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THE ROLE OF POLYSACCHARIDES ON THE GRAPE MUST ULTRAFILTRATION PERFORMANCE

A INFLUÊNCIA DOS POLISSACÁRIDOS DO MOSTO NO DESEMPENHO DA ULTRAFILTRAÇÃO

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SUMMARY

This work addresses ultrafiltration of grape must and the understanding of the membrane/polysaccharides interactions due to the chemical composition of the soluble grape must polysaccharides. The performance of two laboratory-made cellulose acetate membranes was investigated. The membranes have molecular weight cut-off of 96 kDa (CA-400-32) and 31 kDa (CA-400-28). To identify the different polysaccharides in the fractions obtained by ultrafiltration, these molecules were isolated by dialysis, then concentrated, freeze-dried and the polysaccharide composition analysed by Gas-Chromatography with flame ionization detector after acid hydrolysis, reduction and acetylation. Polysaccharides adsorbed on the membranes were also identified and quantified by Gas-Chromatography, after hydrolysis, reduction and acetylation of the known mass of dehydrated membrane. The analysis of the membrane matrix, give evidence that mannoproteins were adsorbed on the matrix of CA-400-32 membrane, which has originated, in some extension, the clogging of the pores. It was concluded that the ramnogalacturonan type II crossed the CA-400-32 membrane easily, however its depletion in the retentate and permeate streams over time may be due to its accumulation on the membrane surface, probably caused by adsorption. Arabinogalactan-proteins and mannoproteins were found in the permeate stream of the CA-400-32 membrane, whilst in the CA-400-28 membrane, ramnogalacturonan type II and the majority of arabinogalactan-proteins and mannoproteins remained in the retentate.

RESUMO

Este trabalho visa a ultrafiltração do mosto e o estudo das interações membrana/polissacáridos devido à composição química dos polissacáridos do mosto. Foi investigado o desempenho de duas membranas de acetato de celulose feitas em laboratório. As membranas têm um limite de exclusão molecular de 96 kDa (CA-400-32) e 31 kDa (CA-400-28). Para identificar os diferentes polissacáridos nas frações obtidas por ultrafiltração, as moléculas foram isoladas por diálise, concentradas, liofilizadas e a composição em polissacáridos analisada por cromatografia gasosa com detector de ionização de chama, após hidrólise ácida, redução e acetilação. Os polissacáridos adsorvidos nas membranas foram também identificados e quantificados por cromatografia gasosa, após hidrólise, redução e acetilação duma massa conhecida de membrana desidratada. A análise da matriz da membrana, mostrou que manoproteínas foram adsorvidas na matriz da membrana CA-400-32, o que originou, em alguma extensão, o entupimento dos poros. Concluiu-se que a ramnogalacturonana tipo II atravessou a membrana CA-400-32 facilmente, no entanto a sua diminuição ao longo do tempo no retentado e permeado pode ser devido à sua acumulação na superfície da membrana, provavelmente causada por adsorção. As arabinogalactanas-proteínas e as manoproteínas foram encontrados no fluxo do permeado da membrana CA-400-32, enquanto na membrana CA-400-28, a ramnogalacturonana tipo II e a maioria das arabinogalactanas-proteínas e manoproteínas permaneceram no retentado.

Key words: ultrafiltration, separation, polysaccharides.
Palavras-chave: ultrafiltração, separação, polissacáridos.

INTRODUCTION

Membrane processes like Microfiltration (MF), Ultrafiltration (UF), Reverse Osmosis (RO), Nanofiltration (NF) and Electrodialysis (ED) are widely used in wine industries, namely to clarify musts and wines, to concentrate them, to eliminate a part of the fermentable sugars, or to achieve tartaric stability.

Despite the fact that these techniques have become an alternative to conventional processes, the complex composition of grape musts and wines cause problems of membrane fouling that are not well understood. However, very often the fouling of ultrafiltration membranes is attributed to the retention of macromolecular components like polysaccharides, and this is very detrimental for the wine quality. The traditional approach that considers the ultrafiltration mainly governed by sieving mechanisms, has been taken over by an approach where simultaneously with these mechanisms, there are other ones resulting from the development of membrane/macromolecules interactions. In fact, taking into account the complex nature of these macromolecules, a multiplicity of complex interactions with the membrane matrix, can occur and lead not only to fouling but also to undesirable aspects like inhibition of their function as protective colloids (Gonçalves *et al.*, 2001; De Pinho, 2010).

Polysaccharides are an important group of macromolecules in grapes and include: arabinogalactan-proteins (AGPs) (Saulnier and

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Brillouet, 1989; Brillouet *et al.*, 1990; Saulnier *et al.*, 1992; Pellerin *et al.*, 1995; Pellerin and Cabanis, 1998; Doco *et al.*, 2000; Vidal *et al.*, 2001; Doco *et al.*, 2003; Vidal *et al.*, 2003), arabinans (Villetaz *et al.*, 1981; Belleville *et al.*, 1993), and rhamnogalacturonan type II (RG-II) (Belleville *et al.*, 1993; Doco and Brillouet, 1993; Pellerin *et al.*, 1996; Doco *et al.*, 1997; O'Neill *et al.*, 2004).

In grape must (unfermented juice) polysaccharides originate from the cell walls of grape berries, belonging two families. The largest one is rich in arabinose and galactose and contains arabinogalactan-proteins (AGPs). An AGP of average molecular weight of 165 kDa (Saulnier and Brillouet, 1989) and another one of 110 kDa (Saulnier *et al.*, 1992) were isolated from the pulp of grape berries. However, in other work, the average molecular weight of five arabinogalactan-proteins fractions separated from a red wine ranged from 180 to 260 kDa (Pellerin *et al.*, 1995).

AGPs have a main chain of galactose units linked by β-(1,3) oside bonds, with short lateral chains of galactose bonded on β-(1,6). In C3, these are replaced by individual arabinose units. AGPs also have a few individual arabinose units on carbon 6 or 4 of the galactose in the main and secondary chains (Brillouet *et al.*, 1990; Pellerin *et al.*, 1995). AGPs are linked to a protein (2 to 10% by weight) characterized by the presence of hydroxyproline. The AGPs are present in wine (Pellerin *et al.*, 1995) at concentrations approximately of 100 to 250 mg/L for red wines and 50 to 150 mg/L for white wines (Pellerin and Cabanis, 1998; Doco *et al.*, 2000). The pulp tissue from grape berry contains two-fold more soluble AGPs than the skin tissue (Vidal *et al.*, 2001). AGPs represent a major proportion (40%) of the total wine polysaccharides, confirming the abundance of AGPs in red wine (Pellerin *et al.*, 1995).

The elucidation of the composition and structure of the macromolecular fraction of the wines aged on lees, showed a depolymerization of the native polysaccharides of the grapes. This modification consisted of a 75% dearabinosylation of arabinogalactan-proteins (AGPs) and a total hydrolysis of the pectic arabinans (Doco *et al.*, 2003).

The arabinans of the same family are only rich in arabinose, they are small polymers with a low molecular weight (6 kDa) and consist of short arabinose chains, bonded in α-(1,5), with ramifications of individual arabinose units on C3 (Villetaz *et al.*, 1981). The concentration of arabinans is approximately 100 mg/L at the end of alcoholic fermentation, and is rarely present in mature wine (Pellerin and Cabanis, 1998; Doco *et al.*, 2003). Nevertheless, an insoluble linear arabinan has been isolated from a red wine (Belleville *et al.*, 1993).

The second family is rhamnogalacturonan type II (RG-II), identified in many plant tissues (Vidal *et al.*, 2000; Ridley *et al.*, 2001). It's a very complex pectic polysaccharide with a low molecular weight – 5,3 kDa determined by high performance size-exclusion chromatography (HPSEC) and 9,8 kDa determined by low-angle laser-light-scattering (LALLS) (Doco *et al.*, 1993) – and composed mainly of rhamnose and galacturonic acid. RG-II consists of a main chain of seven to nine galacturonic acids units with four lateral oligosaccharide chains (Doco *et al.*, 1993; O'Neill *et al.*, 2004). These chains contain arabinose, rhamnose, fucose, galactose, galacturonic and glucuronic acids and also rare sugars as 2-O-methyl-fucose, apiose, 2-O-methyl-xylose, KDO (2-Keto-3-deoxy-D-manno-octulosonic acid), DHA (3-deoxy-D-lyxo-hepyulosaric acid) and aceric acid (3-C-carboxy-5-deoxy-L-xylose) (Doco *et al.*, 1993; Pellerin *et al.*, 1996; Vidal *et al.*, 2000; Ridley *et al.*, 2001; O'Neill *et al.*, 2004). RG-II is resistant to pectolytic enzymes and is found in fruit juice and wine obtained by total liquefaction with pectinases (Doco *et al.*, 1997). RG-II represents about 20% of total red wine polysaccharides (Pellerin *et al.*, 1996). The concentration of RG-II in white wines is smaller (ranging from 30 to 50 mg/L), than in red wines (from 100 to 150 mg/L) (Doco *et al.*, 1997). It was shown that the content of RG-II per kilogram of berries is approximately 250 mg and its content in skin tissue is three-fold higher than that of pulp tissue (Vidal *et al.*, 2001). This result is consistent with the fact that more grape polysaccharides are present in red wine than in white wine (Vidal *et al.*, 2001).

Mannoproteins (MPs) are another major group of polysaccharides in wine and are originated from yeast cell walls. This polysaccharide leads to an enrichment of wine in mannose both in the beginning of alcoholic fermentation and during aging on lees by autolysis of yeasts (Saulnier *et al.*, 1991; Waters *et al.*, 1994; Feuillat, 2003). MPs have a wide range of molecular weights in wines, extending from 5 to more than 800 kDa (Saulnier *et al.*, 1991; Gonçalves *et al.*, 2002). A group of MPs are constituted by about 10% protein and 90% of mannose. The molecular structures of MPs consist of a peptide chain linked to D-mannose units in α-(1→6), α-(1→2) and α-(1→3) (Saulnier *et al.*, 1991; Waters *et al.*, 1994). Its concentration in wines is approximately 100 to 150 mg/L (Saulnier *et al.*, 1991; Waters *et al.*, 1994; Feuillat, 2003). MPs isolated from a white wine represent 32.2% of the total wine polysaccharides (Gonçalves *et al.*, 2002).

The involvement of polysaccharides in many oenological phenomena is due to their physico-chemical properties, when in solution. For instance, their ability to interact with tannins to inhibit their aggregation, maintaining the stability of colour (Riou *et al.*, 2002) and in red wines the increase of smoothness and body (Guadalupe *et al.*, 2010). As already stated, another important property is their ability to act as protective colloids, their contribution to protein stability (inhibition of protein haze in white wines) (Waters *et al.*, 1993) and tartaric stability (inhibition of hydrogen tartrate crystallization,

increasing tartaric stability) (Gerbaud *et al.*, 1997; Moine-Ledoux and Dubourdieu, 2002). Also, an important role of RGII, that is under investigation, and include nutritional benefits, is the complexation of divalent cations, to form specific coordination complexes with heavy metals (O'Neill *et al.*, 1996; Pellerin *et al.*, 1997). Finally, the presence of polysaccharides is determinant in the persistence of bubbles in sparkling wines and in the interaction with aromatic compounds (Chalier *et al.*, 2007).

In order to evaluate the role of these macromolecules in the fouling of filtration membranes, many studies have been done (Belleville *et al.*, 1990, 1992; Cameira dos Santos *et al.*, 1994; Cameira dos Santos, 1995; Vernhet *et al.*, 1997, 1999; Vernhet and Moutounet, 2002; Vernhet *et al.*, 2003; Ulbricht *et al.*, 2009; El Rayess *et al.*, 2011).

Belleville *et al.* (1992, 1993) identified the fouling of an inorganic tubular alumina membrane during red wines microfiltration and attributed it mainly to RG-II, that was strongly absorbed by the membrane and caused a sharp decrease of the permeation flux and a linear arabinan, also induced a decrease in permeate flux. Cameira dos Santos *et al.* (1994) studied wine crossflow microfiltration using polyethersulfone (PES) capillary membranes and concluded that macerated wine is more foulant, probably due to the higher levels of polysaccharides and polyphenols. They also suggested that differences in membrane fouling behaviour between wine samples is not related to its initial polyphenol and polysaccharide concentrations, but with the composition and structure of these foulant molecules. Cameira dos Santos *et al.* (1994) has revealed the involvement of wine polyphenols in the membrane fouling by washing the fouled membrane with acidified methanol and significant increases in permeability were obtained. This fact could be attributed to the elimination of the layers of phenolic compounds because the other wine constituents are insoluble in this solvent. Vernhet *et al.* (1999) isolated wine polysaccharides from a red wine, and microfiltered separately in a synthetic wine to determine their influence on the permeation fluxes of an hydrophilic organic microfiltration membrane (poliethersulfone). The permeation flux decline was differed according to the nature of the polysaccharide tested. According to these authors mannoproteins fractions (30–400 kDa) displayed the strongest decrease on wine filtrability. However, the fouling caused by mannoproteins was reduced in the presence of the pectic polysaccharides, which suggests that the performance of the membrane will depend on intermolecular interactions and interactions between membrane and foulant. Vernhet *et al.* (2002) also studied the effect of wine polysaccharides on three organic membranes, two of them made of PES with different amount of polyvinylpyrrolidone (PVP) and another one made from polyvinylchloride (PVC). They showed that the polysaccharides adsorption was negligible in static membranes for the three membranes. By filtering wines enriched with polysaccharides, no significant differences were observed between the three membranes in terms of amount and nature of the deposited polysaccharides. It was observed a deposition of AGP-II and MPs on the surface of the membrane. They concluded through observation of membrane surfaces by scanning electron microscopy that membrane fouling by polysaccharides is due to their accumulation on membranes, mainly at the pores entrance and on surface. Vernhet *et al.* (1997) demonstrated through adsorption experiments that the polarity of the membrane surface is a limiting factor in wine polysaccharides adsorption. Adsorbed polysaccharides were mainly grape arabinogalactan-proteins and yeast mannoproteins. According to these authors when the polarity of the membrane surface increased polysaccharides adsorption decreased, due to unfavourable polar interactions between hydrophilic polysaccharides and the material. Contrary to polysaccharides, the maximum tannins adsorption occurred on the most polar membrane. The aim of one study by Ulbricht *et al.* (2009) was to pursue the hypothesis that polypropylene (PP) and PES membranes exhibit different levels of adsorption of typical foulants in wine such as polyphenols and polysaccharides, to link the level of adsorption to polymer characteristics and to correlate membrane fluxes with these findings. It was found that polyphenols and polysaccharides are only marginally adsorbed by PP membranes that presents hydrophobic character, but strongly adsorbed by PES membranes that has hydrophilic character. The low adsorption tendency of wine ingredients to PP membranes results in higher fluxes and longer service life of the respective filtration modules in wine clarification.

The present work investigates the ultrafiltration of grape must with two UF cellulose acetate membranes, of different molecular weight cut-offs, for the separation of polysaccharide macromolecules into two streams, that are further processed by the dialysis (allowing the liberation of small molecules like monosaccharides, salts, organic acids and small polyphenols) and identification of its composition in terms of neutral monosaccharides. Cellulose acetate membranes have many advantages. They combine high rejection to solutes with high hydraulic permeability due to its asymmetrical structure and have a good behaviour against fouling, because of the hydrophilic character of cellulose acetate.

Therefore the main objective of this study is to understand how the different grape must polysaccharides interact with UF membranes, due to their specific monosaccharide composition, and how that determines the retention and permeation of each polysaccharide group. Winemakers must also be aware of the risk of eliminating high molecular weight polysaccharides that are not only an integral part of a wine's composition but also of its organoleptic characteristics.

MATERIAL AND METHODS

Grape must samples

Grape must used as source of polysaccharides in UF experiments was prepared from matured grapes of Trincadeira Preta (a red cultivar of *Vitis vinifera*), harvested in 2010 in INIAV vineyard (Oeiras, Portugal), used in Carcavelos DOC wine production. The samples were kept refrigerated and decanted, under 6ºC. Sulfur dioxide was added (300mg/L) to conservation and the grape must was brought to room temperature for the experimental runs.

Preparation of the must and of the must ultrafiltration streams, previous to polysaccharide analysis

Prior to polysaccharide analysis, samples of grape must and aliquots of ultrafiltration streams, were extensively dialyzed against distilled water, concentrated and freeze-dried.

The total colloids are first extracted from must and from aliquots of UF streams by dialysis using a 3500 Da cut-off membrane purchased at Spectrum Laboratories, Inc from CA, USA. This treatment allowed the removal of small molecules such as monomeric sugars, salts, organic acids, low size polyphenols and monomeric anthocyanins. Dialysis was carried on till the permeate conductivity was lowered up to 40 µS/cm (the initial must conductivity is 3260 µS/cm). The dialysis retentate was then concentrated under vacuum at low temperature (40ºC) and freeze-dried to give the fraction called total colloids.

Membranes

Two flat-sheet laboratory made membrane were prepared according to the phase inversion method, of Kunst and Sourirajan (1974), using cellulose acetate (CA) with 39.8%, acetyl content, supplied by Eastman-Kodak (Rochester, NY, USA). Table I displays the casting solution composition and the casting conditions of the membranes designated by CA-400-28 and CA-400-32 with different content of formamide to yield an increasing hydraulic permeability.

TABLE I

Casting solution composition and film casting conditions

Soluções poliméricas e condições de preparação das membranas

Membrane	CA-400-28	CA-400-32
Casting solution (wt %)		
Cellulose Acetate (g)	17	17
Acetone (mL)	55	51
Formamide (mL)	28	32
Casting Conditions		
T of casting solutions (ºC)	20-25	20-25
Evaporation time (min)	0.5	0.5
Gelation medium (1-2h)	Deionized water 0 – 3ºC in all cases	Deionized water 0 – 3ºC in all cases

Membrane characterization

The membranes were characterized in terms of hydraulic permeability (Lp) and molecular weight cut-off. Membranes were first compacted through the recirculation of pure water (conductivity <1 µS/cm) at a transmembrane pressure 20% higher than the operating pressure for 3h, to avoid pressure effects on membrane structure throughout subsequent experiments.

The membrane molecular weight cut-off was determined through the permeation of reference solutes, polyethylenglycols (PEG) supplied by Merck and dextrans supplied by Pharmacia. The rejection coefficients to reference organic solutes are displayed in Table II, after permeation of 0.6 g/L solutions with solutes of increasing molecular weight (PEG and dextrans). The solute rejection coefficient, f, was determined in terms of Total Organic Carbon (TOC) and is defined by the following equation,

$$f = \frac{c_f - c_p}{c_f}$$

where c_f and c_p are the solute concentration in the feed and in the permeate solution, respectively.

The MWCO value is obtained by the intersection of the 91% rejection line with the linear relationship of log (f/(1-f)) versus solute molecular weight. This procedure is represented in Figure 1 for the CA-400-32 membrane and in Figure 2 for the CA-400-28. The membrane MWCO is defined by the molecular weight of a given macromolecule whose rejection coefficient is higher than 91%.

TABLE II

Rejection coefficients obtained for PEG and dextrans macromolecules

Coeficientes de rejeição obtidos para as macromoléculas PEG e dextranas

Membrane	**CA-400-28**	**CA-400-32**
Solute	**f (%)**	**f (%)**
PEG 6000	43	49
PEG 8000	57	48
PEG 10000	67	49, 50
PEG 20000	82	-
PEG 35000	93	63, 68
Dextran 10000	-	52
Dextran 40000	-	73
Dextran 70000	-	85
Dextran 110000	-	94

f (%) - Rejection coefficient

The pure water permeate flux was measured at operating pressures in the ranging from 1 x 10^5 Pa to 3 x 10^5 Pa to yield the hydraulic permeability (L_p) of the two membranes. The membrane hydraulic permeability is given by the slope of the straight line of the pure water permeate flux versus transmembrane pressure. The results obtained are shown in Table III.

TABLE III

Membrane Characterization

Caraterização da membrana

Membrane	**Lp (L/h/m^2/10^5 Pa)**	**MWCO (kDa)**
CA-400-32	17.85	96
CA-400-28	16.97	31

Ultrafiltration experiments in concentration mode to achieve polysaccharide separation

Polysaccharides of the grape must were fractionated by UF in a Lab-unit ("CELFA"), type P-28 (Figure 3). The experiments were run in concentration mode for which the concentrate was recirculated to the feed tank. The membrane surface area was 25.52 x 10^{-4} m^2 and the feed temperature was kept at 25ºC for all experiments.

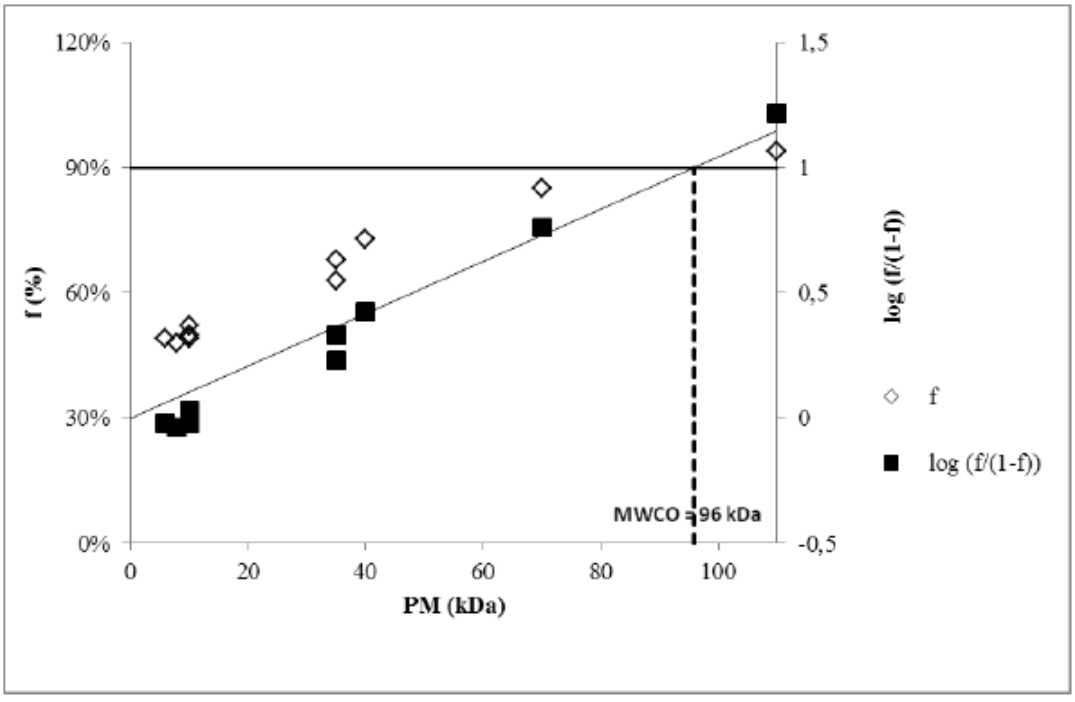

Figure 1 - The MWCO determination for CA-400-32 membrane.

Determinação do limite de exclusão molecular para a membrana CA-400-32.

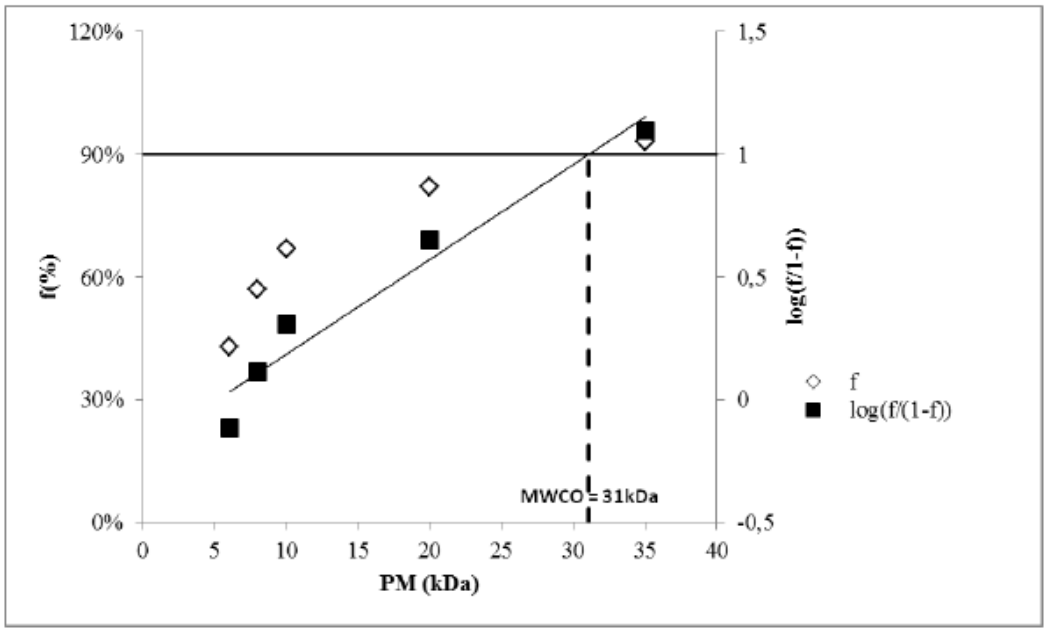

Figure 2 - The MWCO determination for CA-400-28 membrane.

Determinação do limite de exclusão molecular para a membrana CA-400-28.

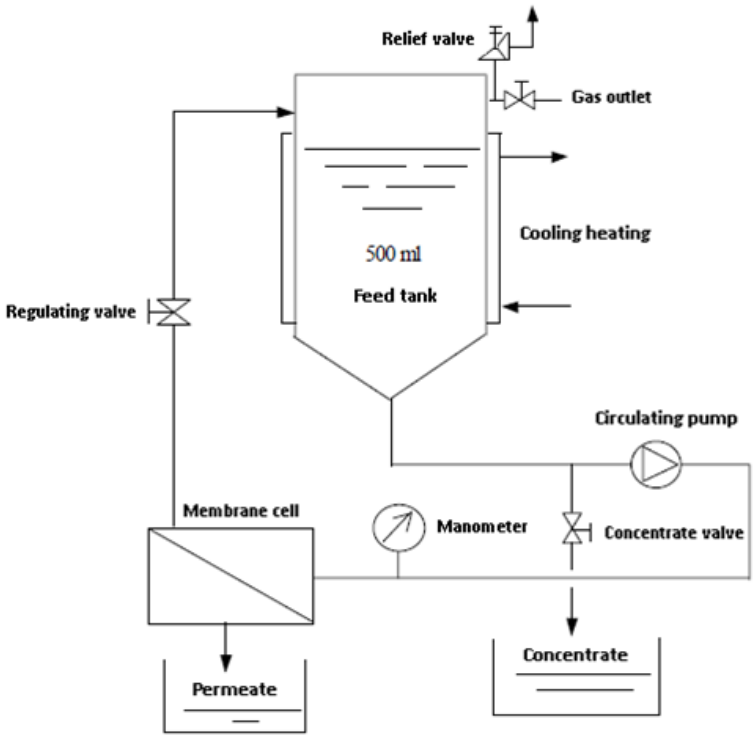

Figure 3 - Schematic diagram of the filtration unit (type P-28 Lab-Unit – CELFA)

Diagrama esquemático da unidade de filtração (tipo P-28 Lab-Unit – CELFA)

The permeation of reference solutions for membrane characterization, as well as permeation of grape must, was performed at a transmembrane pressure of 3 x 10^5 Pa, at a feed flow velocity of 0.67 m/s and at a temperature of 25°C. The permeate samples were collected after the stabilization time. The stabilization time for each experimental run was 10 min. After each essay, membranes were washed with deionized

water and the pure water flux was measured for the verification of any irreversible fouling or damage.

Samples of the feed reference solutions were taken at the beginning and at the end of each experimental run and the average concentration of these two samples were considered the feed concentration. Samples of the feed grape must and of the UF permeates, in the essay with CA-400-32 membrane were taken at the beginning, at the 34th min, at the 49th min, at the 64th min and at the end. Samples of the feed grape must and of the UF permeates in the essay with CA-400-28 membrane were taken at the beginning, at the 60th min, at the 130th min, at the 190th min and at the end.

Membrane cleaning

To recover the initial fluxes, membranes were cleaned, after each essay, first with circulation of deionized water at the temperature of 20ºC for 1h and after with circulation of water at the temperature of 50ºC for 2h. The pure water flux was measured for the verification of any irreversible fouling or damage.

Analytical methods

The feed and the permeate reference solutions concentrations were determined in terms of Total Organic Carbon (TOC). TOC measurements were performed in a Dohrmann Carbon Analyser DC-85A (Dohrmann, Santa Clara, CA, USA) calibrated with potassium hydrogen phthalate solution (2000 ppm). The neutral monosaccharide composition of the feed and permeate of the grape must were determined by Gas Cromatography (GC) after acid hydrolysis with 2M trifluoracetic acid (120ºC, 75 min), reduction, acetylation and extraction of the acetylated alditols (Harris *et al.*, 1984). The separation of alditol acetates was achieved using a fused silica DB-225 (210ºC) capillary column (30 m x 0.25 mm i.d., 0.25 µm film) with H_2 as the carrier gas at a flow rate of 1.3 mL min^{-1} on a Varian Chrompack CP-3800 Gas Chromatograph, equipped with a flame ionization detector (FID) and a capillary split/splitless inlet. Samples were injected in the pulsed split mode with a split ratio of 60:1. The injector and the FID were operated at 250ºC.

Grape must sample was characterized in terms of density, total soluble solids, probable alcohol content, pH, total acidity, total polysaccharides, total polyphenols, conductivity, turbidity and viscosity. Density, total soluble solids, probable alcohol content and conductivity were determined according to Reg. (CEE) 2676/90; pH was determined according to method unique of International Organization of Vine and Wine (OIV); total acidity was determined according to NP-2139/1987. Total soluble solids was carried out by using an Abbe refractometer Atago NAR-1T (Atago Co., Ltd., Tokyo, Japan) and expressed as ºBrix. The pH was measured at 20ºC with a Crison micropH 2002 (Crison, Barcelona, Spain). The total acidity was determined by titration with 0.1N NaOH and results were expressed as g/L tartaric acid. Total polysaccharides of the feed and permeate grape must were determined according to Segarra *et al.* (1995): 4 mL of grape must were precipitated with 20 mL of ethanol; the precipitate was dried and dissolved in 4 mL of water; phenol–sulphuric method, according to Dubois *et al*. (1956), was used to analyze total polysaccharide content and the absorbance is read at 490 nm by using a spectrophotometer; the results were expressed in mg/L of glucose. A calibration curve was previously done. Total polyphenols were determined by total phenolic index, that is a spectrophotometric method (Shimadzu UV, model UV-1700) by measuring the UV absorption at 280 nm, under 1 mm optical path (Somers and Evans 1977) and the results were expressed as mg/L of gallic acid (product of absorbance at 280 nm by the dilution factor of the sample of grape must and using a standard curve). Conductivity was measured at 25ºC with a Crison conductimeter, model 525. Colour intensity was determined by the sum of absorbance at 420, 520 and 620 nm under 1 mm optical path (Sudraud, 1958; Glories, 1984) with a Shimadzu UV, model UV-1700. The tint was measured as the ratio of absorbances A_{420}/A_{520} (Somers and Evans, 1977). Clarity was measured by transmittance at 625 nm using a Shimadzu UV, model UV-1700. Turbidity was determined with a Turbiquant 3000 IR turbidimeter and expressed in nephelometric turbidity units (NTU). Viscosity was measured by using a Brookfield DV-I viscometer (Brookfield Engineering Laboratories Inc., Stoughton, MA, USA).

Quantification of polysaccharides adsorbed on the membrane

A known mass of each anhydrous membrane was cut, weighed, and directly hydrolyzed on a glass tube with 500 µL of TFA. After hydrolysis, the acid was eliminated under a current of air, and the monosaccharide released were reduced, acetylated and separated under the conditions described before.

Reagents for hydrolysis and acetylation

13 M Trifluoroacetic Acid, from Merck, Schuchardt, Hohenbrunn, Germany (99% or greater purity) is used diluted in water at 2 M solution; Acetic Acid (100% puriss.) was obtained from Sigma-Aldrich, Steinheim, Germany; Perchloric Acid (70% puriss. p.a.) was obtained from Sigma-Aldrich, Steinheim, Germany; 1 - Methylimidazole (more than 99% purified by redistillation) was obtained from Sigma-Aldrich, Steinheim, Germany; Ethyl Acetate (minimum assay GC 99.5%) was obtained from Panreac, Barcelona, Spain; Sodium Borohydride (96% PS) was obtained from Panreac, Barcelona, Spain; Acetic Anhydride (99.5% puriss GR for analysis) from Merck, Dermstadt, Germany; Acetone from Panreac, Barcelona, Spain and Chloroform from Merck, Darmstadt, Germany.

Standard sugars

myo-Inositol, L - Rhamnose, L(+) - Fucose, L(+) - Arabinose, D(+) - Xilose, D(+) – Mannose, D(+) - Galactose were obtained from Sigma-Aldrich Steinheim, Germany (99% or greater purity) and D(+) - Glucose Anhydre, from Merck, Darmstadt, Germany.

RESULTS AND DISCUSSION

Characterization of the grape must

The physico-chemical composition of grape must is shown in Table IV.

TABLE IV

Physico-chemical composition of grape must.

Composição físico-química do mosto.

Analytical Parameters	Trincadeira Preta
Density	1.084
Total Soluble Solids (ºBrix)	20.7
Probable Alcoholic Content (%V/V)	11.9
pH	3.45
Total Acidity (g/L tartaric acid)	5.6
Conductivity (mS/cm)	3.12
Total Polysaccharides (mg/L glucose)	4481.5
Total Polyphenols (g/L gallic acid)	410.8
Colour Intensity ($A_{420}+A_{520}+A_{620}$)	0.783
Tint (A_{420}/A_{520})	1.661
Clarity ($\%T_{625}$)	67.4
Turbidity (NTU)	36.10
Viscosity (mPa s)	2.02

The composition of the polysaccharides of grape must expressed in terms of the seven major neutral monosaccharides is summarized in Table V. The arabinose and galactose shown in Table V confirm the presence of arabinogalactan-proteins that are released after the crushing and pressing of the grapes (Vidal *et al.*, 2000). Quantitatively AGPs (Arabinose - 25.21 mg/L and galactose - 26.5 mg/L) are the major grape polysaccharide present in the grape must (Vidal *et al.*, 2000). The arabinose to galactose ratio is 0.95, which is close to 1.0 that is the usually ratio in AGPs (Doco *et al.* 2003).

The content of rhamnose in the grape must, 16.03 mg/L, confirm the presence of RG-II. The composition of RG-II contains fucose and in the grape must small amounts of fucose (4.22 mg/L) were also found. Xilose is a typical constituent of cell walls in the Vegetal Kingdom (Hall *et al.*, 1976) and it is present in grapes and must in the amount of 6.30 mg/L.

TABLE V

Composition of grape must polysaccharides in neutral sugars

Composição dos polissacáridos do mosto em açúcares neutros

Neutral Sugars	Amount in grape must (mg/L)
Rhamnose	16.03
Fucose	4.22
Arabinose	25.21
Xilose	6.30
Mannose	46.58
Galactose	26.5
Glucose	19.61

With the data available, we chose four monosaccharides to assess the main structure of grape must polysaccharides. The identification of 4 constituent neutral sugars was selected to act as markers of macromolecules. Rhamnose acts as a marker of RG-II, arabinose and galactose act as a marker of AGPs, while mannose acts as a marker of mannoproteins.

Permeate flux during ultrafiltration

The variation of permeation flux (J_p) as a function of the volume reduction factor (*VRF*) is displayed in Figures 4 and 5. These Figures show a clear decline of Jp, with the increase in *VRF*, due to increasing fouling effect on the membrane, because of concentration polarization and adsorption on membrane material. The initial permeate flux of 10.65 L/h/m^2 decreased to about 3.5 L/h/m^2, after 278 min of operation, which corresponds to a *VRF* of 1.13 (Figure 4). This permeate flux relative to the initial flux presents a flux reduction of 68%. In Figure 5 the initial permeate flux was 8.22 L/h/m^2 and decreased up to 0.75 L/h/m^2, where the flux reaches a plateau, after 313 min of operation, which corresponds to a value of *VRF*=1.04. The permeate flux relative to initial flux presents a flux reduction of 91%. It can be observed that these curves can be divided into three periods: an initial phase with a rapid decrease of the permeate flux; a second phase with a smaller decrease of the permeate flux that begins around *VRF* = 1.05 (Figure 4) and *VRF* = 1.02 (Figure 5); a third phase with a very small decrease in permeate flux up to steady-state conditions, that occurred after *VRF* = 1.08 (Figure 4) and *VRF* = 1.033 (Figure 5).

The reduction of the flux is probably due to the adsorption of macromolecules in the matrix of the membranes, which is reported in the literature to be caused by polysaccharides and polyphenols (Belleville *et al.*, 1990, 1992; Cameira dos Santos *et al.*, 1994; Cameira dos Santos, 1995; Vernhet *et al.*, 1997, 1999; Vernhet and Moutounet, 2002; Vernhet

et al., 2003; Ulbricht *et al.*, 2009; El Rayess *et al.*, 2011).

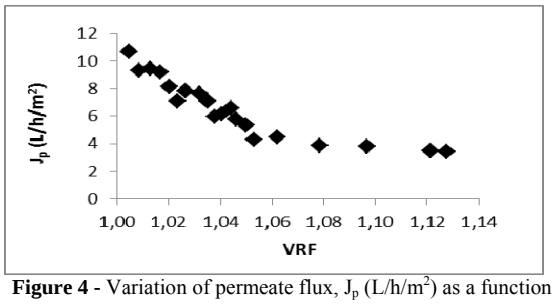

Figure 4 - Variation of permeate flux, J_p (L/h/m^2) as a function of VRF for UF of grape must Trincadeira Preta 2010 with CA-400-32 membrane. (Operating conditions: $\Delta P = 3 \times 10^5$ Pa; T = 25ºC; $A_{CA-400} = 25.52 \times 10^{-4}$m^2; v = 0.67 m/s).

Variação do fluxo de permeado, J_p (L/h/m^2) em função do factor de concentração (VRF) para a ultrafiltração do mosto Trincadeira Preta 2010 com a membrana CA-400-32. (Condições de operação: $\Delta P = 3 \times 10^5$ Pa; T = 25ºC; $A_{CA-400} = 25.52 \times 10^{-4}$m^2; v = 0.67 m/s).

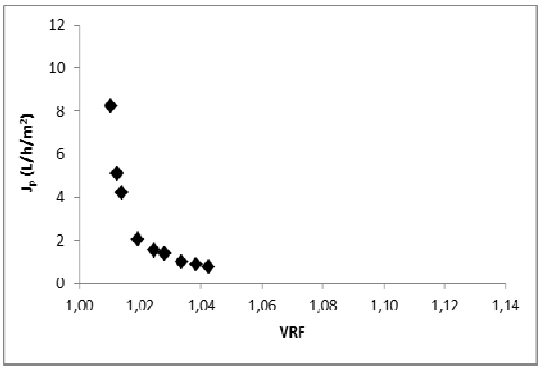

Figure 5 - Variation of permeate flux, J_p (L/h/m^2) as a function of VRF for UF of grape must Trincadeira Preta 2010 with CA-400-28 membrane. (Operating conditions: $\Delta P = 3 \times 10^5$ Pa; T = 25ºC; $A_{CA-400} = 25.52 \times 10^{-4}$m^2; v = 0.67 m/s).

Variação do fluxo fluxo de permeado, J_p (L/h/m^2) em função do factor de concentração (VRF) para a ultrafiltração do mosto Trincadeira Preta 2010 com a membrana CA-400-28. (Condições de operação: $\Delta P = 3 \times 10^5$ Pa; T = 25ºC; $A_{CA-400} = 25.52 \times 10^{-4}$m^2; v = 0.67 m/s).

Quantification of polysaccharides adsorbed on the membrane

The results of the analysis of the membranes as described in the paragraph quantification of polysaccharides adsorbed on the membrane of the Material and Methods section are presented in Table VI and show that mannose and glucose were adsorbed in the CA-400-32 membrane matrix. In fact, Table VI shows the composition in neutral sugars of the membrane matrix (first line), and of the organic matter adsorbed on it (second and third lines). First it was determined the yield of an unused membrane in sugars, to know which glucidic constituents could be liberated (after acid hydrolysis) from the cellulose acetate membrane matrix (CA-400). Secondly it was analyzed the matrix of the CA-400-32 and CA-400-28 membranes after being used in the ultrafiltration of grape must.

The observation of glucose and mannose in the CA-400-32 membrane indicates the adsorption of mannoproteins. The results show that the adsorbed amount of glucose was 17.35 mg/25.52 cm^2 (after removing the 25.78 mg/25.52 cm^2 constituent of the membrane matrix) and 2.04 mg/25.52 cm^2 of mannose. In contrast, the membrane CA-400-28 did not show adsorption of polysaccharides.

Membrane cleaning

The recovery of the pure water permeation fluxes after water flushing of the membranes (description in Membrane cleaning of Material and Methods) was 64% for CA-400-32 and 80% for CA-400-28 membranes. The higher value of 80% of pure water flux recovery for the membrane of lower MWCO is in accordance with the previous result that did not show adsorption of polysaccharides.

TABLE VI

Concentration of the neutral sugars in the membranes (fouled with Trincadeira Preta 2010 must), after acid hydrolysis

Concentração dos açúcares neutros nas membranas (colmatadas com o mosto Trincadeira Preta 2010), depois de hidrólise ácida

Membrane		Monosaccharides detected	
Type	Status	Glucose (mg/25.52cm^2)	Mannose (mg/25.52cm^2)
CA-400	unused membrane	25.78	-
CA-400-32	Membrane used in grape must ultrafiltration	43.13	2.04
		(43.13-25.78) = 17.35	
CA-400-28	Membrane used in grape must ultrafiltration	26.03	-
		(26.03 -25.78)=0.25	

Neutral sugars constituents of the grape must polysaccharides in the two streams

Tables VII and VIII show the results of neutral sugars corresponding to the constitutive monomers of polysaccharides present in the aliquots collected in the two streams (retentate and permeate), during the UF experiment of the grape must (using the membranes CA-400-32 and CA-400-28, respectively).

TABLE VII

Neutral sugars constituents of the grape must polysaccharides during the UF of the grape must Trincadeira Preta 2010 in the retentate and permeate with membrane CA-400-32. (Operating conditions: $\Delta P = 3 \times 10^5$ Pa; T = 25ºC; $A_{CA-400} = 25.52 \times 10^{-4} m^2$; v = 0.67 m/s)

Açúcares neutros constituintes dos polissacáridos do mosto durante a ultrafiltração do mosto Trincadeira Preta 2010, no permeado e retentado com a membrana CA-400-32. (Condições de operação: $\Delta P = 3 \times 10^5$ Pa; T = 25ºC; $A_{CA-400} = 25.52 \times 10^{-4} m^2$; v = 0.67 m/s)

Time (min)	VRF	Rhamnose (mg/L)		Arabinose (mg/L)		Mannose (mg/L)		Galactose (mg/L)		Glucose (mg/L)	
		Retentate	Permeate	Retentate	Permeate	Retentate	Permeate	Retentate	Permeate	Retentate	Permeate
15	1.005	16.0	15.2	25.2	9.3	46.6	10.1	26.5	7.5	19.6	16.7
34	1.009	15.9	15.8	25.1	8.9	45.9	9.4	26.1	7.3	19.3	16.8
49	1.013	11.2	10.9	23.6	7.8	44.3	8.9	24.3	6.2	17.9	15.2
64	1.017	0	11.5	23.3	7.1	44.1	0	23.8	5.1	17.7	14.2
80	1.020	0	0	22.6	6.5	43.8	0	23.2	5.2	16.4	13.4

TABLE VIII

Neutral sugars constituents of the grape must polysaccharides during the UF of the grape must Trincadeira Preta 2010 in the retentate and permeate with membrane CA-400-28. (Operating conditions: $\Delta P = 3 \times 10^5$ Pa; T = 25ºC; $A_{CA-400} = 25.52 \times 10^{-4} m^2$; v = 0.67 m/s)

Açúcares neutros constituintes dos polissacáridos do mosto durante a ultrafiltração do mosto Trincadeira Preta 2010, no permeado e retentado com a membrana CA-400-28. (Condições de operação: $\Delta P = 3 \times 10^5$ Pa; T = 25ºC; $A_{CA-400} = 25.52 \times 10^{-4} m^2$; v = 0.67 m/s)

Time (min)	VRF	Rhamnose (mg/L)		Arabinose (mg/L)		Mannose (mg/L)		Galactose (mg/L)		Glucose (mg/L)	
		Retentate	Permeate	Retentate	Permeate	Retentate	Permeate	Retentate	Permeate	Retentate	Permeate
15	1.010	16.0	0	25.2	0	46.6	5.8	26.5	4,3	19.6	14.2
20	1.013	16.0	0	25.2	0	46.6	5.8	26.5	4,3	19.6	14.2
30	1.014	16.0	0	25.2	0	46.6	0	26.5	0	19.6	0
60	1.019	15.8	0	23.5	0	44.2	0	25.4	3,2	19.2	3.5
100	1.024	15.8	0	23.5	0	44.2	0	25.4	0	19.2	0
130	1.028	15.7	0	23.8	0	43.9	0	25.0	3,8	18.7	0
190	1.033	15.9	0	22.1	0	43.7	0	23.7	0	18.4	0
253	1.038	15.9	0	22.1	0	43.7	0	23.7	0	18.4	0
313	1.042	15.6	0	21.9	0	43.3	0	23.1	0	17.8	3.1

The results in Table VII (membrane CA-400-32) show a decrease of the rhamnose content in the retentate to the point of total disappearance, while the content of other neutral sugars in the retentate remain almost constant. Furthermore the content of neutral sugars in the polysaccharides of the permeate during the UF have a decrease, verifying the complete disappearance of rhamnose in the fractions of permeate collected. So, we can say that rhamnose crosses the membrane and is not adsorbed. Despite the fact that mannose also crosses the membrane, the behaviour of this sugar is different. Mannose was found in the permeate only up to 49 min, but in small quantities, which shows retention and adsorption of the mannoproteins in membrane matrix. In fact, as already discussed, the analysis of the membrane

matrix described in Table VI, give evidence that mannose was adsorbed on the matrix, which has originated, in some extension, the clogging of the pores. The presence of mannose in the permeate stream can be due to the permeation of MPs of low molecular weight, while the retention in the retentate stream due to MPs of high molecular weight. The contents of arabinose and galactose in the permeate stream are lower than those present in the original grape must, and this fact also show the retention of AGP.

In Table VIII, the neutral sugars of the retentate and permeate stream indicate that all the polysaccharides of grape must (RG-II, AGP and MPs) are retained by the membrane CA-400-28. In fact, only small quantities of mannose, galactose and glucose were found in the permeate at the first 20 minutes of the experiment.

CONCLUSIONS

It was concluded that the grape must studied contains various fractions of RG-II, and some of them crossed the CA-400-32 membrane easily. However the disappearance of the fraction with the lowest molecular weight (RG-II) in the retentate and permeate stream of the CA-400-32 membrane, may be due to its deposition on the surface of the membrane, and in the inside of pores. This latter phenomenon, called in literature as "clogging", mainly happens at the entrance of the pores and on its surface, as found in a previous study of polysaccharides by Vernhet and Moutounet (2002).

Due to the fact that we are dealing with polyssacharide fractions of low molecular weigth (5kDa), we can also admit the hypothesis that this accumulation of RG-II on membrane surface is probably caused by the previous adsorption of mannoproteins on the membrane matrix. This hypothesis is supported by the fact that the macromolecules with highest molecular weight (AGPs and MPs), were found in the permeate streams of the CA-400-32 membrane, which means that this membrane don't retain big macromolecules.

By the other hand, it was found that in UF experiments with the CA-400-28 membrane, RG-II and the majority of AGPs and MPs remained in the retentate, which means that this membrane largely retains big macromolecules.

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