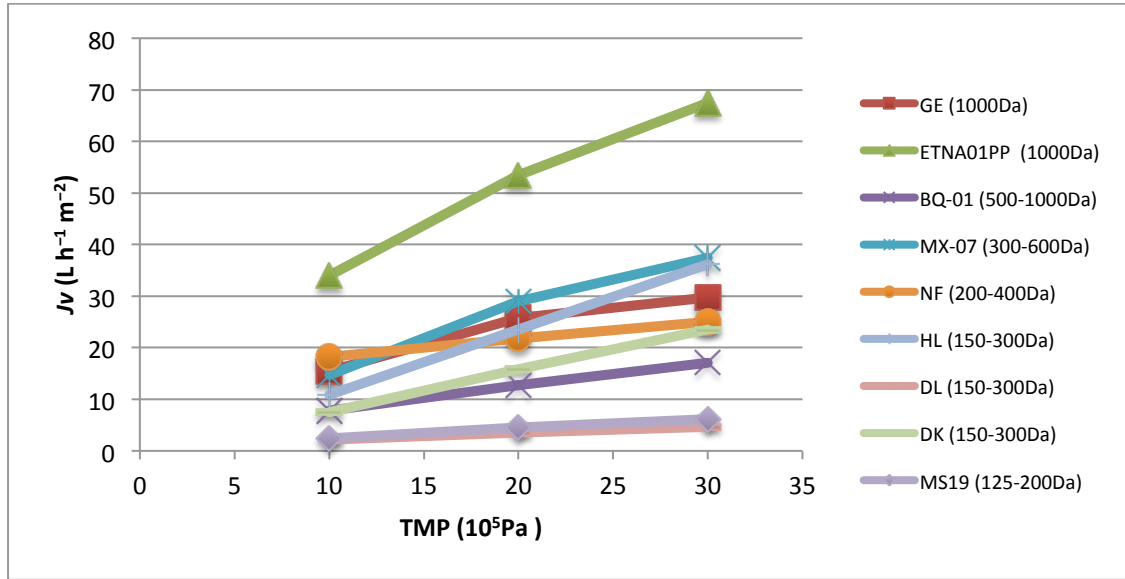


Figure 6. Effect of the transmembrane pressure and MWCO on the steady-state permeate flux for experiments performed at $v = 2 \text{ m s}^{-1}$ and $T = 20^\circ \text{C}$.

Fig. 6 also provides the influence of the MWCO of the membranes on the steady-state J_V . For instance at 30 bar, NF membranes which presented (GE and ETNA01PP), higher value ($67.4 \text{ L h}^{-1} \text{ m}^{-2}$) obtained with the ETNA01PP membrane in comparison to that of the GE membrane ($29.6 \text{ L h}^{-1} \text{ m}^{-2}$) can be only attributed to the different nature of the membranes. However, the hydraulic permeability with pure water of the GE membrane ($L_p = 184 \text{ L h}^{-1} \text{ m}^{-2} 10^5 \text{ Pa}^{-1}$) was lower than that of the ETNA01PP GE membrane ($L_p = 245 \text{ L h}^{-1} \text{ m}^{-2} 10^5 \text{ Pa}^{-1}$). The higher reduction in the permeate flux from pure water to grape pomace extract filtration with the GE membrane is an indication of a greater fouling effect with this membrane, made of polyamide, than with the Fluoro polymer ETNA01PP membrane. This behavior could be explained by the greater hydrophobicity in terms of its contact angle value (65.3°) of the HTNA01PP membrane.

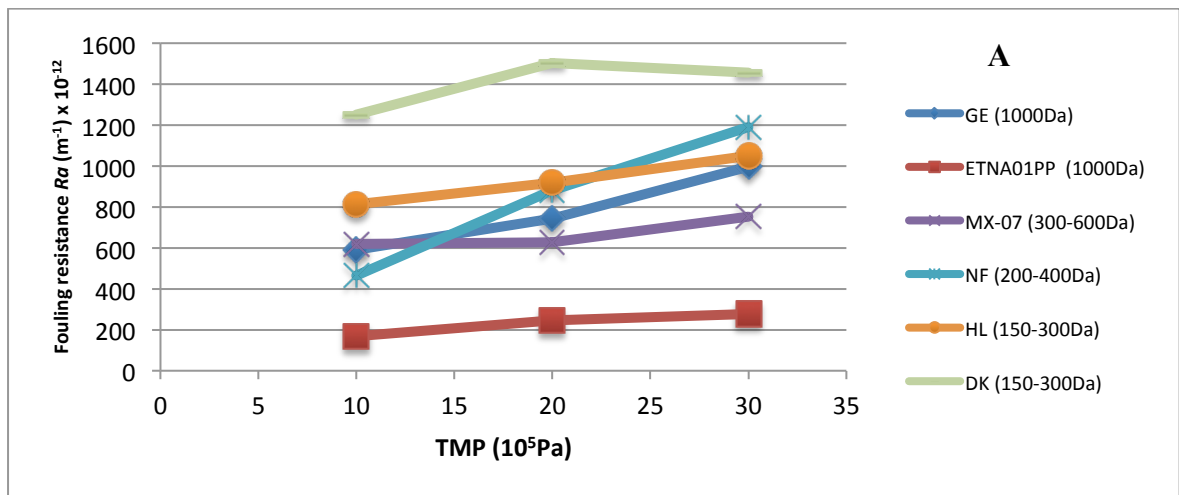
With respect to the influence of the operating temperature, there is a decrease of J_V from 29.95 to $27.04 \text{ L h}^{-1} \text{ m}^{-2}$ when the temperature is increased from 20 to

40 °C (see experiments GE-6, GE-7 and GE-8 in Table 3). The negative effect leads to lower mass-transfer coefficients at higher temperature, contrary to the film model (Hoek, Kim, & Elimelech, 2002). These results can be explained by a greater fouling of the membrane at higher temperatures, which can be due to the formation of a gel layer of pomace extracts (essentially pectins, glucans) at the membrane surface. Similar negative temperature effects were observed by Jiraratananon and Chanachai for passion fruit juice ultrafiltration (Jiraratananon & Chanachai, 1996).

3.3.3.3. Fouling resistance

The total resistance (R_t) could be defined with the results of the filtrate flux for a given pressure. Then, resistance created by fouling and/or concentration polarization (R_a) during grape pomace extract filtration was calculated as the difference between total resistance (R_t) obtained during the filtration experiment and membrane resistance (R_m):

$$R_a = R_t - R_m$$



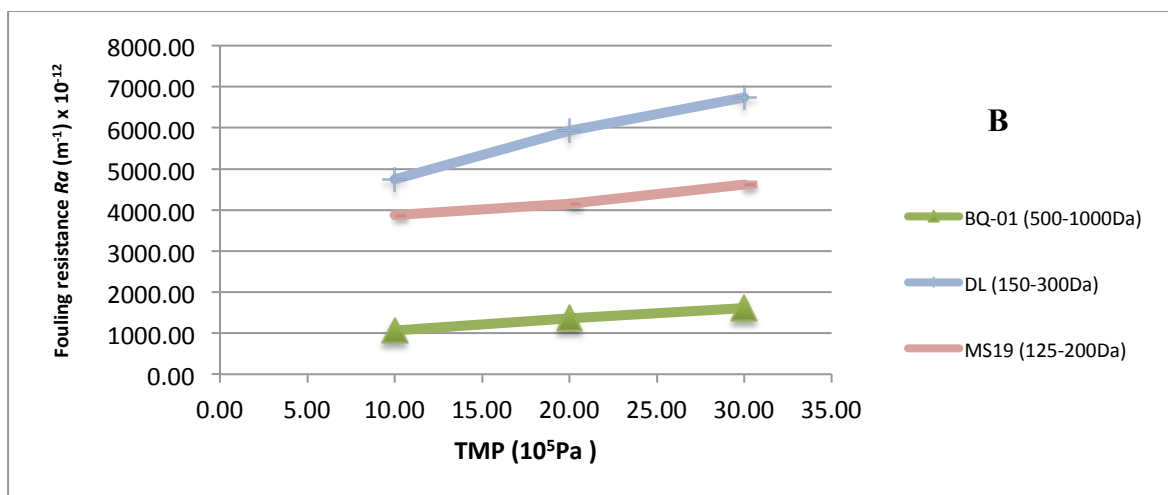


Figure 7. Evolution of fouling resistance for nanofiltration membranes tested: .; on the steady-state permeate flux for experiments performed at $v = 2 \text{ m s}^{-1}$ and $T = 20^\circ\text{C}$ A (Low fouling); B (High fouling)

Fouling resistance (R_a) includes intrinsic membrane fouling resistance, fouling layer resistance and resistance due to concentration polarization phenomena and/or gel layer formation (Bernat et al., 2009; Butylina, Luque, & Nyström, 2006). As the transmembrane pressure increases, fouling resistance increases for all NF membranes tested. When transmembrane pressure varies from 10 to $30 \cdot 10^5 \text{ Pa}$, R_a increases up to 3 times, depending on the membranes.

In most cases, R_a could be separated in two categories: low and high fouling membranes. The higher fouling resistance was observed with the more hydrophilic membrane (DL) having the lesser contact angle (27°). Comparing GE membrane and ETNA01 membranes, the material could explain the more important fouling with the GE membrane made of polysulfone. The same observation was made on the tested ultrafiltration membranes. For the same MWCO (150-300 Da), the DL and HL membranes have very different fouling resistances ($6000 \cdot 10^{12}$ and $900 \cdot 10^{12} \text{ m}^{-1}$) even with the same material (thin film) and similar contact angles (27 and 32 respectively). HL is a typical composite membrane, it consists of three layers: a thin top selective polyamide layer of a few hundred nanometers in thickness (poly(piperazine-amide)), an asymmetric microporous polysulfone support layer, and a polyester non-woven fabric layer for mechanical strength. A mean pore radius of $0.46 \pm 0.08 \text{ nm}$ was obtained by Silva et al., 2016 supposing slit pores in retention measurement. The top active layer of DK and DL consists of three sub-layers, as opposed to the HL top active layer composed of two sub-layers. Furthermore, these

thin film composite (TFC) membranes typically suffer from compaction effects under pressure. At the pH of the extracts tested (pH 3.9), the membranes are positively charged or close to neutral for the HL membrane (isoelectric points (pHi) such as 3.9, 4.8 and 4.0 for HL, DL and DK membranes, respectively), (Chandrapala et al., 2016). This pH of the extracted near the isoelectric point for the HL membrane could explain minimizing interactions between the compounds of the extract and the surface of the membrane. All these parameters (contact angles, composition, pHi, ...) could explain the differences observed with the various membranes tested. It is generally recognized that membrane hydrophobicity/hydrophilicity, pore size (and their distribution) and surface charge may be important factors determining separation performance and the fouling tendency of nanofiltration membranes (Al-Amoudi, 2013). For the other membranes, no correlations were observed between fouling resistance (R_d) and the known parameters: nominal MWCO and contact angle. The structure of the membrane, nature of materials and the different interactions most likely explain the differences observed.

3.3.3.4. Phenolic compounds fractionation

Table 4 shows the composition of the subcritical extract with the main families of molecules, which was used in the NF experiments. The extract was acidic in nature (3.9 ± 0.1) probably due to the wine organic acids and phenolic compounds. Furthermore the extract was rich in total phenols (3309 mg/L) determined at 765 nm. Different phenolic classes like phenolic acids, flavonols and anthocyanins were found in the ranges of 243.7, 46.6 and 153.6 mg/L in the extract. Finally, the majority of the detected flavan-3-ols were found to be polymeric form (153 mg/L), whereas the concentration of respective monomeric compounds was negligible (76,3 mg/L).

Table 4. Characteristics of the grape pomace extracts used as feed liquids. Values represent mean \pm standard deviation (n = 6).

pH		3,9 \pm 0,1
Sugars	mg/L	4096 \pm 217
Total polyphenols	mg/L	3309 \pm 366
Phenolic acids	mg/L	244 \pm 90
Polymeric flavan3-ol	mg/L	153 \pm 8,5
Catechin	mg/L	76,3 \pm 3,8
Quercetin	mg/L	46,6 \pm 5,9
Taxifolin	mg/L	21,3 \pm 2,3
Anthocyanin	mg/L	153,6 \pm 12,4

Table 5 shows the retention percentage of the permeate flux in terms of sugars, flavonoids and anthocyanins for all the NF membranes investigated. The initial feed showed a content of anthocyanins similar to that reported by (Díaz-Reinoso et al., 2010; Díaz-Reinoso et al., 2009; Santamaría et al., 2002) in grape pomace extract.

All the NF membranes investigated presented high average rejections towards polymeric flavan-3-ol (in the range of 59 to 100%) and while for other families macromolecules such Catechin (in the range 23.0–99.4%) the range was variable. Sugar compounds were weakly retained by the several of the majority of the membranes (22.8%) and while the lowest molecular weight membrane showed a high retention of the compounds.

In particular, the membrane, with the lower range MWCO (150-400Da), showed the high average rejection towards flavonoids and anthocyanins (95.4% and 95.9%, respectively) but not phenolic acid. The DL membrane retained all flavonoids and anthocyanins in the retentate side (rejection of 82.4 and 87.2% towards flavonoids and anthocyanins, respectively); in contrast, about 64 % of sugars were

measured in the permeate stream. The Fluoropolymer membrane ENTA01PP showed low average rejections towards all the families of macromolecules in comparison to thin film membranes this may be due to the hydrophobicity of the membranes and the lower fouling.

Sugars on the hand show lower rejection rates to similar molecular weight phenolic acids. With a rejection of the MX-07 membrane towards sugar compounds was 32.7%, while for phenolic acids it was 38,6%. These results were different to that obtained to HL membrane with a higher rejection of sugars 93,6 % in comparison to phenolic acid 74,7%. Thus the use of the HL membrane for fractionation may lead to a certain recovery of phenolic acids in the permeate stream, indicating that this membrane offered the best separation of phenolic compounds from sugars.

Basically, the rejection of NF membranes towards the analysed compounds decreased by increasing the MWCO of the selected membranes. However, the rejection of all selected membranes towards anthocyanins was higher than 52%. This behavior can be explained assuming that anthocyanins, unlike other subgroups of flavonoids with a similar C6-C3-C6 skeleton, have a positive charge in their structure at acidic pH (the pH of the pomace extract is 3.9). At this pH most of the membranes exhibit a positive charge (Boussu et al., 2008). Consequently, the electrostatic repulsion, independent of the MWCO of the selected NF membranes, contributes to the high average rejection of the membranes towards anthocyanins.

In terms of retention these results are very similar to those reported in the NF treatment of orange peel residues with the Osmonics DL membranes (Conidi et al., 2014). Two different NF membranes have been used to recover flavonoids and anthocyanins from press liquor obtained from pigmented orange peels (Conidi et al., 2012). The first (NF70, 180 Da) showed flavonoids and anthocyanins rejection values of greater than 90%, whereas the second (NF200, 300 Da) showed rejections of greater than 85% for these components. The two membranes are made of semi-aromatic piperazine based polyamide skin layer and have different MWCO: 180 and 300 Da which could explain the higher rejection of the compounds with the NF70. Also Diaz et al. (2009) recovered total phenols from fermented grape pomace using two different NF membranes (Nanomax 95- polyamide and Desal DL 2540 with MWCO of 250 and 150-300 Da, respectively). According to this study, the Desal DL

2540 was much more effective: 80% rejection instead 25% for Nanomax. Diaz et al. (2010) also tested NF membranes (Nanomax 95 and Nanomax 50 with an MWCO of 250 and 350 Da, respectively) to recover phenolic compounds from liquors obtained by pressing distilled grape pomace. The highest rejection was obtained using Nanomax 50 (97% compared with 52% for Nanomax 95). In addition, using a fine NF membrane, it is possible to recover more than 95% of polyphenols from olive mill wastewater. For instance, Coskun et al. (2010) used three different NF membranes (NP030, NP010, and NP270) to treat olive mill waste water. According to the results, these membranes were able to remove chemical oxygen demand associated with polyphenol content in terms of retention efficiency and high permeate fluxes. Besides, low-MW polyphenols such as hydroxytyrosol, protocatechuic acid, catechol, tyrosol, caffeic acid, p-coumaric acid, and rutin were concentrated by Cassano et al. (2013), using an NP90 membrane (100% polyphenol rejection).

The above information has shown that array of membrane fractionation is large. Consequently the process could be adapted to produce fractions with different phenolic content and purities thus could be utilized in different applications. Depending on the targeted family of molecules the separation of phenolic seems to be possible with the application of NF membranes. For instance the HL and NF membrane could be used to separate phenolic acids, since were passed into the permeate stream (57% retention), while the catechins and quercetins were partially retained in the concentrate stream by MX07 and BQ01. Likewise, the BQ01 permeate stream sustained the anthocyanins, as the retentions were at 52%. The higher retention of anthocyanins in comparison to catechins and quercetins could explained by the fact that anthocyanins structure with higher positive charges that interacts with membranes (Galanakis et al., 2013). While the GE and the ENTA membranes could be used to separate polymeric proanthocyanidins. Although, the performance parameters of these membrane processing were very satisfying, since permeate flux, were relatively high (average $1.08 \text{ L/h m}^2 10^5\text{Pa}$), significant attention should taken to fouling. Eventually, nanofiltration could be utilized in order to concentrate specific phenolic classes. In particular, the elimination of sugars and water at the same time the retention of phenolic classes using the HL membrane with a permeate flux of ($1.15 \text{ L/h m}^2 10^5\text{Pa}$).

Table 5 Retention coefficients (%) obtained for several parameters of subcritical grape pomace extracts as a function of different nanofiltration membranes

Group of micromolecules	Molecular weight range g/mol	1 kDa ENTA01PP	1 kDa GE	BQ-01 (500-1000Da)	MX-07 (300-600Da)	DK (150-300Da)	NF (200-400Da)	HL (150-300Da)	DL (125-300Da)	MS-19 (125-200Da)
Sugars	180	8,1 ± 1,2	24,1 ± 2,0	47,0 ± 3,1	32,7 ± 2,3	64,2 ± 1,0	69,2 ± 5,7	99,6 ± 0,1	99,5 ± 0,1	99,1 ± 0,3
Total phenolic compounds		46,0 ± 5,9	43,1 ± 3,4	64,6 ± 6,0	74,7 ± 5,0	82,1 ± 2,5	95,1 ± 5,4	97,8 ± 0,5	100,0 ± 0,2	100,0 ± 0,6
Phenolic acids	170-198	25,7 ± 3,9	18,3 ± 2,3	36,4 ± 3,1	38,6 ± 4,0	42,2 ± 5,9	57,5 ± 5,3	74,7 ± 3,3	93,6 ± 0,5	86,6 ± 0,4
Polymeric flavan3-ol	579-867	59,3 ± 4,0	73,8 ± 5,0	89,0 ± 5,0	95,7 ± 5,3	98,6 ± 2,2	100,0 ± 2,5	100,0 ± 0,7	100,0 ± 0,8	100,0 ± 0,7
Catechin pK=4.6	290	22,0 ± 3,0	35,5 ± 3,8	54,6 ± 3,6	42,3 ± 4,1	78,3 ± 4,7	95,3 ± 2,6	98,3 ± 0,4	100,0 ± 0,5	100,0 ± 0,6
Quercetin pK>7	302-508	62,2 ± 4,8	54,9 ± 3,9	70,3 ± 5,6	91,1 ± 2,8	99,2 ± 1,0	100,0 ± 1,0	100,0 ± 0,8	100,0 ± 0,6	100,0 ± 0,7
Taxifolin pK=7,4	450	36,5 ± 4,5	45,0 ± 4,1	61,1 ± 3,5	76,2 ± 4,6	73,9 ± 1,0	100,0 ± 1,3	100,0 ± 0,5	100,0 ± 0,5	100,0 ± 0,2
Anthocyanins	287-639	62,9 ± 7,6	53,6 ± 5,4	52,5 ± 3,2	87,0 ± 7,7	87,3 ± 4,3	100,0 ± 1,0	100,0 ± 0,4	98,4 ± 0,7	96,3 ± 0,4

3.3.4. Conclusion

The current study suggests that the fractionation as well as the recovery of valuable compounds from grape pomace extracts is possible with the utilization of membrane technologies. The separation of these ingredients was mainly governed from the characteristics MWCO of the applied membranes. With regard to grape pomace extract used, the membranes possessing MWCO between 1000 to 500 Da were able to quantitatively recover polymeric proanthocyanidins in concentrate stream and separate it from phenols that pass into the permeate stream. On the other hand, the 600 to 300 Da membranes could also be utilized for the fractionation of monomeric phenolic families. The membranes were able to partially remove the anthocyanins fragments of phenolic acids derivatives and flavonols in the concentrate stream. This process would improve the value of the different families due to their purity. Finally, nanofiltration could be used in order to fractionate and concentrate the grape pomace extracts.

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CHAPTER CONCLUSION

The potential advantages of membrane technologies for the recovery and fractionation of high added value compounds from grape pomace subcritical water extract has been successfully demonstrated. This has including improved product qualities that are generally limited for the characteristics of the membranes by the fouling. Currently, the work on the recovery of bioactive compounds from grape pomace by ultra and nanofiltration is relatively scarce. The results obtained have demonstrated that the use of membrane technologies could bring innovative changes in the recovery of bioactive compounds for future industries. The development of membrane science and technology in order to create more selective membranes for specific components is very important. This work has shown large functionality of the membranes for recovering specific components. It is clear the fundamental the properties and characteristics of the membranes (pore size, affinity, material), and the operating conditions play an important role of the separation of components by ultra and nanofiltration membranes. Finally, the recovery of macro and micromolecules can be carried out by UF and NF membranes.

GENERAL CONCLUSION

As part of VALUXTRACT research project, this work has focused on the study of eco-innovative processes to extract and purify of high value compounds from grape pomace produced in the wine industry. The review highlighted the main alternative technologies applied or potentially utilizable for the extraction of high added value compounds from wine and vine byproducts on the industrial and laboratory scale. We have shown, moreover, the important potential of these methods. This work has focused on the one hand, on the optimization of subcritical water extraction of polyphenols from grape pomace and, secondly, on the assessment of membrane technologies in an overall process of fractionation and recovery of bioactive compounds.

To do this, The first part of our work screened the phenolic compositions of by-products obtained after vinification of different grape varieties, in order to assess their potential content in high added value compounds after the subcritical water extraction. High amounts of anthocyanins and Flavan-3-ols were recovered from fermented grape pomace using differential temperatures with a high variability between by-products. Contrary to anthocyanins, high extraction temperatures (about 200 °C) yielded higher amounts of tannins. Overall, we found that grape pomace antioxidant activity and total polyphenols quantified by Folin Ciocalteu method were not directly related to the main polyphenol content in SWE extracts. The data obtained here using laboratory-scale equipment will be useful to develop an industrial scale SWE process. Finally as observed, grape pomace by-products can be considered as an important source of polyphenols. In this regard, this global characterization may potentially provide the basis for a sustainable process of integrated exploitation of winemaking by-products as potential, inexpensive, and easily available sources of bioactive compounds for the pharmaceutical, cosmetic, and food industries.

Furthermore, the extraction of these molecules would require a specific consideration in order to facilitate their extraction and increase the recovery yield. Different parameters influence the extraction such as temperature, pressure, hydraulic retention time, volume of extraction that are correlated directly to the kinetics of extraction and degradation of specific molecules. The SWE extracts had comparable or higher levels of anthocyanins and ORAC values than extracts obtained using conventional hot water or 50% ethanol. Subcritical water at 100°C appears to be an excellent alternative to organic solvents to extract anthocyanins from grape pomace and possibly other grape processing byproducts. While the optimized subcritical extraction for flavonols was at 175°C, giving a predicted total yield of flavonols of

190mg/g DM from grape pomace. Additionally by adjusting the hydraulic retention time of the extraction, degradation effects were minimized, and optimum extraction could be achieved using 1.7 min. Results obtained also suggest that new antioxidant compounds such as Hydroxymethylfurfural and Furfural were formed at the higher extraction temperatures.

After the subcritical water extraction, extracts produced are rich in several families of molecules. An essential purification step of target compounds prior to industrial use was indispensable. Coupling the subcritical water with membrane processes offers an innovative solution for the purification of these extracts.

Ultrafiltration (UF) was used for the fractionation of phenolic compounds from subcritical water grape pomace extract and their separation from other co-extracted components. Thereby, the extract was assayed in a cross-flow apparatus against eleven membranes with molecular weight ranging from 100 to 2 kDa. The monitoring of the process was carried out by determining performance parameters and retention coefficients of proteins, polysaccharides, sugars, phenolic and anthocyanins classes. Results indicated that solutes retention was affected mainly by severe fouling phenomena due to polar solutes adsorption on membrane surface instead of size exclusion. Indeed, polysulfone membranes were not able to fractionate phenolic classes except for the separation obtained between polymeric and monomeric proanthocyanidins. Membranes lower than 20 kDa retained high percentages (i.e. >60%) of polysaccharides and proteins.

Nanofiltration membranes, on the other hand, were able to fractionate different phenolic classes and sugars, as they were retained at high percentages on the wide range of the membranes utilized. Consequently the process could be adapted to produce fractions with different phenolic content and purities thus could be utilized in different applications. Depending on the targeted family of molecules the separation of phenolic seems to be possible with the application of NF membranes. For instance the HL and NF membrane could be used to separate phenolic acids, since they passed into the permeate stream (57% retention), while the catechins and quercetins were partially retained in the concentrate stream by MX07 and BQ01. Likewise, the BQ01 permeate stream sustained the anthocyanins, as the retentions were at 52%. The higher retention of anthocyanins in comparison to catechins and quercetins could be explained by the fact that anthocyanin have a higher structure positive charge that interacts with membranes. The GE and the ENTA membranes could be used to separate polymeric proanthocyanidins. Although, the performance parameters of these membranes were very satisfying, since permeate flux, were relatively high (average 1.08 L/h m² 10⁵Pa), significant attention should be taken to fouling. Eventually, nanofiltration

could be utilized in order to concentrate specific phenolic classes. In particular, the elimination of sugars and water and at the same time the retention of phenolic classes using the HL membrane with a permeate flux of $(1.15 \text{ L/h m}^2 10^5 \text{ Pa})$.

Perspectives

After this work, several aspects of the impact of technology on the extraction and purification of bioactive molecules studied remain to be elucidated:

- Assess the impact of coupling alternative technologies, for example PEF which was used in the project as a pretreatment technique.
- Further study the contribution of thermal on damage of thermolabile phenolic compounds during the extraction.
- Additional analysis membrane resistances by determining irreversible fouling obtained by flow measurements after membrane cleaning, to conclude the retention mechanisms and clogging of the different membranes: adsorption and internal fouling from the deposit.
- Further consideration of the impact of diafiltration on the selected VRF to improve the separations of compounds.
- Expose the industrial potential of the different fractions produced, in the case of the project as oenological tannins or for other applications in the cosmetic or pharmaceutical industry.

Technologically, it would be interesting:

- To study the feasibility of a treatment SWE using semi-continuous or continuous method on an industrial scale, by using a countercurrent extruder as an example. It would be also important to validate the technical feasibility of the implementation of the membrane technologies on a larger scale.
- To extend the study SWE to other varieties of grape pomace or other plant biomass (shoots, stems ...) to validate the value of this technology across a wine distillery.

Résumé général

Une bio raffinerie est un ensemble industriel complexe permettant la transformation de la biomasse agricole, forestière et algale en une variété de produits bio-sourcés commercialisables (ingrédients et suppléments pour alimentation humaine et animale, biomolécules, agro-matériaux) et / ou de la bioénergie (biocarburants, électricité, chaleur). La bio raffinerie cherche à valoriser l'ensemble de tous les composants de la biomasse. Pour ce faire, la bio raffinerie nécessite des étapes de prétraitement, de fractionnement/purification et de conversion de la matière première pour la production optimisée de produits à haute valeur ajoutée. Pour être économiquement viable et adapter une perspective de développement durable, la bio raffinerie doit satisfaire à une double exigence: la compétitivité de ses coûts de production et l'utilisation des produits et des procédés respectueux de l'environnement, sans production de déchets additionnels (impact minimal sur l'environnement).

Une distillerie est un exemple de bio raffinerie, qui cherche à valoriser les sous-produits récupérés à partir du processus de vinification.

Les raisins (*Vitis vinifera* L.) sont l'un des fruits les plus cultivées dans le monde avec une production annuelle de 58 millions de tonnes en 2012 (FAOSTAT 2012). Environ 80% de la production sont utilisés pour la vinification. Les principaux résidus sont formés après l'étape de pressurage. A ce stade, le marc est riche en alcool. Selon la réglementation européenne (règlement CE 555/2008 de la Commission du 27 Juin 2008), ces "sous-produits" doivent être éliminés de manière respectueuse de l'environnement. Pour les vignerons français, l'Etat oblige soit:

- au compostage, méthanisation ou de l'épandage des sous-produits de tout ou partie de leurs résidus sur leurs propres terres
- à la livraison des marcs de raisin dans une installation de méthanisation, compostage, ou une distillerie (n ° 2014-903 décret du 18 août 2014, Art D. 665-34.-I.).

En France, environ 50 distilleries collectent les sous-produits dans un rayon moyen de 50 km de distance autour de leur site et permettent la récupération d'environ

850 000 tonnes de marc de raisin chaque année (Institut français de la vigne et du vin, Novembre 2013).

Jusqu'à présent, les distilleries assurent le rôle de traitement de la totalité de la charge polluante des marcs de raisin sur le territoire national, pour des raisons de qualité (limitation des sur-pressurages des raisins et de la qualité du vin) et des règlements (lutte contre la fraude et garantir la réglementation douanière). Cependant, le Décret n ° 2014-903 18 Août, 2014 abolie l'obligation de livrer les sous-produits à la distillerie, menaçant ainsi la fourniture de matières premières. La compétitivité et la rentabilité de l'industrie de la distillerie est basée, par conséquent, sur l'amélioration et la modernisation des processus de traitement. Le levier principal d'amélioration est l'extraction et la purification de composés à haute valeur ajoutée à partir des sous-produits. Le secteur a donc tout intérêt à se déplacer vers une approche de type «bio raffinerie » maximisant les moyens d'utilisation des sous-produits.

Aux distilleries, les marcs sont transformés en divers sous-produits (figure 1) de plus ou moins forte valeur ajoutée (alcool, huile de pépins de raisin, engrais, acid tartic, colorants etc.).

Ces sous-produits sont utilisés comme matières premières dans les différents secteurs (industries de l'agriculture, l'viticulture, chimiques, cosmétiques et alimentaires). Ce processus permet une valorisation des sous-produits (compost, aliments pour animaux, produits chimiques ...) et / ou de l'énergie (bioéthanol, biogaz ...).

En raison des équipements industriels, la distillation et l'extraction de l'acide tartrique sont actuellement les principales méthodes de valorisation dans la distillerie. Cependant, l'extraction des composés phénoliques peut être intégrée dans le processus de valorisation.

Elle permettrait une diversification des activités de distillerie grâce à l'intégration d'une nouvelle étape, le fractionnement de la biomasse végétale pour extraire des composés à haute valeur ajoutée. Les marchés de ces produits sont nombreux: le vin, les produits alimentaires (colorants, conservateurs naturels), la santé (compléments alimentaires, médicaments), les cosmétiques (antioxydants naturels) ou l'industrie chimique (colle adhésive verte).

Cependant, pour des extraits dans une industrie (à savoir Naturex, BERKEM, CHR Hansen, DIANA Ingrédients, Oenofrance...), la distillerie doit proposer des

extraits avec une composition phénolique particulière ouvrant ainsi des marchés spécifiques. Indéniablement, l'application éventuelle d'un extrait de plante est essentiellement déterminée par la composition phytochimique qui est particulièrement dépendants de la matière première utilisée et la méthode de fabrication de l'extrait.

C'est dans ce contexte global de récupération de bio-composés et en minimisation des impacts environnementaux que s'inscrit le projet de recherche VALUXTRACT. L'objectif global du projet VALUXTRACT est la récupération de composés à haute valeur ajoutée à partir de déchets solides de la vinification par l'emploi de méthodes «vertes» dans le but de produire des extraits pour les applications œnologiques principalement, mais aussi pour les aliments, les cosmétiques et les industries pharmaceutiques.

* * *

Cette thèse a été réalisée dans le cadre du projet européen "Valuxtract", financé par l'Agence Nationale de la Recherche française (ANR) dans le cadre du 1^{er} appel transnational de ECO-innova (ERA-NET, ANR-12-INOV- 0001-04). Il a été mené au sein de l'Unité de Recherche Œnologie situé à l'Institut des Sciences de la Vigne et du Vin, Villenave d'Ornon - France. Elle a été réalisée sous la direction du Professeur Martine Mietton-Peuchot.

Une partie de ce travail a été réalisé en étroite collaboration avec l'Université de Changins - Haute Ecole de viticulture et œnologie, l'Université Hochschule Geisenheim - Institut für Œnologie, l'Université de Compiègne - Laboratoire Transformations Intégrées de la Matière Renouvelable et le laboratoire Phenobio - Martillac.

Le manuscrit se compose de cinq publications organisées en trois chapitres, (en presse ou soumis au moment de la rédaction) qui reflètent le fruit des résultats obtenus:

Le premier chapitre présente une vue d'ensemble sur l'extraction et la purification de composés à haute valeur ajoutée à partir des sous-produits de la chaîne de vinification en utilisant des procédés / technologies alternatives / non-conventionnelles.

Le deuxième chapitre est composé de deux publications liées à l'optimisation de l'extraction de composés à haute valeur ajoutée à partir de marc de raisin en utilisant l'eau sous critique. La première publication présente les résultats de l'étude portant sur

l'optimisation des rendements d'extraction des composés phénoliques utilisant des matières premières multiples.

La seconde publication décrit l'optimisation du processus d'extraction du marc de raisin par l'eau sous critique. Les principaux résultats de l'optimisation et de la sélectivité de ce procédé sont décrits en détail.

Le chapitre trois compile deux publications qui traitent du fractionnement et de la concentration de composés à haute valeur ajoutée à partir d'extraits par procédés membranaires.

Ce chapitre se concentre sur l'étude du fractionnement de l'extrait obtenu par ultrafiltration avec pour but de séparer des macromolécules pour obtenir un extrait riche en composés phénoliques. La dernière publication se concentre sur l'utilisation de la nanofiltration pour le fractionnement des différentes familles de composés phénoliques.

CHAPITRE 1: ETAT DE L'ART

Tout au long du projet Valuxtract un chapitre de livre a été écrit "Yammine, S., Ghidossi, R. & Mietton-Peuchot, M., 2014. Extraction et purification des composés phénoliques issus des sous-produits de la vinification. Dans Y. El Rayess, ed. Vin: Composition phénolique, classification et bienfaits pour la santé. NOVA éditeurs scientifiques, pp. 313- 330 ". En outre, avec les partenaires du projet, une review, a été rédigée pour faire un état des lieux de toutes les publications traitant de ce sujet. La review soumise évoque les principales technologies appliquées ou potentiellement utilisables pour l'extraction de composés à haute valeur ajoutée du vin et des sous-produits de la vigne à l'échelle industrielle et laboratoire. Pour fournir une présentation globale de chaque technologie utilisée, tous les paramètres du procédé et leurs limites sont discutés. Les principales approches telles que l'extraction par liquide sous pression, l'extraction assistée par ultrasons, l'extraction micro-ondes assistée par solvant, l'extraction par fluide supercritique ou sous critique, par champs électriques pulsés (CEP) et par décharges électriques hautes tensions (DEHT) sont principalement traités. Ces technologies sont encore en cours de développement, et jusqu'à présent peu ou pas d'upscaling industrielle n'ont été remarqués. Par conséquent, ces technologies sont actuellement étudiées et sont des sujets qui font l'œuvre de nombreux articles scientifiques.

Article #1

La valorisation des déchets de cave est très prometteur, puisque le raisin est l'une des cultures de fruits le plus produit avec une production mondiale annuelle de 58 millions de tonnes en 2012. Environ 80% est utilisé pour la vinification et il a été estimé que 13 à 20% des sous-produits (ce qui représente environ 5-8 millions de tonnes de matières potentiellement exploitables), sont générés après le processus de vinification. D'autres estimations font état de valeurs plus élevées (jusqu'à 14,5 millions de tonnes uniquement en Europe). Cela représente sans aucun doute une énorme quantité de matière à partir de laquelle les composants à haute valeur ajoutée pourraient être extraites. Les déchets solides de raisin sont particulièrement riches en polyphénols et leur utilisation peut s'appliquer dans divers domaines tel que les cosmétiques, la nutraceutiques, les produits chimiques et les industries agro-alimentaires. Au cours de ces dernières années, un intérêt croissant s'est porté sur les

polyphénols pour leurs bienfaits potentiels sur la santé (prévention des maladies cardiaques et des cancers). Leur extraction à partir de déchets de cave et de leur purification présentent un intérêt particulier pour produire des extraits à forte valeur ajoutée.

Les composés phénoliques sont habituellement extraits par la procédure d'extraction classique. La variabilité naturelle des procédés de pré-transformation des matières premières (séchage, broyage, etc.) pourrait être déterminante pour la quantité et la composition de l'extrait. Par exemple, des températures élevées peuvent conduire à la dénaturation de composés ciblés et un broyage à une augmentation significative des composants indésirables lors de l'extraction. Ainsi, les procédés de pré-transformation classiques diminuent la sélectivité et / ou l'efficacité du procédé d'extraction. La sélectivité des procédés d'extraction dépend aussi de l'affinité moléculaire entre le solvant et le soluté pendant l'étape de diffusion solide-liquide. Cependant, la toxicité, la sécurité environnementale et la faisabilité financière doivent également être prises en compte dans la sélection d'un solvant pour l'extraction du composé à haute valeur ajoutée. A la fin de ces procédés, une étape de purification est nécessaire pour obtenir des extraits avec une grande pureté en composés phénoliques. L'adsorption sur résine est couramment utilisée à l'échelle industrielle. L'inconvénient majeur de cette technique est l'utilisation d'une grande quantité de solvant qui doit ensuite être évaporée.

Les pertes de certains composés, la faible efficacité de production, des procédures fastidieuses et énergivores (chauffage prolongé, agitation, utilisation de grands volumes de solvant ...) peuvent être rencontrés en utilisant ces procédés classiques d'extraction. Les tendances récentes dans ces techniques d'extraction ont largement mis l'accent sur la recherche de solutions qui minimisent l'utilisation de solvant et d'énergie. Ainsi, des techniques alternatives ont été étudiées pour améliorer les rendements globaux d'extraction en composés phénoliques et donc diminuer les coûts de fonctionnement. Ces techniques comprennent:

- les procédés de pré-traitements alternatifs: ultrasons, champs électriques pulsés et décharges de hautes tensions,
- l'extraction non-conventionnelle par solvant sous haute pression: extraction par fluide supercritique et extraction par eau sous-critique et,

- les technologies de purification alternatives, telles que les procédés membranaires.

Bien que beaucoup d'études expérimentales ont été réalisés et particulièrement axées sur l'amélioration de l'ensemble des procédés d'extraction, aucune de ces technologies alternatives n'est actuellement utilisée à l'échelle industrielle. Le présent document vise à décrire les mécanismes impliqués par ces technologies alternatives et ainsi résumer le travail accompli sur ces améliorations. Dans cette review, il est montré le stade de développement de chaque technologie, soulignant la nécessité et les défis à surmonter dans le domaine de la recherche pour une mise en œuvre industrielle dans le processus global d'extraction. Une comparaison critique des techniques classiques et de substitution sera décrite pour le prétraitement des matières premières, la diffusion de polyphénols et la purification de ces composés à haute valeur ajoutée. Cette review vise à donner au lecteur des réponses clés (coûts, avantages, inconvénients) pour l'aider dans le choix des technologies de remplacement à des fins d'extraction.

La récupération de composés à haute valeur ajoutée à partir des sous-produits de vinification se développe rapidement et la clé d'une extraction/purification réussie est la mise au point de méthodes performantes permettant de conduire à des extraits qui répondent aux normes de qualité des consommateurs. Plusieurs de ces nouvelles technologies présentées, comme l'extraction par eau sous-critique et la purification par procédés membranaires ont déjà montré leur efficacité. Cependant, comme présenté dans cette review, la mise à l'échelle de ces techniques n'est pas aussi simple à réaliser dans la pratique et dépend surtout des propriétés fonctionnelles des composés ciblés.

Après toutes ces considérations, les restrictions de rendement et la balance énergétique doivent également être étudiés. Pour le procédé de prétraitement, les technologies PEF et HVED ont montré leur efficacité avec une faible consommation d'énergie par rapport à d'autres techniques. Cependant, d'autres recherches sont nécessaires pour comparer l'énergie utilisée dans les différentes technologies d'extraction et de purification.

ENJEUX ET CONSIDERATIONS

Pour rester compétitifs, les distilleries ont intérêt à faire évoluer leur activité vers une approche de bioraffinerie intégrée, notamment grâce à la multiplication du nombre de produits issus de la valorisation du marc de raisin, en particulier les produits à haute valeur ajoutée tels que les polyphénols. Ces produits représentent de nouvelles opportunités dans les différents secteurs en fonction de leur composition phytochimique et de leur pureté.

La méthode actuelle d'extraction de composés phénoliques présente certains inconvénients tels qu'une durée d'extraction conséquente et la nécessité de l'emploi d'une grande quantité de solvants organiques, qui sont nocifs pour la santé humaine et causent un stress environnemental. La régénération du solvant organique utilisé pour cette extraction est également un inconvénient majeur. De ce fait, de nombreuses équipes de recherche travaillent activement dans le développement de technologies d'extraction alternatives ainsi que dans la mise au point de procédés de purification de composés à haute valeur ajoutée à partir de sous-produits de raisin, dans un objectif commun de réduire l'utilisation de solvants. Pour ce faire, ces technologies doivent:

- **Améliorer l'efficacité de l'extraction des composés phénoliques,**
- **Limiter la dégradation des composés thermolabiles,**
- **Réduire les étapes énergivores (régénération du solvant, séchage, concentration,...),**
- **Améliorer la qualité des extraits par purification.**

Une méthode alternative efficace, économique, respectueuse de l'environnement, sûre et rapide, est ainsi nécessaire pour pallier à ces inconvénients. Les techniques les plus courantes, récemment décrites par ailleurs, comprennent l'extraction par fluide supercritique (par exemple, le dioxyde de carbone), l'extraction par liquide sous pression ou l'extraction accélérée par solvant, ainsi que l'extraction de l'eau sous-critique. Parmi ces techniques, l'extraction par eau sous-critique, utilisant de l'eau comme agent d'extraction, est l'une des méthodes les plus intéressantes parce que l'eau est non-inflammable, non toxique, économique et respectueuse de l'environnement. Ces caractéristiques correspondent de manière adéquate aux trois critères mentionnés ci-dessus. À l'heure actuelle, peu d'études comparatives de ces

technologies ont porté sur l'aspect qualitatif de l'extraction et/ou ne permettent pas de conclure sur la sélectivité du processus en raison du choix des paramètres de fonctionnement.

D'autre part, les extraits produits sont riches en plusieurs familles de molécules. Une étape de purification avant l'utilisation industrielle des composés cibles est essentielle. En raison de sa flexibilité, le couplage de l'extraction par eau sous-critique avec un procédé membranaire offre une solution pour la purification de ces extraits. Ce procédé présente l'avantage d'avoir un faible impact environnemental car il utilise de petites quantités de solvants, des températures peu élevées et des pressions d'utilisation basses, répondant ainsi aux critères ci-dessus.

Dans ce contexte, les objectifs de ce projet de thèse sont:

- **Une meilleure compréhension de la variabilité des sous-produits, via l'application de l'extraction par eau sous-critique sur ceux-ci.**
- **Comparer l'efficacité de l'extraction par eau sous-critique sur la composition phytochimique des extraits obtenus afin d'élargir les connaissances sur l'amélioration de l'extractibilité des composés ciblés.**
- **Évaluer l'utilisation de l'ultrafiltration pour d'améliorer la pureté globale de composés phénoliques extraits à partir de marc de raisin.**
- **Tester la nanofiltration pour fractionner et/ou concentrer différentes familles de composés.**

CHAPITRE 2 EXTRACTION PAR EAU SOUS-CRITIQUE DE COMPOSES A HAUTE VALEUR AJOUTEE A PARTIR DE MARC DE RAISIN

Une alternative intéressante aux méthodes d'extraction classiques est l'obtention de composés phénoliques par l'utilisation de l'extraction par eau sous-critique (SWE). Cette technologie utilise de l'eau à des températures comprises entre 100 et 374 °C et à une pression suffisante pour maintenir l'eau à l'état liquide. La température critique et la pression de l'eau sont respectivement 374 °C et 22,1 MPa (Moran & Shapiro, 2006). Dans des conditions "sous-critiques", les liaisons hydrogènes intermoléculaires se rompent et la constante diélectrique de l'eau diminue. La constante diélectrique de l'éthanol et de l'eau pure à la température et la pression ambiante sont 27 et 79, respectivement. Lorsque la température augmente jusqu'à 250 °C, la constante diélectrique de l'eau est réduite à 27, ce qui est comparable à la constante diélectrique de l'éthanol.

D'autre part, les sous-produits de raisin sont une source importante et relativement peu coûteuse d'une large gamme de polyphénols, y compris monomères et oligo-proanthocyanidines ainsi que d'anthocyanes variés qui fournissent des avantages économiques importants. Ce contenu varie en fonction des conditions de croissance de la plante et subit également des modifications en fonction des processus de vinification mis en œuvre. Cet aspect a retenu une grande partie de notre attention dans ce chapitre en raison de la grande variabilité des données de la littérature. Ainsi, le but de ce travail était de caractériser la composition phénolique d'extraits de marc de raisin d'origines variées par eau sous-critique. Le marc traité est issu de quatre cépages différents de *Vitis vinifera* (Chardonnay, Cabernet franc, Merlot, Dunkelfelder). Cette sélection a pour but d'identifier les propriétés intéressantes de ces cépages pour une utilisation en tant qu'ingrédients fonctionnels et de les comparer à des températures d'extraction différentes par SWE. La sélection des variétés analysées dans ce chapitre est basée sur la recherche d'une grande diversité phénolique.

D'autre part, différents paramètres influencent l'extraction comme la température, la pression, le temps de séjour hydraulique, le volume d'extraction. Ces paramètres sont directement corrélés à la cinétique d'extraction et à la dégradation de molécules spécifiques.

Ainsi, le chapitre se compose de deux publications consacrées à la présentation des résultats d'extraction de différentes familles polyphénoliques par extraction par eau sous-critique dans des conditions opératoires variées. Lors de l'extraction des polyphénols de raisin de la variété Dunkelfelder par eau sous-critique, une étude paramétrique a été mise en œuvre pour optimiser les conditions opératoires d'extraction et comparer les résultats à une extraction classique par solvant organique.

Article # 2

L'évaluation détaillée des teneurs totales en composés phénoliques et tanins totaux, la composition monomérique et oligomérique en flavan-3-ol, le profil des proanthocyanidines et le potentiel antioxydant des extraits de marc par eau sous-critiques provenant des quatre variétés de raisin (*Vitis vinifera* L.) a été menée. Des différences significatives ($p < 0,05$) de la teneur en composés phénoliques totaux, teneur en tanins totale et la capacité antioxydante de l'extrait de marc de raisin par eau sous-critique ont été observées entre les différents cépages étudiés. Pour la première fois dans la littérature, la composition des quatre cépages en flavan-3-ol a été décrite pour la totalité de leur fraction extraite par eau sous-critique. La composition phénolique et la capacité antioxydante des marcs de raisin extraits ont été comparées. La caractérisation globale de ces extraits par eau sous-critique fournit une base pour l'exploitation intégrée de ce sous-produit de la vinification en tant que source potentielle de composés bioactifs facilement disponibles et peu coûteux pour les industries pharmaceutiques, cosmétiques et alimentaires en utilisant une méthode d'extraction «verte».

Article # 3

L'eau, un solvant peu coûteux et respectueux de l'environnement, est un solvant idéal pour l'extraction industrielle de composés phénoliques mais son utilisation est limitée en raison de la faible efficacité de l'extraction à basse température. Dans cette étude, l'extraction par eau sous-critique (SWE) de marc de raisin a été étudiée au travers de quatre variables indépendantes : la pression (25-100 bar), la température (100-200 °C), le temps de séjour hydraulique et le volume d'extraction. Les résultats ont été comparés avec des extractions classiques par solution aqueuse à 50% (v/v) d'éthanol. Les niveaux d'anthocyanes et les valeurs ORAC des extraits SWE sont

comparables ou plus élevés à ceux obtenus par eau chaude classique ou solution à 50 % d'éthanol. L'eau sous-critique à 100 °C semble être une excellente alternative aux solvants organiques pour extraire les anthocyanes à partir de marc de raisin et éventuellement d'autres sous-produits de transformation du raisin. La température d'extraction sous-critique optimale pour les flavonols a été déterminée à 175 °C, ce qui induit un rendement total prévu de flavonols de 190 mg/g MS de marc de raisin. En outre, en réglant le temps de séjour hydraulique de l'extraction, les effets de dégradation ont été réduits au minimum. Une extraction optimale peut ainsi être réalisée à un temps de séjour hydraulique de 1,7 minutes. Les résultats obtenus suggèrent également que les nouveaux composés anti-oxydants tels que l'hydroxyméthylfurfural et le furfural se sont formés à des températures plus élevées d'extraction.

Conclusion du chapitre

L'extraction par eau sous-critique est un procédé d'extraction relativement récent (début de 1995) avec un succès considérable pour différentes applications analytiques dans les pays développés.

La première partie de notre travail a permis de déterminer les compositions phénoliques des sous-produits obtenus après vinification des différents cépages, afin d'évaluer leur contenu potentiel en composés à haute valeur ajoutée après l'extraction par eau sous-critique. La comparaison de plusieurs sous-produits de vinification avec leurs raisins respectifs a fourni des preuves que le marc est une source très riche en antioxydants, flavan-3-ols et anthocyanes. La répartition quantitative et qualitative des polyphénols dans le marc de raisin a montré des différences significatives par le biais de variétés et millésimes.

Cette étude de caractérisation du marc de raisin de Dunkelfelder, Merlot, Cabernet Franc et Chardonnay a mis en évidence que le Dunkelfelder a présenté les fractions les plus intéressantes en raison de leur teneur en polyphénols plus élevées en termes de flavan-3-ols et anthocyanes. Ces extraits ont également démontré les plus hautes capacités antioxydantes au travers de quatre tests différents. Par conséquent, ces variétés ont été choisies pour une optimisation plus poussée par extraction par eau sous-critique.

La deuxième partie du travail a été d'évaluer l'impact des différents paramètres de fonctionnement sur le rendement global d'extraction et d'en tirer un ensemble de conditions qui décrivent le fonctionnement «optimal» dans le cas de l'extraction de substances naturelles telles que le marc de raisin.

L'influence des paramètres d'extraction, tels que la température, le temps de séjour hydraulique, et la pression a également été évaluée. Les résultats ont montré que la température et le temps de séjour hydraulique sont des paramètres critiques à prendre en compte lors de l'étude d'un procédé d'extraction par eau sous-critique.

La technologie "verte" d'extraction par eau sous-critique présente de nombreux avantages par rapport à l'extraction par solvant classique, en particulier la réduction du temps d'extraction et la quantité de solvants organiques nécessaires. Ainsi, de ce point de vue, l'eau sous-critique se révèle être une très bonne alternative à l'extraction par solvant pour les substances naturelles du marc de raisin.

CHAPITRE 3: FRACTIONNEMENT DES DIFFERENTES FAMILLES DE MOLECULES PHENOLIQUES A PARTIR D'EXTRAITS DE MARC PAR DES PROCEDES MEMBRANAIRES.

Dans les chapitres précédents, nous avons montré que les extraits préparés par extraction à l'eau sous-critique sont riches en plusieurs familles de molécules. Une étape de purification des composés cibles est essentielle avant leurs usages industriels. Pour cette étape de purification, le couplage de l'eau sous-critique avec des procédés membranaires représente une solution innovante.

Depuis 30 ans, les technologies membranaires sont utilisées avec succès dans l'industrie agro-alimentaire. Elles sont par exemple retrouvées dans l'industrie laitière, dans la préparation des jus de fruits ou en œnologie. Leurs principaux avantages par rapport aux autres méthodes de purification conventionnelles sont : absence de transition de phase, faibles besoins énergétiques, sélectivité et productivité élevées, transposition à l'échelle industrielle facile.

Théoriquement, la séparation membranaire des molécules cibles peut sembler simple car elle est basée sur un mécanisme de tamisage moléculaire. Néanmoins, le seuil de coupure d'une membrane (MWCO) ne constitue pas une barrière absolue. Les interactions entre la membrane et les molécules cibles jouent un rôle important sur la sélectivité. Par exemple, le caractère hydrophobe de la surface de la membrane peut influencer le pouvoir de solvation des solutés. Un autre problème réside dans le fait que l'extrait est formé de petites et grandes molécules sous forme de colloïdes. Par exemple, les polyphénols lient les protéines dans notre extrait. Cela signifie que selon les caractéristiques structurales des macromolécules, de petites molécules peuvent être récupérées dans le concentrât malgré un poids moléculaire inférieur au MWCO.

Compte tenu des éléments présentés ci-dessus, le présent chapitre explore mécanismes de séparation membranaires et la purification des composés cibles provenant de différents extraits naturels préparés à partir d'extraction de marc de raisin par eau sous-critique.

Le chapitre est organisé en deux parties correspondant à deux publications. La première s'intéresse à la séparation des composés d'intérêt des macromolécules par ultrafiltration (Article 4). La seconde concerne le fractionnement par nanofiltration

des différentes familles de polyphénols afin d'accroître la valeur ajoutée de l'extrait (Article 5).

Article # 4

Le but du travail de recherche présenté est d'étudier la possibilité d'utiliser l'ultrafiltration (UF) pour fractionner les composés phénoliques des extraits par un traitement à l'eau sous-critique (SWE) de marc de raisin et les séparer des autres composants co-extraits. Pour ce faire, des filtrations tangentielles de l'extrait ont été réalisées en utilisant 11 membranes de seuils de coupure compris entre 100 kDa et 2 kDa. L'efficacité des traitements membranaires a été évaluée en déterminant les coefficients de rétention des protéines, des polysaccharides, des sucres, des composés phénoliques et des différentes classes d'anthocyanes. Les résultats ont mis en évidence que la rétention des solutés est principalement affectée par des phénomènes de colmatage sévère liés à l'adsorption de solutés polaires sur la surface de la membrane. En effet, la surface filtrante des membranes utilisées constituée de polysulfone n'est pas capable de fractionner les différentes familles de composés phénoliques à l'exception de la séparation entre les monomères des polymères de proanthocyanidine. A partir d'un seuil de coupure de 20 kDa, les membranes testées ont une capacité de rétention élevée (supérieure à 60%) des protéines et des polysaccharides.

Article # 5

Des essais de filtration de l'extrait de marc de raisin en concentration ont été réalisés dans un équipement de filtration tangentielle de laboratoire. Neuf membranes commerciales de NF de seuils de coupure approximatifs compris entre 1000 Da et 150 Da ont été testés. Au delà de la nature et du MWCO des membranes de NF employées, les expériences de filtration de l'extrait naturel de marc de raisin ont été réalisées en faisant varier les principaux paramètres opératoires : pression transmembranaire, vitesse d'écoulement tangentielle et température. L'évolution des volumes de perméat cumulatifs et des flux de perméat au cours temps de traitement ont été analysés. L'effet des conditions de fonctionnement mentionnées sur le flux de perméat a été discuté. L'efficacité des traitements de filtration a été déterminée par l'évaluation des facteurs de rétention de plusieurs familles de polyphénols.

Enfin, les membranes possédant MWCO entre 1000 à 500 Da permettent de collecter quantitativement des polymères de proanthocyanidines dans le concentrât et d'isoler des phénols dans le perméat. Les membranes de MWCO compris entre 600 et 300 Da peuvent également être utilisées pour le fractionnement des familles de monomères phénoliques. La membrane a permis d'éliminer partiellement à la fois des fragments d'anthocyanes de dérivés d'acides phénols et des flavonols dans le concentrât.

Conclusion du chapitre

Les avantages potentiels des technologies membranaires pour la récupération et le fractionnement de composés à haute valeur ajoutée à partir d'extraits naturels de marc de raisin préparés par traitement à l'eau sous-critique ont été démontrés avec succès. Ceci comprend également l'amélioration de la qualité des extraits; le principal facteur limitant étant le colmatage des membranes. Actuellement, les travaux de recherche sur la purification des composés bioactifs issus du marc de raisin par ultra et nanofiltration est relativement rare. Les résultats obtenus ont démontré que l'utilisation des technologies membranaires pourrait apporter des changements novateurs dans la purification des composés bioactifs pour les industries futures. Le développement de la science et des technologies membranaires afin de créer des membranes plus sélectives pour des composants spécifiques est très important. Ce travail a montré la grande fonctionnalité des membranes pour récupérer des composants spécifiques. Il est clair que les propriétés fondamentales des membranes (taille des pores, affinité, matériel), et leurs conditions d'exploitation jouent un rôle important sur la séparation des composés par des membranes d'ultra et nanofiltration. Enfin, la purification de macro et molécules simples peut être effectuée par UF et NF membranes.

CONCLUSION GÉNÉRALE

Dans le cadre du projet de recherche VALUXTRACT, le travail de recherche a porté sur l'étude de procédés éco-innovants pour extraire et purifier à partir de marc de raisin des composés à haute valeur ajoutée.

La revue bibliographique a mis en évidence les principales technologies alternatives appliquées ou potentiellement utilisables pour l'extraction de composés à haute valeur ajoutée des sous-produits de la vigne et du vin à l'échelle du laboratoire et à l'échelle industrielle. Par ailleurs, nous avons montré, le potentiel important de ces méthodes. Ce travail a porté d'une part sur l'optimisation de l'extraction des polyphénols de marc de raisin par traitement à l'eau sous-critique et, d'autre part, sur l'évaluation des technologies membranaires dans un processus global de fractionnement et de purifications des composés bioactifs.

Pour ce faire, la première partie de notre travail a consisté à déterminer la composition phénolique de sous-produits obtenus après vinification de différents cépages, afin d'évaluer leur contenu potentiel en composés à haute valeur ajoutée après l'extraction de l'eau sous-critique (SWE). Des quantités élevées d'anthocyanes et de flavanes-3-ols ont été récupérées à partir de marc de raisin fermenté en utilisant des températures différentielles avec une grande variabilité entre les sous-produits. Contrairement aux anthocyanes, des températures d'extractions élevées (environ 200°C) ont donné des quantités plus élevées de tanins. Dans l'ensemble, nous avons constaté que la teneur en polyphénols dans les extraits de SWE n'est pas directement liée à l'activité antioxydante du marc de raisin ni à sa concentration en polyphénols totaux quantifiés par la méthode de Folin Ciocalteu. Les données obtenues ici en utilisant l'équipement en laboratoire seront utiles pour développer un processus de SWE à l'échelle industrielle. Enfin, comme observé, les sous-produits du marc de raisin peuvent être considérés comme une source importante de polyphénols. À cet égard, cette caractérisation globale peut potentiellement constituer la base d'un processus d'exploitation durable intégrant l'utilisation des sous-produits de la vinification comme sources potentielles, peu coûteuses et facilement disponibles de composés bioactifs pour les industries pharmaceutiques, cosmétiques et alimentaires.

En outre, afin de faciliter leur extraction et augmenter le rendement de purification un examen spécifique de ces molécules est nécessaire. Différents paramètres influencent l'extraction tels que la température, la pression, le temps de

séjour hydraulique, le volume d'extraction. Ils sont directement corrélés à la cinétique d'extraction et à la dégradation de molécules spécifiques. Les extraits SWE ont des concentrations en anthocyanes et des valeurs ORAC comparables ou supérieures à des extraits obtenus à l'aide d'eau chaude classique ou 50% d'éthanol. Le traitement à l'eau sous-critique à 100°C semble être une excellente alternative aux solvants organiques pour extraire les anthocyanes à partir du marc de raisin ou éventuellement d'autres sous-produits de transformation du raisin. Les conditions optimales pour l'extraction à l'eau sous-critique des flavonols est à 175°C avec un rendement total de flavonols de 190 mg/g MS de marc de raisin. En outre en réglant le temps de séjour hydraulique de l'extraction, les effets de dégradation ont été réduits au minimum. Une extraction optimale peut être obtenue en utilisant un temps de séjour hydraulique de 1,7 minutes. Les résultats obtenus suggèrent également que les nouveaux composés anti-oxydants tels que l'hydroxyméthylfurfural et de furfural sont formés au niveau des températures d'extraction plus élevées.

Après l'extraction par eau sous-critique, les extraits produits sont riches en plusieurs familles de molécules. Une étape de purification des composés cibles avant usage industriel est indispensable. Le couplage de l'eau sous-critique avec des procédés membranaires propose une solution innovante pour le fractionnement et la purification de ces extraits.

L'ultrafiltration (UF) a été utilisée pour le fractionnement des composés phénoliques de l'extrait issu du traitement à l'eau sous-critique de marc de raisin et leur séparation d'autres autres composants co-extraits. De ce fait, l'extrait a traité en filtration tangentielle avec onze membranes de seuils de coupure compris entre 100 kDa et 2 kDa. L'efficacité des traitements membranaires a été évaluée en déterminant les coefficients de rétention des protéines, des polysaccharides, des sucres, des composés phénoliques et des différentes classes d'anthocyanes. Les résultats ont mis en évidence que la rétention des solutés était principalement affectée par des phénomènes de colmatage sévère liés à l'adsorption de solutés polaires sur la surface de la membrane. En effet, la surface filtrante des membranes utilisées constituée de polysulfone n'est pas capable de fractionner les différentes familles de composés phénoliques à l'exception de la séparation entre les monomères des polymères de proanthocyanidine. A partir d'un seuil de coupure de 20 kDa, les membranes testées

ont une capacité de rétention élevée (supérieure à 60%) des protéines et des polysaccharides.

D'autre part, les membranes de nanofiltration, ont permis le fractionnement des différentes classes phénoliques et des sucres, qui ont été retenus à des pourcentages élevés par l'ensemble des membranes utilisées. Par conséquent, le procédé peut être adapté pour produire des fractions à des teneurs et puretés en composés phénoliques différentes qui pourraient donc être utilisés dans différentes applications. En fonction de la famille de molécules ciblées la séparation de composés phénoliques semble être possible grâce à l'utilisation des membranes de NF. Par exemple, les membranes HL et NF peuvent être utilisées pour séparer les acides phénols, car ils passent dans le perméat (57% de rétention), tandis que les catéchines et quercetins sont partiellement retenus dans le concentrât par MX07 et BQ01. Le perméat de BQ01 isole partiellement les anthocyanes dont la rétention était de 52%. La plus grande rétention des anthocyanes par rapport aux catéchines et quercetins pourrait être expliquée par le fait que les anthocyanes ont une structure chargée positivement plus élevée qui interagit avec les membranes. Les membranes GE et ENTA peuvent être utilisées pour séparer les polymères de proanthocyanidins. Bien que, les paramètres de fonctionnement de ces membranes ont été très satisfaisants, puisque le flux de perméat, est relativement élevés (en moyenne $1,08 \text{ L/h.m}^2$, 10^5 Pa), une grande attention doit être portée au colmatage. Finalement, la nanofiltration pourrait être utilisé afin de concentrer les classes phénoliques spécifiques. En particulier, l'élimination des sucres et de l'eau et en même temps la rétention des composés phénoliques à l'aide de la membrane HL avec un flux de perméat satisfaisant ($1,15 \text{ L/h.m}^2$, 10^5 Pa).

Perspectives

Après ce travail, plusieurs aspects de l'impact de la technologie sur l'extraction et la purification de molécules bioactives étudiés restent à élucider:

- Évaluer l'impact du couplage avec d'autres technologies alternatives, par exemple les procédés électrotechniques qui ont été utilisés dans le projet comme une technique de prétraitement.

- Étudier davantage l'influence de la chaleur sur les dommages des composés phénoliques thermolabiles lors de l'extraction.
- Evaluer la contribution du colmatage réversible et irréversible par des mesures après différents nettoyages. .

Technologiquement, il serait intéressant:

- d'étudier la faisabilité d'un traitement SWE continu ou semi-continu à l'échelle industrielle, en utilisant une extrudeuse à contre-courant par exemple. Il serait aussi important de valider la faisabilité technique de la mise en œuvre des technologies de membranaires à une plus grande échelle.
- Pour prolonger l'étude de l'extraction par SWE à des marcs de raisin issus d'autres cépages ou à d'autre biomasse des végétales de la vigne (pousses, tiges ...) pour valider la valeur de cette technologie.