Purification of Grape Proanthocyanidins by Membrane Ultrafiltration

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***Abstract*—The objective of this study was to maximize the permeate flux (J) in the purification by UF of a grape seed extract, by evaluating the effect of operating variables: transmembrane pressure and tangential velocity on J and on the extracts chemical characteristics. Concentrations of total phenols, mean degree of polymerization (mDP), and average molecular weights (aMW) were compared. Flat membranes of polyvinylidene fluoride (PVDF) with a molecular weight cut off (MWCO) of 10 (kDa) and 1 (kDa) were used in an Alfa Laval equipment LAbUnit M10. The pressure was tested at 2, 3, 4 and 5 (bar) at a constant speed of 0.9 (m/s) and the tangential velocity was evaluated at 1.2, 1.3 and 1.4 (m/s) at 5 bar pressure and constant temperature (20 ℃). The transmembrane pressure and tangential velocity tests confirmed a direct relationship of these variables with J for both membranes. The J for 1kDa membrane was more sensitive to changes in pressure, with a maximum J of 15.18 (L/m^2h), while that for 10 kDa membrane J reached a maximum value of 37.50 (L/m^2h), both at 20 ℃, 5 bar and 1.3 (m/s). The purification by UF reduced the mDP of the extracts from 7.15 up to 1-3 units of flavan-3-ols, corresponding to dimmers and trimmers in the permeate. To maximize J, the phenolics concentration, and minimize the mDP, carrying out the UF process with 10kDa membrane to 5 bar and 1.3 m/s would be optimal.**

***Index Terms*—ultrafiltration, grape seed, phenolic compounds, purificaction, flavan-3-ols, permeate flux.**

I. Introduction

Flavan-3-ols compounds are secondary metabolites in most plants, seeds, flowers and fruits; their structures vary from monomers (for example, catechin and epicatechin) to polymers, also known as proanthocyanidins (PAs) [1]. In grapes (*Vitis vinifera*), grape seed holds 60% of PAs, containing higher concentrations of monomeric, oligomeric and polymeric flavan-3-ols, with lower degree of polymerization than those found in grape skin [2].

It is well known that PAs have numerous bioactive properties. They present anti-mutagenic [3], anti-microbial [4], antitumor [5], cardio protective and enzyme modulating properties [6], among others. PAs extracted from *V. vinifera* have demonstrated the ability of inhibiting the angiotensin-converting enzyme (ACE), decreasing the tension of blood vessels and blood volume, thus lowering blood pressure and proving to be a potential treatment for hypertension and congestive heart failure [7]. The mean degree of polymerization (mDP) of PAs as well as the amount of aromatic hydroxyl groups determines these bioactive properties [8]. However, high molecule sizes can limit cellular adsorption of these compounds, and thus limit the bioactive properties above mentioned [9].

Therefore, it is necessary to select PAs with a low mDP (mDP $\leq$ 3) (to facilitate cellular adsorption), which has led to the study of different techniques such as size exclusion chromatography (SEC) [10]. Although it provides high purity extracts with rapid run times and increased resolution, the maximum average permeate flow is low, limiting the use of SEC as an industrial and profitable process for the purification of grape seed PAs.

Membrane ultrafiltration (UF) has proven to be a novel and effective phenol purification process. The UF process of grape seed extracts is recent and still in study. Reference [11] concentrated grape seed extract using a 0.45 μm Millipore type GS membrane and a 0.22 μm Millipore type HA membrane, retrieving 11,4% of the seeds weight with the 0.22 μm membranes. Other authors have studied the use of UP150 150 kDa and UV050 50 kDa membranes to produce grape seed extract, retrieving 87% and 91% of polyphenols, respectively [12].

The objective of this work was to maximize the permeate flow of PAs grape seed by UF, studying the influence of operative parameters such as transmembrane pressure, tangential velocity and MWCO, using fluoropolymer composite membranes in order to achieve a PAs rich grape seed extract with bioactive properties.

II. Material and Methods

A. Plant Materials

Pais grapes (*Vitis vinifera)* were harvested from Cerro Negro, Quillon, Chile, on March 2012. They were then weighed, passed through a stripping process to separate the branches and placed in a 5L glass beaker which was filled with 4L of distillated water. The sliced grapes were manually stirred in the water for 1 minute to allow the seeds to separate from the pulp and then the mixture was left resting. Using a 4.08 mm mesh, the seeds were separated from the pulp, placed in a glass beaker and weighed.

B. Effect of Seed Preconditioning in the Extraction

Before the extraction, three types of conditioning were studied on the seeds: **F**, immediate extraction from the

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freshly seed ; **FM**, milling to 1-2 mm of the freshly seed in a domestic mill; **LM**, seed lyophilization and milling to 1-2 mm Then, samples were stored at -18 ℃ in sealed bags wrapped in kraft paper with silica gel. The extraction was carried out in 250 mL flasks wrapped in kraft paper, with a solid/liquid (S/L) ratio of 1/20 and acetone: MilliQ water (2:1, v,v), at 180 rpm agitation in a orbital shaker (New Brunswick Scientific G-24, USA) for 2 h. Afterwards, the solvent was removed by evaporation at 35 ℃ and vacuum in the Bibby Rotary Evaporator (RE100, USA). Finally, the extracts were centrifuged, uniformly diluted to 100 mL, bubbled with Nitrogen (N_2) and 1 mL aliquots were extracted from each one for total phenols analysis.

C. Effect of Solid/liquid (S/L) Ratio in the Extraction

The effect of the S/L ratio was also studied in the extraction of lyophilized milled seeds, expressed in grams of seed per milliliter of solvent (g seed/mL solvent), by evaluating the total phenols concentration. Five S/L ratios were analyzed: 1/20, 1/25, 1/33, 1/50 and 1/100. The extractions were carried out in 250 mL flasks with acetone: MilliQ water (2:1, v,v) as solvent, in the orbital shaker (New Brunswick Scientific G-24, USA) at 180 rpm agitation at room temperature for 15 h. Afterwards, the solvent was removed at low temperature (35 ℃) and pressure in the Bibby Rotary Evaporator (RE100, USA). Finally, the extracts were centrifuged, uniformly diluted to 100 mL, bubbled with Nitrogen (N_2) and 1 mL aliquots were extracted from each one for total phenols analysis. At the end of this stage an extraction protocol was determined, as resumed in Table I.

TABLE I. GRAPE EXTRACTION CONDITIONS

Parameter	Condition
Preconditioning Seed	Freshly milled seeds (FM)
S/L ratio	1:20
Solvent	Acetone: MilliQ Water 2:1
Equipment	Glass Reactor (BIOFLO Model c30, New Brunswick Scientific, USA), bubbled with N_2, sealed with parafilm and wrapped in kraft paper
Volume	1.5 L
Agitation/time	180 rpm/15 h

After the extraction, the grape seed extracts were vacuum filtered in a Kitasato flask using filter paper (Xingxing Moderate 102, China). Then, the solvent was removed by evaporation at 35 ℃,vacuum in the Bibby Rotary Evaporator (RE100, USA) and centrifuged in a Haraeus Sepatech Suprafuge 22 centrifuge for 10 min at 4294*g*. The supernatants were taken to 500 mL with destillated water and stored in glass flasks at -18 ℃ in the dark, until UF was performed.

D. Ultrafiltration Process

The extracts were submitted to an initial stage of Microfiltration (MF) to avoid the immediate obstruction of the UF membranes. The equipment used in the MF and UF was an Alfa Laval LabUnit M10 (Denmark), consisting of four plates united by bolts onto a metal support that allows the operation of 4 membranes in series with tangential filtration, with a membrane area of 0.0336 m^2. The feeding of the module was done using an ECO pump type GA4-KDT-TTU of 4-7 L/min capacity and the temperature was regulated with water through a heat exchanging process of 0.076 m^2 exchanging area. The temperature in all cases was maintained constant at 20 ℃. For the MF process the LabUnit M10 was equipped with 4 FSM 0.45PP fluoropolymer membranes of 0.45μm pores.

The influence of MWCO, transmembrane pressure and tangential velocity in the UF process of seed grape extract was studied. The J and the chemical characteristics of the extracts were analyzed. Two flat membranes were used in the UF: ETNA01PP (MWCO=1kDa) and ETNA10PP (MWCO=10kDa) (Alfa Laval, Lund, Sweden) made from fluoropolymer composite (Polyfluoro vinylidene "PVDF" covered with cellulosic polymer supported on polypropylene). The effect of transmebrane pressure on J was studied by operating at 2, 3, 4 and 5 bars at a constant velocity of 0.9 m/s with total recirculation. The effect of tangential velocity on J was evaluated, manipulating the pump's potentiometer as follows: 40%, 50%, 70% and 80%, which corresponds to 0.9, 1.2, 1.4 and 1.3 m/s, respectively. The working pressure was kept constant at 5 bars. All samples were then characterized according to the following chemical analysis.

E. Chemical Analysis

The raw extract ,permeate and retentate samples were characterized using the following analysis: Total Phenols by the Folin-Ciocalteau method [13]; modified by [14] and the phloroglucinolysis method to estimate the mean degree of polymerization (mDP) of the samples [15], [16].

F. Statisical Analysis

All of the analyses were done in triplicate and the results were analyzed by one way ANOVA.The homogeneity of variance between the groups was tested using the Levene test with a significance level set at 5%. To determine whether there was a significant difference between means, multiple range tests were done through Fischer ś minimum significant differences method (MSD) with a 95% confidence. All of these analyses were performed using the STATGRAPHICS Centurion XVI 16.1.15 software.

III. RESULTS AND DISCUSSION

A. Effect of Seed Preconditioning in the Extraction

Fig. 1 presents the comparison of total phenol content, normalized per gram of seed used, obtained after the extraction of preconditioned fresh seed (F), fresh powder (FM) and lyophilized powder (LM), respectively.

The total phenolics content of FM was 36% higher than F and 57% higher than LM. Although LM considered the grinding step, the concentration of total phenol obtained was less than the concentration of FM.

Since seeds were dehydrated, a higher initial mass was necessary to obtain the same S/L ratio in the extraction, which was not convenient. Also, it's important to note that a higher energy requirement is necessary for freeze drying and the processing time extends. Therefore, only the pre-grinding as seed treatment was established for further extractions to larger scale.

Other authors such as Refs. [11], [17], [18] have also reported that milling the vegetal material benefits the phenol extraction, since the mass transfer favors the grinding of the sample due to the raise in the area/volume ratio

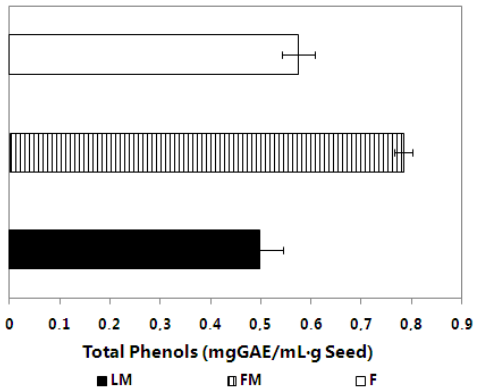

Figure 1. Effect of different pre-treatments of the seed on the total phenol concentration of the extracts.

B. Effect of Solid/liquid (S/L) Ratio on the Extraction

In Fig. 2 the influence of solid/liquid (S/L) ratio on the total phenol content of the extracts is presented. The values were normalized by the grams of seed used in each case.

The statistical analysis showed that the reasons 1/20 (12.5 g seed) and 1/33 (7.5 g seed) have no significant differences in the total phenol concentration ($p<0.05$). Nevertheless, the smaller ratio (1/20) was selected because it will result in lower costs in larger scale operations.

The results obtained here are in agreement with Ref [11], which studied S/L ratios between 1/10 and 1/4, founding an optimum for the phenolic concentration to 1/5 ratio. They showed that higher amounts of solid decrease the phenol content. A possible explanation for this might be that the solvent is unable to effectively penetrate the ground seed.

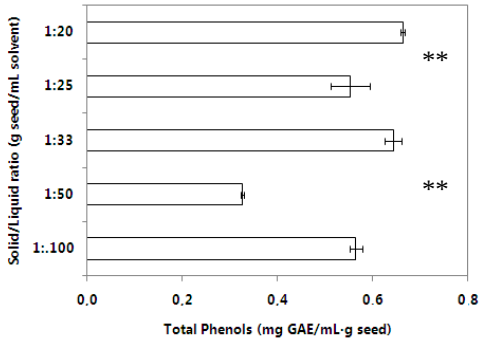

Figure 2. Effect of solid/liquid ratio on the total phenol concentration of the extracts.

C. Ultrafiltration Studies

The J of both membranes was tested as function of the transmembrane pressure. Results obtained for ETNA01PP and ETNA10PP membranes are shown in Fig. 3 and 4, respectively.

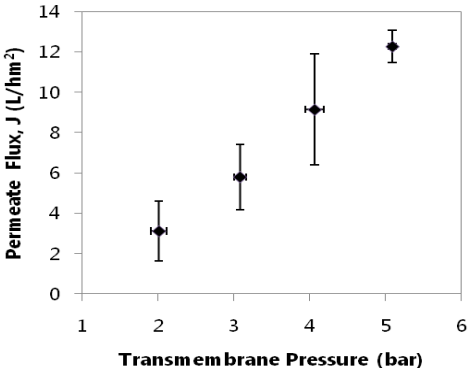

Figure 3. Average permeate flux (J) to different transmembrane pressures, tangential speed 0.9 (m/s), ETNA01PP membrane at 20 ℃.

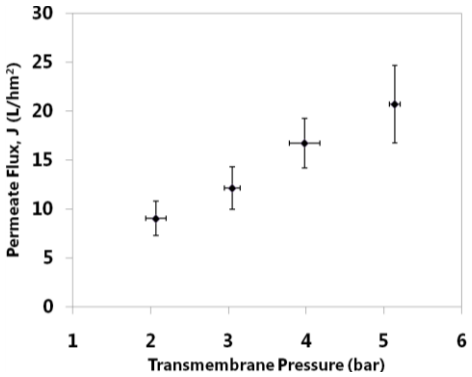

Figure 4. Average permeate flux (J) to different transmembrane pressures, tangential speed 0.9 (m/s), ETNA10PP membrane at 20 ℃.

The J for the ETNA01PP (1kDa) membrane remained between 1.55 L/m^{2}h and 12.50 L/m^{2}h at pressures of 2.1 and 5.1 bar, respectively, reaching the maximum J at the highest pressure tested ($p<0.05$). Thus, the transmembrane pressure has an effect on J and a direct relationship between both variables was observed, which would indicate that in these conditions the polarization or gel layer is not present. It is important to note that the variability among experiments could be explained by the chemical cleaning of the membrane, which would increase the pore size, leading in some cases, to changes in J

The J for ETNA10PP membrane (10 kDa) ranged from 7.14 L/m^{2}h to 23.21 L/m^{2}h at pressures of 2.1 and 5.1 bar, respectively. Similar to the observed with the other membrane, the maximum J was also obtained at the highest pressure. In comparison with the membrane of 1 kDa (to compare Fig. 4 and 5), the J was less sensitive to the change on the transmembrane pressure.

Ref. [19] ultrafiltered grape juice "concord" with a 10 kDa membrane PVDF at a pressure of 4 bar, obtaining J values of 3.61 L/m^{2}h, and 5.39 L/m^{2}h when the feed concentration was reduced to half. . Both J values were lower than those obtained in this study. Possible explanations for this are different hydrodynamic (not

reported speed), an initial different concentration and/or higher molecular size of the extract, or a greater chemical affinity of grape juice with the PVDF membrane due to the presence of sugars in the juice.

Another aspect, physicochemical more than hydrodynamic, that affects the J of both membranes is the adsorption of the molecules in the membranes. Ref. [20] studied the adsorption of the flavan-3-ols on membranes of polyethersulfone (PES) and polyvinylpyrrolidone (PVP). Note that the adsorption of these molecules on these materials was mainly caused by Lifshitz -van der Waals force and polar interactions, without neglecting electrostatic. They also found that the mDP, the polarity of the membrane material and the increased amount of phenolic rings promote the adsorption, even 10 hours after start the experimentation. The PVDF, which is the material of the membranes of this study, had large polarity in its –CF2- group, which may be the cause for the adsorption of molecules in the membrane, also it could cause a higher concentration of phenols in the retentate (which will be reviewed in the next section).

The J of both membranes was tested as function of the tangential velocity and the results for ETNA01PP and ETNA10PP membranes are shown in Fig. 3 and 4, respectively.. An initial decay of J was observed, which reached a steady state, in most cases after 40 min.

Results obtained for ETNA01PP and ETNA10PP membranes are shown in Fig. 3 and 4, respectively. The J value obtained with the 1 kDa membrane at 4 bars (constant pressure) and tangential velocity of 0.9 m/s, showed an increase of 20%, 35% and 52%, when tangential velocity was increased to 1.3, 1.4 and 1.2 respectively (data not shown); while at pressures of 5 bar (Fig. 5), J values showed an increase of 2%, 9% and 24 % when tangential velocities were increased to 1.3, 1.4 and 1.2, respectively, which was not considered significant ($p<0.05$). Although J was enhanced, is not recommendable to increase the tangential velocity, at least not with operating pressures of 5 bar. Thus, the operation with the 1 kDa membrane, in terms of influence on J , was more sensitive to changes in transmembrane pressure than in tangential velocities.

On the other hand, the comparison of J values obtained with the 10 kDa (Fig. 6) membrane at pressures of 5 bars and tangential velocity of 0.9 m/s with the J values obtained when tangential velocity was increased to 1.2, 1.3 and 1.4 m/s, showed an increase of 47%, 81% and 55%, respectively, being this increase more significant for the 10 kDa membrane, where the maximum J was near 39 K/m^2h.

Ref [21] ultrafiltered wine must and compared the effects of different tangential velocities on J into a tubular hollow fiber membrane made of polysulfone. They showed that when tangential velocity was increased from 350 to 550 L/h, the J value increased from 13 L/m^2h to 14 L/m^2h, respectively, corresponding to a 7% of increment. This result is consistent with the results obtained in this study, in which J increased a 9% (from 12.26 L/m^2h to 13.39 L/m^2h), when tangential velocity was increased

D. Chemical Analysis of the Samples

In Fig. 7 the levels of total phenols in the feed, permeate and retentate for both membranes at different tangential velocities are presented. The concentration of phenols in the retentate was higher than in the permeate at all conditions studied, which produced a retention over 70% for all membranes. The clogging and the absorption of molecules over the membrane surface or in the pore can cause this inconvenient.

Others authors [22] working also with seed extracts have found retentions of 43%, 71% and 78% for membranes of 0.15 μm (fluoro polymer), 150 kDa (PES) and 50 kDa (PVDF), which is in agreement with the retention of this study.

Ref. [11] concentrated seed extract using 0.45 um Millipore type GS membrane and 0.22 um Millipore type HA membrane, obtained a total phenols concentration in the retentate higher than in the feed, indicating a high percentage of retention (over 80%). Although the extract obtained by them was not identical to this study, -since they uses a higher solid/liquid ratio-, it is expected that to the MWCO 10 kDa and 1kDa membrane the retention will be also high.

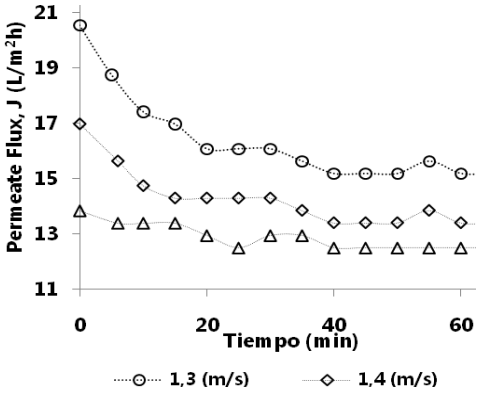

Figure 5. Permeate flux (J) to different tangential speed, to 5 bar, ETNA01PP membrane at 20 ℃.

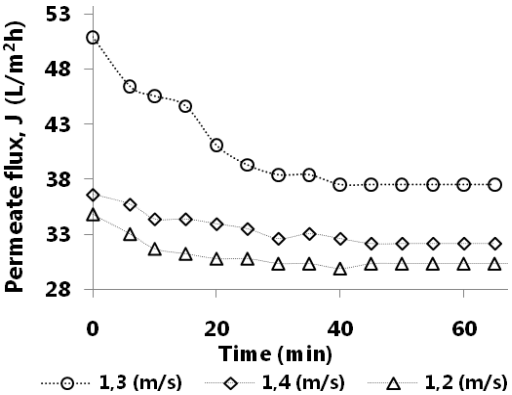

Figure 6. Permeate flux (J) to different tangential speed, to 5 bar, ETNA10PP membrane at 20 ℃.

The mDP indicates how many times the flavan-3-ols basic unit is repeated in the polymer; to get an adequate bioabsorption of these molecules, the mDP should be less than 3 units [23]. The raw grape seed extract had a mDP of 7.15±0.03 and after the MF the value was reduced to

4.39±0.19. With the UF process the mDP of the permeate was reduced to 1-3 units, being the UF process favorable to the reduction of mDP samples. No effect of membrane type was observed ($p<0.05$) as can be seeing in Fig. 8. Only the tangential velocity has influence over the mDP, being lower the values to less speed used in the process. The aMW is proportional to the sample´s mDP, thus a reduction in its values was also observed after the UF.

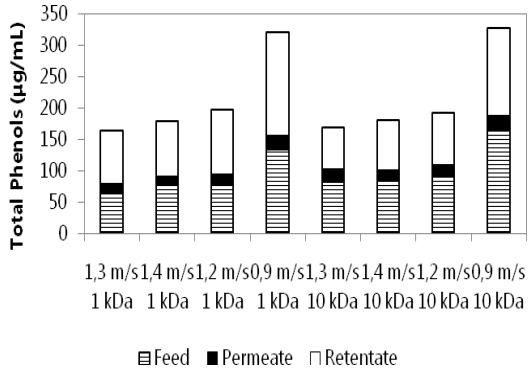

Figure 7. Total phenol concentration of the feed, permeate, and retentate to several tangential velocities for ETNA01PP and ETNA10PP membranes at 20 ℃.

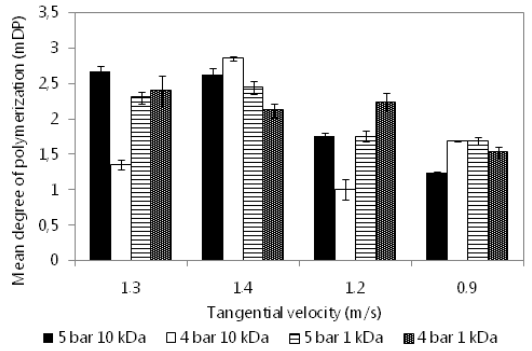

Figure 8. Comparison of the mean degree of polymerization (mDP) of both membranes permeates to different tangential velocity of 1.3, 1.4, 1.2 and 0.9 (m/s) respectively, and pressures of 5 and 4 bar.

IV. Conclusions

Changes on the transmembrane pressure and the tangential velocity had a direct relationship over J. The changes of these variables were more sensitive to 1 kDa membrane than 10 kDa membrane. The maximum J achieved was 15.18 permeate L/m^{2}h for 1 kDa membrane and 37.50 L/m^{2}h for 10 kDa, both at 20 ℃, 5 bar and 1.3 m/s. The concentration of phenols in the permeate was lower than on the retentate, for both membranes in all operating conditions. The membranes studied have a high retention rate for seed extract, which was minimized at higher tangential velocities. Both membranes 10 kDa and 1 kDa reduced the mDP of the permeates, which is favorable to the bioabsorption of the extracts, but given the higher density of flow obtained, working with 10 kDa membrane resulted more suitable.

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