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Graduated in Biotechnology Engineering

FRACTIONATION OF HYDRO-ETHANOLIC EXTRACTS FROM GRAPE POMACE THROUGH MEMBRANE PROCESSING: THE EFFECT OF MEMBRANE AND EXTRACTING MEDIA ON PROCESS PERFORMANCE

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in Membrane Engineering

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Abstract:

Grape pomace are generated as waste in juice and winemaking industry in huge quantities. Studies have shown that nutrient extracts from these particular plant matrices garner myriad of benefits in health and nutraceuticals sector. Moreover, the newer recognition of monomeric and oligomeric phenolic compounds as bioactive molecules of commercial value such as gallic acid, catechin, epicatechin, quercetin and resveratrol gives scope for its extensive study and extraction from the pomace. The need for optimizing the biocompatible solvent extraction of ethanolic-water composition is realized and the kinetics of extraction time is demonstrated. The work focusses on novel use of membrane technology for separation and fractionation of lower flavan-3-ols from the crude extract. Four different membranes of varied pore size, chemical nature and material were chosen. Of these, Duramem 900, a class of Organic Solvent Nanofiltration membrane showed lower rejections of monomeric and oligomeric phenolic compounds. This membrane was then subjected to the comparative study of nanofiltration and diananofiltration.

Keywords: grape pomace; solvent extraction; phenolic compounds; organic solvent nanofiltration; diafiltration

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1. Introduction:

Grapes have a long and abundant history. During the ancient Greek and Roman civilizations, grapes were revered for their use in winemaking. Grape (*Vitis* spp.) is one of the most economically important plant species due to its diverse uses in production of wine, grape juice and other food products [1]. It is cultivated in all continents in the temperate regions where sufficient rain, warm and dry summers as well as relatively mild winters are normal climatic patterns. The qualities of grape products are characterized by their metabolic compositions [2].

Grape fruit contains various nutrient elements, such as vitamins, minerals, carbohydrates, edible fibers and phytochemicals. The unique combination of phytochemicals in grapes includes a variety of bioactive compounds such as simple phenolics, flavonoids, anthocyanins, stilbenes, proanthocyanidins, and vitamin E [1,3,4]. In excess of 500 compounds, including 160 esters, have been identified to be present in wines with active role in the formation of their organoleptic properties [5]. Simple phenolics in grapes are derivatives of hydroxycinnamic acid (p-coumaric, caffeic, sinapic and ferulic acids) and hydroxybenzoic acid (gallic, gentisic, protocatechuic and p-hydroxybenzoic acids) [1,5]. Gallic acid has been shown to possess various therapeutic properties, including antioxidant, anti-cancer, anti-inflammatory, antifungal and antiviral activities [6–9]. However, polyphenols including flavonoids, stilbenes and proanthocyanidins are the most important class of biologically active compounds in grapes. Grape is one of the richest sources of polyphenols among fruits. Flavonoids represent a widespread and common group of natural polyphenols produced by the phenylpropanoid pathway [10,11]. They confer UV-protection, determine flower coloration, attract pollinators, and act as tissue protectors in case of pathogen attack or oxidative damage [12]. These classes of phenolics are the most abundant biologically active phytonutrients among the polyphenols found in grapes, possessing cardioprotective, neuroprotective, antimicrobial and antiaging properties [13-16]. Most of the flavonoids are found primarily in the outer epidermal cells (the grape skin), whereas about 60%–70% of total polyphenols are stored in grape seeds [1,4].

Phenolic compounds are also regarded as preservatives against microbes and oxidation for food [17, 18]. What's more, *in vivo* assays showed that phenolic compounds are bioavailable [19, 20]. Therefore, besides wine and juice, grape diet supplements would be promising functional foods worthy of popularization.

Nevertheless, more than 70% of grape polyphenols remain in the pomace (a byproduct of wine/grape juice processing), which becomes a valuable source of health promoting nutraceuticals [21]. Therefore, the wide range of pharmacological effects of grapes and grape products on human health is due to the fact that those additives are sources of unique combinations of nutraceuticals.

However, some reports have also shown that at higher concentrations the effect of polyphenolic compounds on health was negative and some structures in particular promoted the negative effects [22]. In addition, some high molecular weight phenolics could not be absorbed [23, 24]. Thus, researchers are focused in the monomeric and oligomeric phenolic compounds as they are of paramount importance to human health.

Winemaking industry takes the major share of the grape plantations. However, tonnes of grape pomace are generated as waste and its disposal poses a serious challenge for wineries [25]. The production of wine involves the harvesting and pressing of grapes to extract juice for fermentation. During this process of pressing, the seeds, skins and stems are left out as waste. This waste of grape pomace adds to cost for its removal and requires effective treatment to avoid the initiation of environmental hazards, such as contamination of ground and surface water and also gives foul odour [26]. The winery waste has shown to increase the chemical oxygen demand (COD) and biological oxygen demand (BOD) within wastewater streams. This increase of BOD and COD levels arise due to the presence of lipids and pollution loads in higher quantities and other organic substances such as tannins, sugars, pectins, polyalcohols and polyphenols [27]. For this reason, wineries started using this waste of grape pomace as animal feeds or fertilizers. Nevertheless, owing to the presence of health benefits of the phenolic compounds present in this winery waste, these compounds can be extracted for production of additive-free, safer and maturally processed products. Commercially, it can be viable in response to the changing market of customers using “natural” products [28]. Thus, the work commercially highlights the botanical extracts which are subset of natural extracts that seem to be the fastest growing nutaceutical ingredients segment. An annual world demand increase of this market is estimated to increase by 8.9% through 2015. Furthermore, the antioxidant category of over $ 1.5 billion market of cosmeceutical ingredients market has garnered widespread commercial importance [29].

Solvent extraction has stood against time for being a traditional, simple and cost-effective technique. Literature data reports the influence of different extraction solvents, techniques on the content of natural antioxidants in extracts [30, 31]. The efficiency of solid-liquid extraction is strongly dependent on plant matrix used [30-32]. Solvents, such as propanol, methanol, ethanol, acetone, and ethyl acetate have been

commonly used for the extraction of phenolics from fresh product [31, 33]. The properties of extracting solvents are shown to affect the measured total phenolics content (±25% variation) and antioxidant capacity (up to 30% variation) in fruits and vegetables [31]. Of these, ethanol-water mixtures are of interest to us as they biocompatible and are classified with GRAS status and thus, widely used for the recovery of nutraceuticals [34]. The hydro-ethanolic mixture has been reported to have a beneficial effect on extraction of phenolic compounds from grapes [35]. On the contrary, extractions performed with pure water or ethanol also seem to suffer with low efficiencies especially in the case of anthocyanins and total phenolics from grape by-products when compared to ethanol-water mixture [31,33,36]. Thus, experiments of solid-liquid extraction were performed using the ethanol-water bio compactible mixtures at different compositions to optimize the most effective hydro-ethanolic extracting solvent and also the extracting time for the selected grape pomaces.

The simple steam distillation and vacuum distillation are the traditional approaches used for obtaining the biologically active compounds, extracted from natural products. However, they generally require an increased temperature and high energy consumption. The former is inappropriate for heat-sensitive products. Furthermore, the evaporation of the solvent at times accompany a loss of compounds of low molecular weight which can be at times be evaporated along with them [37]. Another method described in literature, particularly for propolis extract concentration is lyophilization process [38]. However, this method shows some of the disadvantages of the previously mentioned processes, e.g. involves a large amount of energy, comprises incubation at about 70 ◦C [39], etc.

A membrane separation process can be utilized as an alternative of the approaches mentioned above. In the cases of substances with a molecular mass less than 1000 Dalton, nanofiltration has received a great attention as a process with reduced operation cost, carried out at ordinary temperature [40-42]. The last is very important because most of the species extracted from the natural products are very unstable at elevated temperature.

Organic solvent nanofiltration (OSN) is a pressure driven membrane based separation technique where an incoming feed stream is separated into two components referred to as permeate (solvent and solutes able to pass through the membrane) and the retentate (solvent and solutes retained or rejected by the membrane). In nanofiltration molecules ranging between 200–1000 g mol^{-1} can be separated and the mechanism is thought to be mainly the differences in stearic effects. In addition to steric exclusion membrane-solvent interactions, pressure, feed concentration, temperature and charge of the solute can influence the membrane performance, and such factors can hence be used to fine tune the separation.

OSN is a non-thermal technique and can offer potential advantages over conventional unit operations, such as distillation, with regards to energy-efficiency. Additionally the risk of heat sensitive solutes getting degraded and unwanted side-reactions during OSN operation is small [43].

Perhaps the best known and most successful application of OSN to date has been in the MAXDEWAX process developed by W. R. Grace and ExxonMobil and employed for crude-oil dewaxing at the 72 000 barrels per day scale. Applications have been proposed for a variety of industries including refining – e.g. hydrocarbons separation, food industry – e.g. deacidification of vegetable oil , fine-chemical and pharmaceutical synthesis – e.g. organometallic catalyst separation and solvent exchange, etc [44].

In 2000, Nwuha investigated the membrane extraction of the bioactive components of green tea in an organic solvent using different nanofiltration membranes [45]. A series of tests with various membranes using higher ethanol concentrations (e.g. 80%) gave highest rejection of catechins with lower rejection of caffeine for G-10 and G-20 membranes, respectively. There are also multiple reports by Tylkowski et al on the extraction of flavonoids and polyphenols using solvent resistant membranes (duramem 200 and 500) from Sideritis spp (Mursalitza tea) and Propolis (resinous product from beehives) with high rejection (>90%) [46].

The technique of continuous diafiltration (also referred to as constant volume diafiltration) involves washing out the original buffer salts (or other low–molecular weight species) in the retentate (sample) by adding solvent to it at the same rate filtrate is generated. As a result, the Liquid is added at the same rate as filtrate is generated, and when the volume of filtrate collected equals the starting retentate volume, 1 Diavolume has been processed. Using continuous diafiltration, over 99.5% of a 100% permeable solute can be removed by washing through multiple volumes with the solvent or buffer of choice. It takes very high volumes of solvent to completely wash a partially permeable molecule through the membrane than it does to remove a 100% permeable molecule from a mixture [47].

Diafiltration is a perspective approach for successful fractionation and following concentration of multicomponent solutions such as plant extracts. For instance the dual membrane diafiltration is a promising method for membrane separation and concentration. It comprises a separation step, where lower molecular weight compound is separated from higher molecular weight compound, followed by a solvent recovery step, where the lower molecular weight compound is retained by the membrane, allowing the solvent to be recycled into the first stage. Diafiltration was used to separate caffeic from rosemarinic acid, extracted from rosemary. Membrane concentration of antioxidants from Castanea sativa leaves aqueous extracts by diafiltration with two UF membranes was reported, where the ultrafiltration–

diafiltration process with PES membranes increased the antioxidant properties of the concentrated streams [48,49].

This work aims to combine the understanding of science of phenolic compounds and utilization of membranes to separate and fractionate the discrete classes of phenolic compounds. In this tarmac of study, the optimization of the extracting solvent is evaluated for the release of phenolic compounds from the grape pomaces and subjected to membrane processing. 4 different membranes (tight ultrafiltration membrane of Nadir UP_005 and organic solvent nanofiltration membranes of Solsep NF 010306, Duramem 500 and Duramem 900) are screened for the separation and subsequent fractionation of monomeric and oligomeric flavan-3-ols from the polymeric flavan-3ols and later the extracts are characterized by various methods. Finally, a comparison in the performance of organic solvent nanofiltration with diananofiltration is presented.

2. Materials and Methods:

2.1 Materials

The reagents used were ethanol (Panreac, Spain, 99.5% purity), methanol (Pancreac, Spain, 99& purity, Folin-Ciocalteu reagent (Panreac, Folin-Ciocalteu DC), HCl, H_2SO_4, diethy ether, ethyl acetate, acetone were purchased from Merck (Darmstadt, Germany), gallic acid monohydrate (Sigma–Aldrich, ≥99%), Vanillin (Merck, Darmstadt, Germany), Sep-Pak Plus C18 cartridges WAT 036810 and WAT 036800 were obtained from Waters (Milford, MA).

The **grape pomace** is the solid material obtained from crushing and destemming the grapes followed by fermentation and finally pressing the wine, as described in Brazinha et al [50]. The grape pomace from grapes *Vitis vinifera* variety Tempranillo (known worldwide and very common in Portugal) was kindly provided by Esporão S.A. from the winery Herdade do Esporão, Reguengos de Monsaraz, Alentejo (south of Portugal).

The **flat sheet membranes** used in this work and their characteristics are shown in **Table 1**. As this work aims at separating small molecules, specifically the oligomer and monomeric flavan-3-ols from crude grape pomace extracts, all selected membrane are nanofiltration membranes with small pores appropriate for separating small molecules, except Nadir UP 005 which is a very tight ultrafiltration membrane. The membranes are also all stable in ethanol. Nadir UP005, although used for processing aqueous solutions, was reported to be stable in a solution of 70% ethanol in water. The others membranes are organic solvent nanofiltration membranes, suitable for processing solutions with pure ethanol as solvent.

Table 1. Characteristics of the membranes studied

Membrane	Producer	Membrane material	Retention data	Maximum temperature (ºC)	Maximum pressure (bar)
Nadir UP 005	MICRODYN-NADIR GmbH	PES on PE/PP	5000[(a)]	95	5
Solsep NF010306	SolSep BV	Not available	(b)	150	40
Duramem 500	Evonik	Modified	500[(c)]	50	20
Duramem 900	Evonik	Polyimide	900[(c)]	50	20

(a) MWCO (MW at which 90% rejection is obtained) when the processing solution has water as solvent

(b) when the processing solution has ethanol as solvent, an oily molecule with MW around 1000 Da are retained 80% and a colorant with MW around 500 Da are retained higher than 99%

(c) MWCO of styrene oligomers dissolved in acetone

Legend: MWCO molecular weight cut-off, PES Polyethersulfone, PE/PP Polyethylene/Polypropylene

2.2 Experimental Procedure

2.2.1. Grape pomace pre-treatment

The raw material, grape pomaces was dried for 72 h in a hot air oven at 37°C and then milled to a fine powder form using a small grinding coffee mill.

2.2.2. Different methods of Extraction of Phenolic Compounds from Grape Pomace

2.2.2.1 Optimization of the hydro-ethanolic extracting solvent in solid-liquid extractions using Soxhlet experimental unit

The Soxhlet experimental unit of extraction method was used to determine the most effective composition of hydro-ethanolic extracting solvent for extracting maximized phenolic compounds, by using different extracting media (aqueous solutions of 20% wt ethanol, 40% wt ethanol, 60% wt ethanol and 80% wt ethanol). In these Soxhlet experiments, the balloon containing the extracting media was immersed in an oil bath maintained at a temperature of 120 °C, a sufficiently high value to promote the evaporation of extraction media, with constant stirring at 350 rpm (IKA RCT Basic, IKA, Germany). The solids were placed in the Soxhlet apparatus. The time of extraction was 8 hours and the weight ratio of the extracting medium and the dried and milled grape pomace was 8:1.

2.2.2.2 Bourzeix method

The phenolic compounds from dried and milled grape pomace were extracted using the method described by Bourzeix et al. [51]. The solids (7.0 g) were immersed in 20 mL of methanol, 1 g/L of ascorbic acid was added to avoid oxidation and the resulting suspension was stored at -24 °C for 24 h. Later, the supernatant was decanted, and the residue was extracted with 20 mL of methanol/water (80/20, %v/v) for 4 h. In the next stage, the supernatant was again decanted and the residue was treated with 20 mL of methanol/water (50/50, % v/v) for another 4 h. Furthermore, the process was repeated with 20 mL of distilled water at -24 °C for 15 h. Finally, the residue was extracted with 20 mL of acetone/water (75/25, % v/v). The nitrogen atmosphere was maintained throughout the extraction. Care was taken not to disturb the suspension. At the end, all the extracts were combined and reduced to 1:8 ml ratio of raw material and

extracting solvent by using rotary evaporator for maintaining constant ratio in all the methods of extraction.

2.2.2.3 Solid-liquid extraction at 40°C

For ensuring maximum extraction of phenolic compounds including those that are thermosensitive, a simple solid liquid extraction was performed. The dried and milled grape pomace solids along with the extracting media were placed in an airtight balloon which was immersed in an oil bath (IKA RCT Basic, IKA, Germany). The temperature of the bath was maintained at 40 °C with constant stirring at 350rpm. The extraction was performed for a period of 72 h in the dark. The weight ratio of the solids to the extracting media was maintained at 1:8 throughout all extraction experiments.

2.2.3. Extracts pretreatment before membrane processing

The extracts obtained were centrifuged for 45 minutes, at 20ºC and 8000 rpm, for removing suspended solids. Nitrogen was passed through the samples before freezing them at -20°C for storage before further use.

2.2.4. Membrane Processing

The **membrane unit** used was operated in a dead-end mode with a gas control unit (METCell, Membrane Extraction Technology, UK) and flat membranes with an effective membrane area of 51.4 cm^2. The control of the temperature in the concentrate was carried out by immersing the membrane module in a controlled temperature bath and permeate was measured by an electronic balance (Kern 572, Kern, Germany).

The **membrane experiments operated in the concentration mode** were carried out at 40ºC; the feed vessel was stirred at 450 rpm, processing 150 mL of crude grape pomace extract. The membranes were conditioned prior to processing the crude extracts, at a higher transmembrane pressure than the value used when processing crude extracts. The transmembrane pressures were of 3 bar for Nadir UP 005, 25 bar for Solsep NF010306, 8 bar for Duramem 500 and 8 bar for Duramem 900. The crude extracts were processed until a *Final Concentration FC* (-) of 6 was reached. This signifies the ration between retentate and initial feed.

A nanofiltration experiment operated in the diafiltration mode (**diananofiltration**) was carried out at the same temperature, under the same stirring conditions, using Duramem 900 at the same transmembrane pressure of 8 bar. The crude extracts were processed until a *diavolume D* (-) of 2 (ratio of the solvent

added to the initial feed solution) was reached. In diafiltration operation, the feed volume should always be constant, so the amount of solvent added during each experiment is equal to the amount of permeate.

The permeability Lp (t) is related to volume V (t), time (t) and membrane area (A), pressure (P) was calculated through the following equation:

$$\text{Lp(t) [L/m}^2\text{/h/bar]} = \frac{V(t)}{A * t * P} \quad (1)$$

The observed rejection of phenolic compounds as function of the concentration factor in the membranes studied. The observed rejection is calculated through the following equation:

$$R_i = 1 - \frac{c_{i,perm}}{c_{i,feed}} \quad (2)$$

2.3. Analytical Methods

2.3.1 Measurement of total phenolic content by the method of Folin-Ciocalteu, the Glories' method and the spectrophotometric test at 280 nm

The content of the extracts in total phenolic compounds was determined by the **method of Folin-Ciocalteu** proposed by Slinkard et al with slight modification [52]. Briefly, 1.58ml of water, standard or sample extract (20 µl) and 100 µl of FC reagent were mixed. Then, 300 µl of Na_2CO_3 solution (7.5%, w/v) was added and allowed to stand for 30 min at 40°C digestor. Absorption was measured at 765 nm in a spectrophotometer. Gallic acid was used as a standard and the Total Phenolic Content was expressed as Gallic Acid equivalent (GAE) in mg/g of raw material.

Total phenolic content was also measured using a modified version of the **Glories' method** [53,54] described in Mazza et al. [55]. Briefly, the method consisted of mixing 0.25 mL of sample with 0.25 mL of 0.1% HCl in 95% ethanol and then further addition of 4.55 mL of 2% HCl. The absorbance of the solution was then read at 280 nm to measure the total phenolics. The results were also represented as Gallic Acid equivalents.

Somer's et al proposed a **spectrophotometric test at 280 nm**, in which the total phenolic content corresponds to a reading of the sample upon dilution at 280 nm, much simpler than the previous methods

presented [56]. Hence, the grape pomace samples were diluted and analyzed in Deuterium Lamp at 280 nm. The results were also represented as Gallic Acid equivalents.

2.3.2 Measurement of anthocyanins and polymerization index by Somer's Method

Somer's et al had extensively studied the anthocyanins equilibria and established a relatively easier method for the quantitative and qualitative determination of various aspects of anthocyanins [56]. In our work, the measurement of total anthocyanins was calculated. In brief, the method consisted of centrifuging the samples for 10 mins at 4000 rpm. 0.1ml of sample was mixed with 10 ml of 1M HCl and kept for 3 hours in water bath at 25°C. Samples were then measured in spectrophotometer at 520 nm using 1M HCl as reference solution. Later, 5µL of sodium disulphite ($N_2O_5S_2$) was then injected into the sample and then read again at 520nm using water as blank solution.

The total anthocyanins were calculated using the following formula:

$$\text{Total Anthocyanins (mg/L of extract)} = \left[A_{520}^{HCl} - \left(\frac{5}{3} * A_{520}^{N_2O_5S_2}\right) * 20 \right] \quad (3)$$

2.3.3 Measurement of proanthocyanins by the Tannin Power Method

The tannin-specific activity (TSA) of grape pomace extract was determined by nephelometry as described by De Freitas and Mateus [57]. This method is based on the characteristic property of proanthocyanins to interact and precipitate proteins. The extract solutions supplemented with condensed tannins were diluted to 50 times with filtered model solution (12% ethanol, 5.0 g/L tartaric acid, pH 3.20). Later, 4.0 mL of this solution were transferred to a test tube and mixed with 150 µL of Bovine Serum Albumin (BSA). The test tube was kept in the dark for 30 min. The extract solutions with and without BSA were measured for its maximum turbidity in a turbidometer. The difference of the values corresponds to the overall turbidity of the sample. The TSA is expressed in turbidity units NTU/mL of pomace extract and is determined by the following expression, where 0.08 corresponds to the dilution factor of wine:

$$\text{Turbidity (NTU/ml)} = \frac{Turbidity_{sample}}{0.08} \quad (4)$$

2.3.4 Measurement of the monomeric, oligomeric and polymeric flavan-3-ols by the vanillin assay with a prior fractionation of the monomeric, oligomeric and polymeric fractions in C18 Sep-Pak Cartridges

The extracts of feed, retentate and permeate were **fractionated by Waters C18 Sep-Pak cartridges** (Waters, Milford, MA) by following the method described by Sun et al. [58]. Depending on the intensity of the colour of permeate, either 5 ml or 10 ml of permeate and owing to dark colour of feed and retentate; 5ml of feed and retentate were concentrated to dryness using a rotary evaporator at <30 °C. After evaporation, the residue was dissolved in 20 mL of phosphate buffer pH 7. If required, the pH was adjusted to pH 7.0 with NaOH or HCl solutions. Two C18 Sep-Pak cartridges were connected in series (top, Waters Sep-Pak Plus tC18 environmental cartridge; bottom, Waters Sep-Pak Plus C18 cartridge). The cartridges were conditioned with methanol (10 mL), distilled water (20 mL), and phosphate buffer pH 7.0 (10 mL). After the cartridges were conditioned, the samples were then passed at a flow rate not higher than 2 mL/min. Later, 10 mL of phosphate buffer at pH 7.0 was passed to eliminate the Phenolic acids. The cartridges were then dried with N_2 for 90 mins. The elution of monomeric and oligomeric flavan-3-ols (fractions FI + FII) was ensured by passing 25 mL of ethyl acetate, followed by the elution of polymeric proanthocyanidins (fraction FIII) with 15 mL of methanol. The ethyl acetate fraction (fractions FI + FII) were dried in rotary evaporator and then redissolved in 3 mL of phosphate buffer, pH 7.0, and finally redeposited onto the same series of cartridges preconditioned as described above. The cartridges were again dried with N_2 for 90 mins, and monomers (FI) were separated from oligomers (FII) by sequential elution with 25 mL of diethyl ether and 15 mL of methanol. The three fractions (FI, FII and FIII) were evaporated to dryness under vacuum and redissolved in 3 mL of methanol. Sample fractionation was performed in duplicate.

The **determination of the total flavan-3-ol content of the monomeric, oligomeric and polymeric fractions** by vanillin assay was performed according to the method described by Sun et al. [59]. A 2.5 mL portion of H_2SO_4/methanol (25/75, % v/v) solution and 2.5 mL of 1% (w/v) vanillin in methanol were added to 1 mL of the sample. For blank solution, only methanol was added instead of vanillin. For FI fractions, the absorbance at 500 nm was read after a reaction time of 15 min at 30 °C using a Unicam UV-vis UV4 spectrophotometer (Unicam, Cambridge, U.K). For FII and FIII fractions, upon the addition of vanillin solution in sample at room temperature, it was quickly placed in spectrophotometer until the maximum absorbance value at 500 nm was reached. Quantification was performed by means of standard curves prepared from monomers (for FI), oligomers (for FII), and polymers of flavan-3-ol (for FIII) as

previously described [59]. The fractionated phenolic content is related to the volume of methanol for diluting the dry sample V_{rs} (ml), absorbance at 500 nm, volume of extracting solvent V_{ext} (ml), Slope of standard curve of various fractions 'b' **[F1- 0.0081, F2- 0.0046, F3 - 0.0037]**, initial volume of sample evaporated in rotavapour T_{sample} (ml) and initial quantity of powdered extract taken M_{sample} (g). The formula used to calculate the different fractions is:

$$\text{Fractionated Phenolic Content (mg/g)} = \frac{V_{rs} * Abs * V_{ext}}{b * T_{sample} * M_{sample} * 1000} \quad (5)$$

3. Results and Discussions:

3.1 Optimization of the hydro-ethanolic extracting solvent

The effect of the hydro-ethanolic extracting solvent and the time of extraction on the extraction efficiency of total phenolic compounds using Soxhlet experimental unit were studied. Kinetics were performed on the production of extracts at the same weight ratio of extracting medium to grape pomace of 8, during 8 hours, at different extracting media (20% wt, 40% wt, 60% wt and 80% wt of ethanol in water).

The extracts were analysed after each cycle of Soxhlet (each hour) in terms of the content in total phenolic compounds measured by the method of Folin-Ciocalteu and the Glories' method (the most common methods) and the spectrophotometric test at 280 nm (the simplest method). Owing to the phenomenal chemical diversity of the phenolic compounds present, there is no method that offers accurate measurement of the total phenolic content [56].

It was interesting to note in **(figure 1)** that there indeed exists reliability while comparing these three methods for measurement of total phenolics. The bars represent the standard deviation of total phenolics with respect to Gallic Acid Equivalent (mg/g of dried pomace). It can be seen that the variation of standard deviation is in the range of 0.74 – 4.9 mg/g of dried pomace upon comparison of all the three tests performed affirming the similarity in results amongst the techniques used. Thus, direct spectral reading at 280 nm can be considered as the appropriate method for measurement of total phenolic content as it is very rapid and simple compared to the complexities in preparation of sample required for Folin-Ciocalteau and Glories tests.

From the **(figure 1)**, it is interesting to see that lower ethanol composition serves better extraction leading to a greener method. Nevertheless, two important things to note are that **40% wt ethanol** serves as the optimized extracting solvent with a greater margin compared to 20% wt, 60% wt and 80% wt ethanol. Also, the extraction time leads to near stability after 7-8 hours by Soxhlet experiment and thus, distinguishes itself as a faster method of extraction to obtain crude feed for further membrane processing.

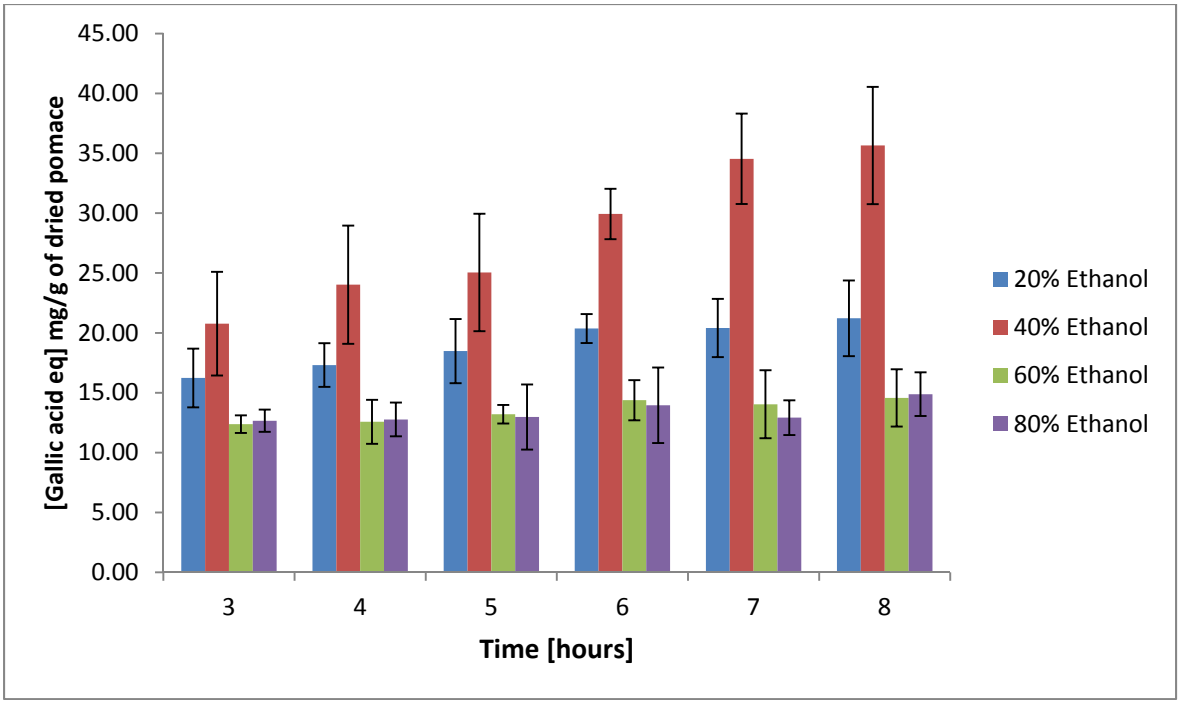

Figure 1.Optimization of extracting solvent and extraction time using a Soxhlet unit for different compositions of ethanol-water mixtures

Legend: The standard bars represent the standard deviation of values obtained by Folin Ciocalteau test, Glories test and Spectrophotometric test at A_{280} for measurement of total phenolic content [GAE] mg/g of dried pomace.

3.2. Comparison of different methods of extraction

The researchers specifically from the field of Agronomy and Enology use the classical Bourzeix's method for extraction of phenolic compounds from the pomaces [58,60,61,63]. This well established method is proved time and again for its effective extraction capability by using majorly methanol and also acetone as extracting solvents. However, one of the important facets in the framework of this work was to incorporate ethanol-water mixture because of its biocompatible nature. Hence, a cross study of comparison was done for 3 different methods of extraction namely, solid-liquid hydro-ethanolic extraction with Soxhlet, classical Bourzeix's method and solid-liquid hydro-ethanolic extraction at 40°C. The comparison of its capability of extraction of different classes of phenolic compounds was analysed by using the following measurements: total phenolic content by spectrophotometric analysis, total anthocyanins measurement [56] and proanthocyanins [57]. The results are discussed in **(figure 2)**.

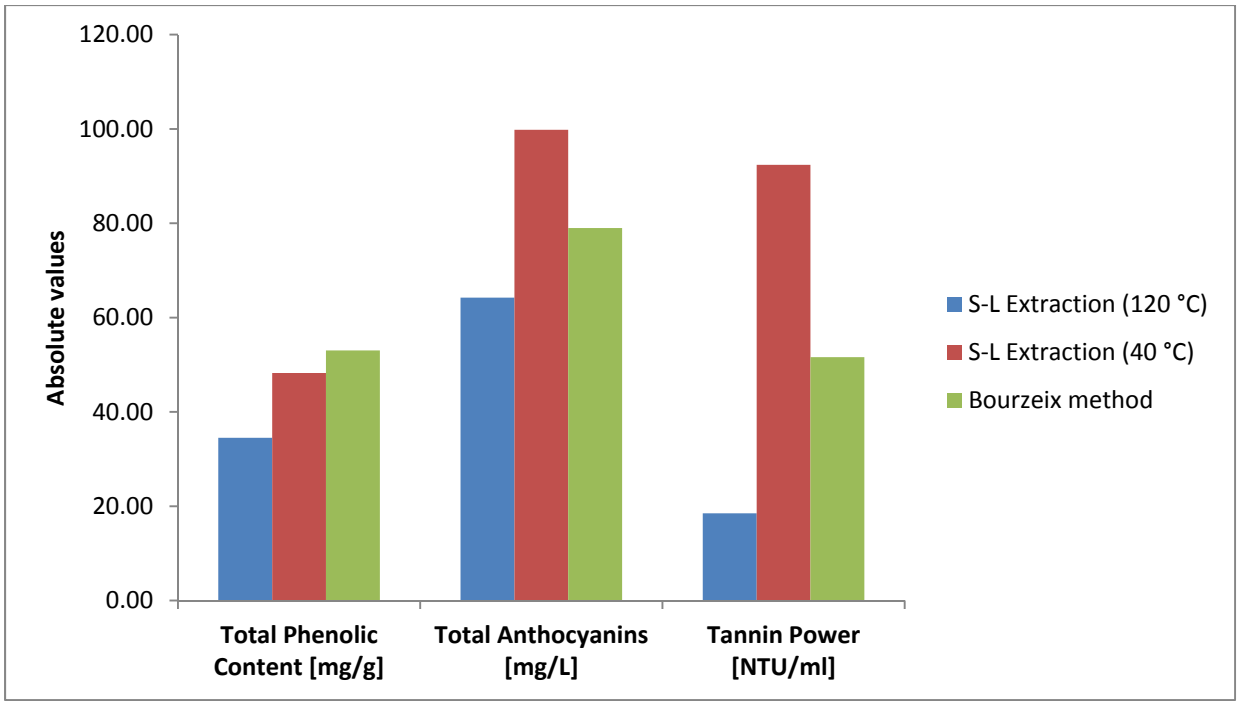

Figure 2. Comparison of different methods of extractions: concentrations of different classes of phenolic compounds (target compounds) in grape pomace extracts obtained by different methods of extraction

As seen in **(figure 2)**, for the measurement of total anthocyanins and proanthocyanins, it is remarkable to observe that solid-liquid hydro-ethanolic extraction distinguished itself as the most appropriate method of extraction. The fact that its total phenolic content value was slightly lesser than Bourzeix's method shows that the use of the total phenolic compounds as an indication of the presence of proanthocyanins has to be taken cautiously; total phenolic compounds present in a sample may be due to others classes of phenolic compounds, such us non-flavonoids like phenolic acids and stilbens.

As expected, the solid-liquid extraction using Soxhlet experimental unit was least effective in comparison with the other methods as reports confirmed that temperature above 45°C proved progressive destruction for not only anthocyanins (acylated monoglucosides of anthocyanins [56] but also proanthocyanins (trimer procyanidins) [64]. However, the advantage of using this Soxhlet unit for solid-liquid extraction is the extraction time that requires only 8 hours compared to 3 days for solid-liquid hydro-ethanolic extraction at 40°C and 5 days for Bourzeix's method. Furthermore, the feed solutions obtained from 40% wt ethanol extracting solution at 40°C and from Soxhlet were subjected to fractionation of monomeric (FI), oligomeric (FII) and polymeric (FIII) fractions of phenolic compounds enriched in flavan-3-ols and the absolute values obtained were compared as shown in **(Table 2)**.

Table 2. Comparison of the fractionated phenolic compounds at different temperatures

Solid-Liquid Extraction at 40% wt ethanol	Fractionated Phenolic Compounds (mg/g)		
	Monomeric fractions FI (mg/g)	**Oligomeric fractions FII (mg/g)**	**Polymeric fractions FIII (mg/g)**
(with soxhlet)	0.37	0.69	6.10
(without soxhlet) 40°C	0.40	2.23	11.93

The above values in the table corresponds to the flavan-3-ols profiles obtained by Monagas et al in a similar study to identify the individual amounts of fractionated phenolic compounds obtained from grape skins [60]. Thus, the present work incorporates the Soxhlet unit for fast screening of membranes as the feed solution can be easily obtained for membrane processing in lesser time. However, owing to its reliable, biocompatible and effective method of extraction of different classes of phenolic compounds, **solid-liquid hydro-ethanolic extraction at 40°C** (ethanol 40% wt) was chosen as the most appropriate extraction method.

3.3 Screening of Commercial Organic Solvent Ultra and Nanofiltration membranes

With the objective of permeating the monomeric and oligomeric phenolic compounds to separate them from the crude grape pomace extract, different membranes with different pore size, material and chemical nature were processed with extracting solution at 40°C and 400 rpm in Metcell. The transmembrane pressures were of 3 bar for Nadir UP 005, 25 bar for Solsep NF010306, 8 bar for Duramem 500 and 8 bar for Duramem 900.

The **permeabilities** of the different membranes and their **observed rejections** of total phenolic compounds measured by direct spectral reading at 280 nm over the Final Concentration FC are shown in **(Figure 3 and Figure 4)** respectively. The interpretations of these graphs add as supporting parameters for screening of membranes.

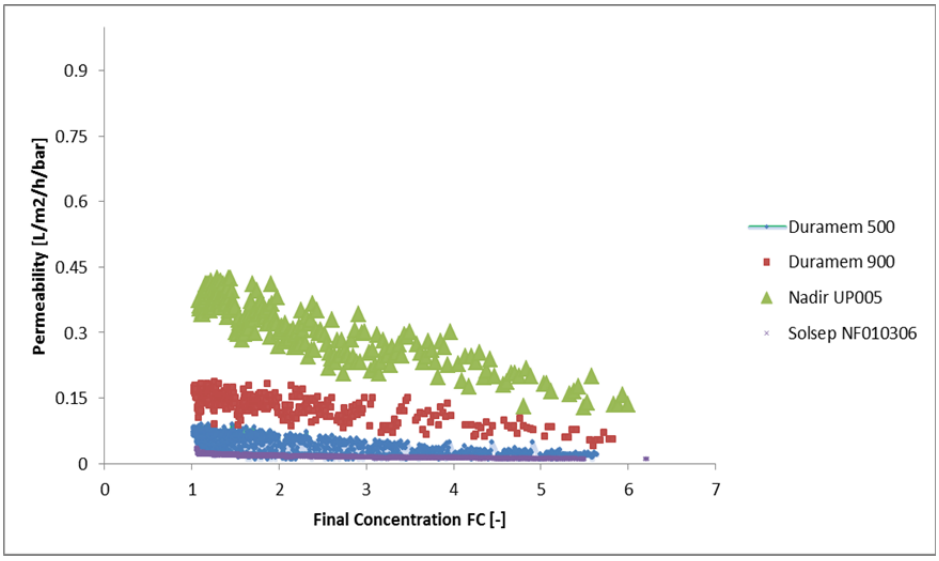

Figure 3. Permeability vs. Final Concentration for Nanoiltration membrane experiments

Legend: FC [-] is the ratio between initial feed and retentate

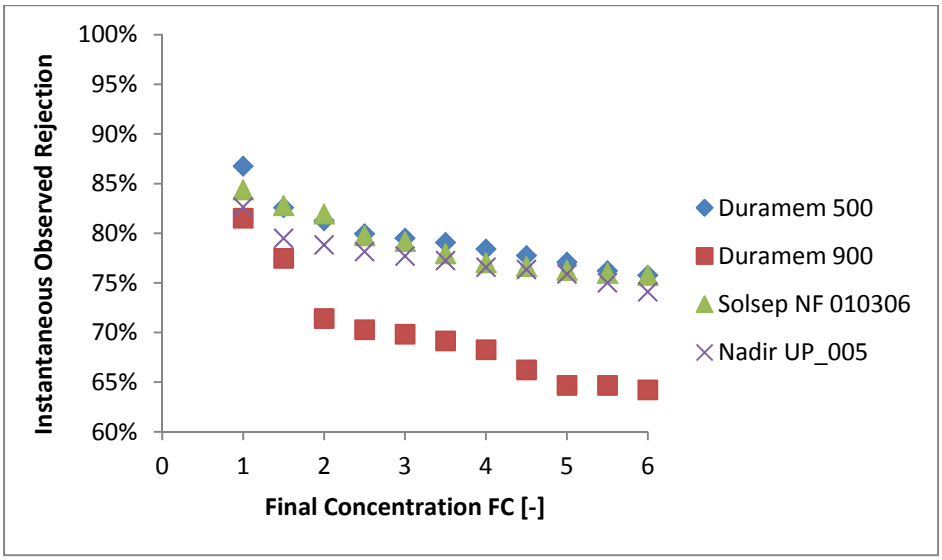

Figure 4. Instantaneous Observed Rejection vs. Final Concentration FC [-]

Legend: FC [-] is the ratio between initial feed and retentate

It can be seen in the above figures that permeabilities are related to the pore size of the membranes. The higher the pore size, the better is the permeability value. Since the feed solution of crude extract contain higher percentages of polymeric phenolic compounds which are bigger in size, the 500 dalton membranes are prone to fouling and its subsequent lower permeability. Furthermore, the rejections of phenolic molecules are almost similar for the three membranes. Nevertheless, Duramem 900 has distinguished

itself to permeate more molecules. However, the permeating molecules are monomeric, oligomeric or polymeric can be confirmed by the experiments that follow below. Also, the membrane conditioning was done effectively with 40% wt ethanol to all the membranes and after the processing is completed, the membranes showed significant closing of global masses balances at the initial feed and final combined retentate and permeate.

3.3.1 Comparison of the Global Observed Rejections of Anthocyanins and Proanthocyanins

The Somer's and the Tannin Power methods were performed in the same way as discussed before and the global retentate and global permeate streams of all the four membranes were analyzed. The results of measurement of total anthocyanins and proanthocyanins and their rejections are represented in **(Table 3)**.

Table 3. Measurement of Total Anthocyanins and Total Proanthocyanins and their Global Observed Rejection Coefficients for the different membranes

Membrane	Anthocyanins			Proanthocyanins		
	Retentate	Permeate	Observed Rejection	Retentate	Permeate	Observed Rejection
Nadir UP_005	400.23	12.12	96.97 %	82.63	3.50	95.76 %
Solsep NF 010306	478.97	52.52	89.03 %	101.25	5.88	94.19 %
Duramem 500	645.39	2.02	99.68 %	105.75	10.75	89.83 %
Duramem 900	591.90	18.20	96.92 %	116.38	8.13	93.01 %

In the above table, it can be seen that Solsep NF 010306 is relatively rejecting lesser anthocyanins which are polymeric flavan-3-ols which is not good for our objective of work. Proanthocyanins are known to exist both in oligomeric and polymeric form and hence, its rejection value interpretation can be done after performing fractionation of individual classes of phenolic compounds.

3.3.2 Fractionation of Phenolic Compounds by C18 Sep-Pak Cartridges

For having a concrete screening of the membranes, the individual fractionated amounts of monomeric, oligomeric and polymeric flavan-3-ols are of importance. Polymeric proanthocyanins with degree of polymerization (DP>5) are very difficult to resolve by HPLC techniques when compared to simple oligomers. Therefore, purification techniques such as normal-phase HPLC, adsorption chromatography on Fractogel TSK HW-40 and C_{18} Sep-Pak cartridges are able to fractionate monomeric, oligomeric and

mixtures of polymeric phenolic compounds [61,62]. Thus, in our study, C_{18} Sep-Pak cartridges were incorporated and the results are depicted in **(Table 4)**.

Table 4. Fractionated phenolic compounds of different membranes

Membrane	Retentate			Permeate		
	Monomeric fractions FI (mg/g)	Oligomeric fractions FII (mg/g)	Polymeric fractions FIII (mg/g)	Monomeric fractions FI (mg/g)	Oligomeric fractions FII (mg/g)	Polymeric fractions FIII (mg/g)
Nadir_UP005	0.51	2.51	4.72	0.13	0.25	0.36
Solep_NF06	0.87	1.14	5.27	0.03	0.04	0.16
Duramem 500	1.05	1.00	5.76	0.03	0.05	0.07
Duramem 900	0.43	1.39	8.46	0.29	0.33	0.53

From the above figure, it can be seen that Duramem 900 made up of modified polyimide, hydrophobic nature with apparent MWCO 900 daltons is relatively the most appropriate membrane. It has retained a lot of polymeric phenolic compounds and allowed to permeate higher monomeric and oligomeric fractions compared to other membranes. A graph of global observed rejection in **(figure 5)** for FI, FII and FIII fractions of different membranes states that Duramem 900 can be chosen as basis for the screening procedure.

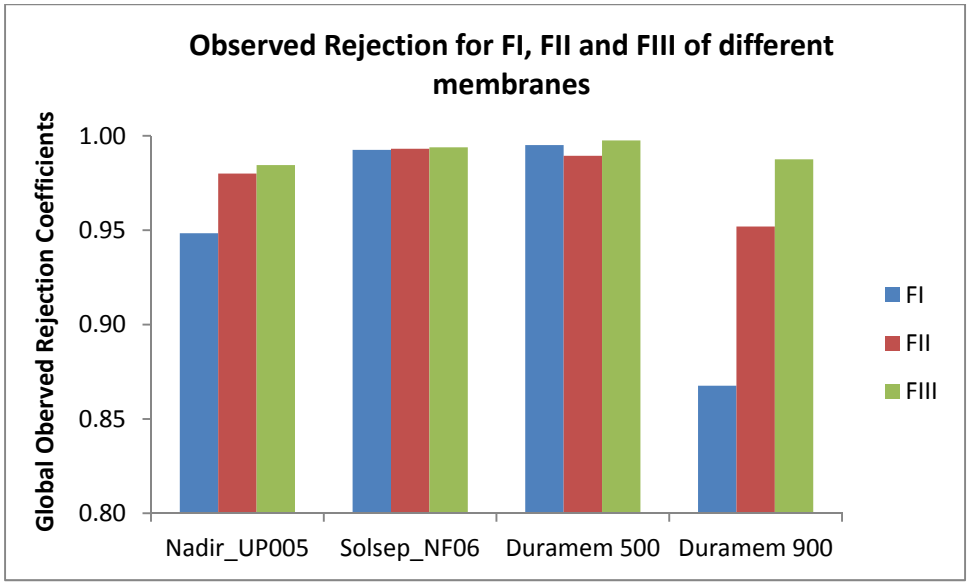

Figure 5. Comparison of Global Observed Rejection Coeffcients of FI, FII and FII by different membranes

Legend: FI – monomeric flavan-3-ols, FII – oligomeric flavan-3-ols, FIII – polymeric flavan-3-ols

3.4 Nanofiltration vs. Diananofiltration

Feed obtained by solid-liquid extraction with 40% wt ethanol using soxhlet experimental unit was used for fast screening of most appropriate membrane. However, once the Duramem 900 membrane was selected, it was processed with feed extract obtained by solid-liquid extraction with 40% wt ethanol for 3 days. Once the most effective extraction method and most appropriate membrane were chosen, it was subjected to Nanofiltration and Diananofiltration membrane processing. With the objective of study of diananofiltration, unfolding of differences in values of all the characterization results would ensure the advantageous usage of diananofiltration for commercial purposes.

3.4.1 Comparison of Permeabilities for nanofiltration and diananofiltration

The Duramem 900 was subjected to nanofiltration with membrane processing conditions same as the previous one. Later on, with second flat sheet Duramem 900 membrane, diafiltration was performed which is based on keeping uniform volume of feed throughout the experiement for two diafiltration volumes. The diluent used was the same solution used for membrane conditioning which is 40% wt ethanol.

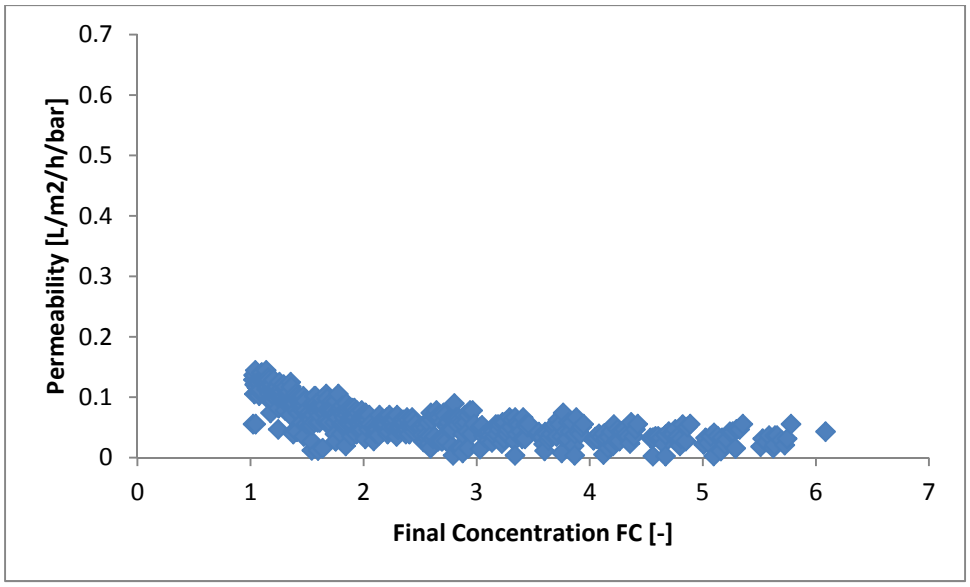

Figure 6. Permeability vs. Final Concentration for Nanofiltration experiment

Legend: FC [-] is the ratio between initial feed and retentate

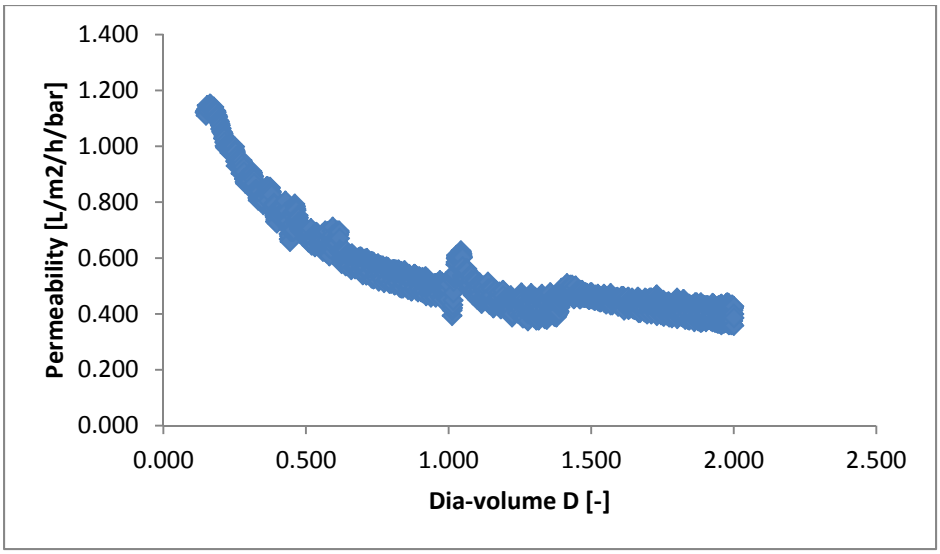

Figure 7. Permeability vs. Dia-volume for diananofiltration experiment

Legend: D [-] is the ratio of solvent added to initial feed solution

From the figures 6 and 7, it can be seen that the permeability of the nanofiltration is much lesser than diananofiltration. In both the filtration techniques, the gradual decrease of permeability over time is the result of the fouling mechanism that occurs. However, the diafiltration has constant initial volume and is kept diluted with the diluent and hence the fouling is controlled to a greater extent. This is one of the major advantages of using diafiltration technique when compared to the normal filtration. This phenomenon plays a vivid role in the rejection of molecules. Further characterization and fractionation results give a better understanding of this concept.

3.4.2 Comparison of the Global Observed Rejections of Anthocyanins and Proanthocyanins

The Somer's and the Tannin Power methods were performed in the same way as discussed before and the global retentate and global permeate streams were analyzed. The results of measurement of total anthocyanins and proanthocyanins and their rejections are represented in **(Table 5)**.

Table 5. Measurement of Total Anthocyanins and Total Proanthocyanins and their Global Observed Rejection Coefficients for nanofiltration and diananofiltration

Membrane	Total Anthocyanins [mg/ml]			Total Proanthocyanins [NTU/ml]		
Duramem 900	Retentate	Permeate	Observed Rejection	Retentate	Permeate	Observed Rejection
Nanofiltration	508.53	20.20	96.02 %	196.25	3.00	98.47 %
Diafiltration	68.67	22.22	94.61 %	39.25	3.13	98.67 %

Since the feed is continuously diluted in diafiltration keeping the uniform volume, the final higher values of anthocyanins and proanthocyanins in nanofiltration experiments are because of its FC [-] to be 6. It can be seen that anthocyanins are highly rejected in both the filtration techniques. However, a higher rejection in proanthocyanins in both the filtration modes is not entirely desirable as proanthocyanins can occur in both oligomeric and polymeric forms and the former are interested in our work. Hence, for better judgement of appropriate method for filtration, the retentate and permeate streams containing phenolic compounds were fractionated by C_{18} Sep-Pak cartridges.

3.4.3 Fractionation of Phenolic Compounds by C18 Sep-Pak Cartridges

The monomeric (FI), oligomeric (FII) and polymeric (FIII) fractions obtained from retentate and permeate streams of nanofiltration and diananofiltration techniques were fractionated and the results are seen in **(Table 6)**.

Table 6. Fractionated phenolic compounds of different membranes

Duramem 900 membrane	Retentate			Permeate		
	Monomeric fractions FI (mg/g)	Oligomeric fractions FII (mg/g)	Polymeric fractions FIII (mg/g)	Monomeric fractions FI (mg/g)	Oligomeric fractions FII (mg/g)	Polymeric fractions FIII (mg/g)
Nanofiltration	0.51	3.63	16.44	0.26	0.84	0.35
Diafiltration	0.22	4.02	11.38	0.30	1.12	0.15

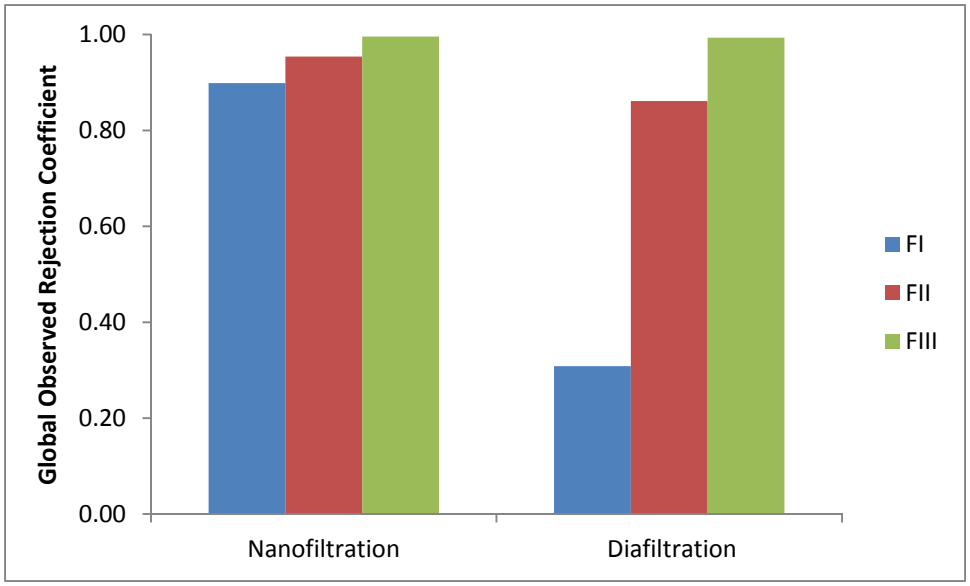

Figure 8. Comparison of Observed Rejection of FI, FII and FII by Nanofiltration and Diananofiltration

Legend: FI – monomeric flavan-3-ols, FII – oligomeric flavan-3-ols, FIII – polymeric flavan-3-ols

From the figure we can see that with diafiltration mode of operation is relatively effective compared to the nanofiltration from the perspective of fractionating monomeric and oligomeric phenolics into the permeate stream. Thus, the higher rejection of monomeric and oligomeric flavan-3-ols in the nanofiltration process in the dead end system can be attributed to the fact that there is a formation of solute film consisting mainly of the higher molecular weight fractions which contributes to the resistance of permeation of the desired monomeric and oligomeric fractions.

4. Conclusion:

The study derives strength for the research activity from the 'biobased' economy which mimics the natural ecosystems – reuse, recover and regenerate; the areas which will be explored to find a value addition to the thousands of tons of grape pomace residue generated every year by the juice and winemaking industry. Additionally, it incorporates prudently the use of membrane technology as a clean and effective way of separation and fractionation of bioactive molecules known to have myriads of benefits in the health sector.

In the present work, optimizations of several parameters were carried out that play a significant role in achieving the desired concentration and fractionation of monomeric and oligomeric flavan-3-ols. The primary step was to efficiently extract the phenolic compounds from the solid parts of the grape pomace. In this context, one of the key issues of this work was to keep the process commercially attractive by having biocompatible solvents for extraction. Thus, studies were carried out to access the optimum composition of hydro-ethanolic extracting media that has maximum extracting capability. Results showed that 40% wt ethanol was the most effective mixture and corroborates the fact of a having a greener method of extraction.

Furthermore, there was a comparative study of the three different methods of extraction. Kinetics of extraction in Soxhlet was studied and optimum extraction time was 8 hours. As it was a quicker method, it was employed in fast screening of membranes. However, solvent extraction at 40°C was more effective in the extraction of phenolic compounds from the industrial waste of grape pomace.

The total phenolics were measured by using 3 different methods namely the Folin Ciocalteau test, Glories test and Spectrophotometric test at 280 nm. It was remarkable to observe the similarity in results with Gallic Acid Equivalents having standard deviation range of 0.79-4.9 mg/g of dried pomace. Thus, the latter method was well accepted for being simple, fast and reliable.

The crude extracts were subjected to process screening with 4 different types of membrane. The observed rejection coeffiecients in the Nadir UP_005, Solseo NF 010306, Duramem 500 and Duramem 900 were found to be in the range of 65% to 80% at FC [-] of 6. The measurement of anthocyanins and proanthocyanins at the retentate and permeate streams were done to obtain a supporting data for interpretation of screening procedure. Finally, the monomeric, oligomeric and polymeric fractionation of flavan-3-ols by C18 Sep-Pak cartridges revealed that Duramem 900 was most appropriate membrane for separation and fractionation.

At the end, the most effective method of extraction and the most appropriate membrane was subjected to comparative studies of organic solvent nanofiltration and diananofiltration. It was clearly illustrated in diafiltration studies that reduced fouling; better permeability and diluted feed streams attributed the fact of lower rejections of monomeric and oligomeric phenolic compounds and higher rejection of polymeric fractions in comparison to the nanofiltration. It could be interesting to carry out the study at pilot scale and also access the bioactivity of the extracted flavan-3-ols.

Further study:

The monomeric and oligomeric fractions of flavan-3-ols obtained from the C18 Sep-Pak cartridges will be injected into HPLC equipment loaded with few standard samples of commercially available monomers and oligomers. This will give a better perspective to identify the individual monomers and oligomers permeated in the nanofiltation and diananofiltration techniques. After the results are obtained, the chemical nature and sizes of the distinctive molecules will be related to that of the Duramem 900 membrane.

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