IMPACT OF WHITE WINE MACROMOLECULES ON FRACTIONATION PERFORMANCE OF ULTRAFILTRATION MEMBRANES

Johannes de BRUIJN[1,*], José MARTINEZ-OYANEDEL[2], Cristina LOYOLA[1], Francisco LOBOS[2], Javier SEITER[2] and Ricardo PÉREZ[1]

1: Departamento de Agroindustrias, Universidad de Concepción, Avenida Vicente Méndez 595, Chillán, Chile
2: Departamento de Bioquímica y Biología Molecular, Universidad de Concepción, Concepción, Chile

Abstract

Aim: The aim of this study was to investigate the performance of ultrafiltration (UF) membranes to achieve a selective separation of macromolecules from Sauvignon blanc wine into stable and unstable fractions and to characterize the main compounds separated.

Methods and results: The macromolecules from two Sauvignon blanc wines (Curicó Valley and Casablanca Valley, Chile) were separated by a cascade of UF membranes into three nominal fractions (10-30, 30-100 and 100-300 kDa). These fractions were characterized by native and SDS electrophoresis and membrane performance was evaluated by protein rejection and transmission. Separation by UF allowed the concentration of thermally unstable proteins in the 10-30 kDa retentate fraction, increasing heat induced haze by 8.9 fold, while heat stable glycoproteins were concentrated into the 100-300 kDa retentate fraction, reducing heat induced haze by 5.3 fold compared to unfiltered wine. The retention of high macromolecular species by the UF membrane with a 100 kDa molecular weight cut-off contributed to increased protein aggregation in the filtered wines.

Conclusion: The concentration and purification of anti-hazing compounds by membrane filtration seem to be a new technology to improve the protein stability of white wines.

Significance and impact of the study: Specific wine proteins are responsible for wine instability, resulting in haze formation in white wines. On the other hand, glycoproteins prevent protein aggregation and precipitation, thereby improving wine stability. Fractionation of wine macromolecules by UF membranes may help us to improve our knowledge about the contribution of specific proteins and glycoproteins to the haze stability of white wines.

Key words: ultrafiltration, fractionation, white wine, haze, protein, glycoprotein

Résumé

Objectif: L'objectif de cette étude a été d'étudier la performance des membranes d'ultrafiltration pour obtenir une séparation sélective des macromolécules de Sauvignon blanc en fractions stables et instables et de caractériser les principaux composés.

Méthodes et résultats: Les molécules de deux vins de Sauvignon blanc (Curicó Valley and Casablanca Valley, Chile) ont été séparées par une série de membranes d'ultrafiltration en trois fractions pour les masses 10-30, 30-100 and 100-300 kDa. Les fractions ont été caractérisées par électrophorèse native et SDS. La performance des membranes a été évaluée par le rejet ou la transmission des protéines. La séparation par ultrafiltration a permis la concentration des protéines instables à la chaleur dans la fraction 10-30 kDa, tandis que les glycoprotéines stables à la chaleur ont été concentrées dans la fraction 100-300 kda. La rétention de macromolécules par la membrane d'ultrafiltration de 100 kDa contribue à l'augmentation de l'agrégation des protéines dans le vin filtré.

Conclusion: La concentration et la purification de composés anti-troubles par filtration sur membrane semblent être une nouvelle technologie pour améliorer la stabilité protéique des vins blancs.

Signification et impact de l'étude: Les protéines spécifiques du vin sont responsables de son instabilité, résultant en la formation de troubles dans les vins blancs. Les glycoprotéines empêchent l'agrégation des protéines et des précipitations, ce qui améliore la stabilité du vin. Le fractionnement des macromolécules du vin par des membranes d'ultrafiltration peut nous aider à améliorer nos connaissances sur le rôle des protéines et des glycoprotéines spécifiques à la stabilité des troubles des vins blancs.

Mots-clés: ultrafiltration, fractionnement, vin blanc, trouble, protéine, glycoprotéine

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*Corresponding author: jdebruij@udec.cl

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INTRODUCTION

Although wines contain low concentrations of proteins and glycoproteins, which typically vary from 15 to 230 mg per litre, these compounds play an important role from a technological point of view (Ferreira *et al*., 2001). Indeed, proteins greatly affect the quality of wine by contributing to its sensorial and foam characteristics (Dambrouck *et al*., 2005; Cilindre *et al*., 2007; Salazar *et al*., 2010). Additionally, specific wine proteins, mainly grape pathogenesis-related proteins, are responsible for protein instability, resulting in haze formation in white wines (Waters *et al*., 2005). Protein denaturation leads to their aggregation and flocculation giving a hazy suspension with precipitates, which is a considerable drawback in bottled wines (Waters *et al*., 2005). On the other hand, glycoproteins derived from yeast lyses may prevent protein aggregation and precipitation, having an anti-hazing effect (Moine-Ledoux and Dubourdieu, 1999; Dupin *et al*., 2000; Brown *et al*., 2007; Lomolino and Curioni, 2007).

Membrane separations such as ultrafiltration (UF) may be used to fractionate wine macromolecules and to study their contribution to protein stability; however, fractionation of wine macromolecules by UF membranes is a complex process. Both operational conditions and wine characteristics seem to be critical for membrane-protein and protein-protein interactions. Transmission and rejection of protein molecules by the membrane depend on the system hydrodynamics (shear rate at the membrane surface, transmembrane pressure, membrane resistance, membrane porosity and morphology, surface charge and hydrophobicity (Cui, 2005 and Saxena *et al*., 2009)) the electrostatic charge and isoelectric point of proteins, as well as the ionic species and pH value of the wine (Zulkali *et al*., 2005; Sarkar *et al*., 2009; Rohani and Zydney, 2010).

Therefore, the aim of the present study was to investigate the performance of UF membranes to achieve a selective separation of macromolecules from Sauvignon blanc wine into stable and unstable fractions and to characterize the main compounds separated.

MATERIALS AND METHODS

1. Wines

Sauvignon blanc wines were elaborated in 2009 by an industrial winery according to standard white winemaking procedures. After harvest, grapes from the Curicó Valley (CV) and the Casablanca Valley (CBV) (Chile) were pressed without skin maceration, followed by settling down of must. After alcoholic fermentation, the wines were racked and the SO_2 level was adjusted. After removal of suspended solids by settling, centrifugation and filtration, the wines were bottled and stored in glass jars prior to UF (De Bruijn *et al*., 2009).

2. Ultrafiltration

Wine fractionation was performed by a cascade of membranes using a pilot scale ultrafiltration unit (Millipore, Bedford, USA) in batch mode under cross-flow conditions. The system was equipped with a Pellicon 2 Cassette and C screen, a peristaltic pump, two pressure gauges (located at the feed entrance and retentate exit), and two sampling valves (located at the retentate and permeate exits). We tested four Ultracel PLC (composite regenerated cellulose) membranes (PLC MK; PLC HK; PLC TK; PLC GC) having different nominal molecular weight cut-off (NMWCO) (10, 30, 100 and 300 kDa) and a surface area of 0.5 m^2. Transmembrane pressure was set at 0.95 bar, while recirculation rate was set at 222 L/h. Wine temperature was maintained in the range of 10-14°C. Two minutes after the start of each UF experiment, permeate samples were collected periodically in a graduated cylinder to measure total permeate volume, temperature, pressure and recirculation rate. At the end of each run, the permeate samples were mixed and weighted (Gibertini, TMB 25 AR, Novate, Italy), followed by physicochemical analyses. Cleaning-in-place of the membrane module was performed according to manufacturer's recommendations using solutions of 0.1 M NaOH (Merck, Darmstadt, Germany) and 0.2% (w/v) Tergazyme (Alconox, White Plains, USA) until the original water flux was restored.

3. Analytical methods

The total soluble protein concentration of wine samples was determined by staining with Coomassie Brilliant Blue reagent and reading absorbance at 595 nm (Bradford, 1976). Haze stability of wines was determined by the heat test according to Moine-Ledoux and Dubourdieu (1999) using a Hach 2100P turbidimeter (Hach, Loveland, USA). Gel filtration chromatography of wine samples was carried out using a Superdex 75HR 10/30 column (Supelco, Bellefonte, USA) coupled to a fast protein liquid chromatography system equipped with a L-6210 intelligent pump connected to a L-4250 UV-VIS detector (Merck-Hitachi, Tokyo, Japan) (Esteruelas *et al*., 2009). Native gel electrophoresis of retentate fractions was performed according to Hillier (1976) in a Mini Protean-3 apparatus (Bio-Rad, Hercules, USA). Gels were stained with Coomassie Blue R-250 for protein detection and with periodic acid Schiff for glycoprotein detection. Retentate fractions were also analyzed by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis according to Laemmli (1970) in a Mini Protean-3 apparatus (Bio-Rad, Hercules, USA). Gels were stained with Coomassie Blue R-250 and the relative protein

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content of each band was determined by densitometry using ImageJ software (version 1.44b).

4. Indicators of membrane performance

At each fractionation step, the volumes of feed V_0 (m^3), permeate V_p (m^3) and retentate V_r (m^3) were measured together with the protein concentration of the feed C_0 (kg/m^3), permeate C_p (kg/m^3) and retentate C_r (kg/m^3) samples. Protein loss was then quantified from the following mass balance:

$$C_0 \times V_0 = C_p \times V_p + C_r \times V_r + Loss \quad (1)$$

The performance of the UF membranes to separate and concentrate wine proteins into several fractions can be evaluated from the following separation indicators (Cheryan, 1998):

Rejection: $$R = 1 - \frac{C_p}{C_r} \quad (2)$$

Observed sieving coefficient: $$S_0 = \frac{C_p}{C_0} \quad (3)$$

RESULTS AND DISCUSSION

1. Membrane performance

A considerable percentage of wine proteins were lost after each fractionation step with both Curicó Valley (CV) (table 1) and Casablanca Valley (CBV) (table 2) wines. During fluid recirculation some of the wine proteins may precipitate in the feed and retentate part of the membrane system due to pumping. Some fraction may form insoluble aggregates that precipitate on the membrane surface, thereby increasing secondary membrane resistance, or within the membrane pores, thereby diminishing membrane permeability and selectivity (Cassano *et al.*, 2008). Especially if transmembrane pressure exceeds critical pressure, membrane fouling takes place, resulting in the formation of an external dynamic fouling layer and the increase of overall resistance to permeation, thus affecting the separation characteristics of the membrane (Bacchin and Aimar, 2005 and Bacchin *et al.*, 2006). Although a strong decline of membrane permeability can be observed for the filtration of Sauvignon blanc by using UF membranes with NMWCO of 100 kDa and 300 kDa, the permeability was almost constant for UF membranes with NMWCO of 10 kDa and 30 kDa (figures 1

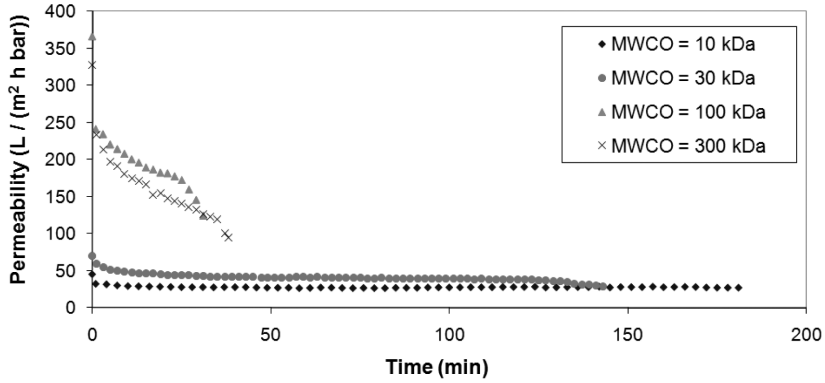


Figure 1 - Evolution of permeability during the fractionation of Casablanca valley (CBV) wine using membranes with molecular weight cut-off (MWCO) of 300 kDa, 100 kDa, 30 kDa and 10 kDa.

Table 1 - Membrane performance during protein fractionation of Curicó valley wine.

Membrane (MWCO)		Protein concentration in feed C_0 (mg/L)	Protein concentration in permeate C_p (mg/L)	Protein concentration in retentate C_r (mg/L)	Volume of feed V_0 (L)	Volume of permeate V_p (L)	Volume of retentate V_r (L)	Loss (%)	Haze[a] (ΔNTU)	Rejection R	Observed Sieving coefficient S_0
10 kDa	Init	9.3			27.9						
	Final		5.1	96.4		27.5	0.59	24.3	389	0.95	0.55
30 kDa	Init	15.1			29.1						
	Final		9.3	57.0		27.9	0.76	30.9	148	0.84	0.62
100 kDa	Init	27.3			30.4						
	Final		15.1	22.1		29.5	0.48	45.1	5	0.31	0.55
300 kDa	Init	31.4			31.5						
	Final		27.3	33.1		30.8	0.35	13.8	46	0.17	0.87

a) retentate

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(CBV wine) and 2 (CV wine)). However, the permeability of the Ultracel PLC membranes of 100 kDa and 300 kDa was still superior compared to data reported in other filtration studies of white wines using polypropylene, polyarylsulfone or polysulfone hollow fibre membranes with a 0.2 μm cut-off (Czekaj *et al.*, 2001 and Ulbricht *et al.*, 2009). On the other hand, the main loss of wine proteins took place at the end of the fractionation process while using the 10 kDa NMWCO membrane. This suggests a minimum participation of wine proteins in membrane fouling, either the precipitation of wine proteins on the feed and retentate side without affecting filtration performance. Fouling colloids are mainly constituted of carbohydrates and polyphenols in the case of red and rosé wines (Belleville *et al.*, 1992; Vernhet *et al.*, 2003; Ulbricht *et al.*, 2009). Similar decline of membrane permeability during the fractionation of CV and CBV wines (figures 1 and 2, respectively) suggests similar adsorption and fouling behavior of the membranes in both cases.

An increased loss of proteins from CV wine compared to CBV wine was found after fractionation. Proteins from CBV wine seem to be less susceptible to denaturation and aggregation than proteins from CV wine. The presence of a higher amount of protein-stabilizing compounds in the CBV wine may stabilize the wine proteins of the 30-100 kDa, 100-300 kDa and >300 kDa retentate fractions. Yeast mannoproteins improve the protein stability of white wines (Moine-Ledoux and Dubourdieu, 1999; Dupin *et al.*, 2000; Brown *et al.*, 2007; Lomolino and Curioni, 2007). Organic acids commonly found in wines also exhibit a stabilizing effect upon the haze potential of wine proteins (Batista *et al.*, 2010). Therefore, the stability of wine proteins may decrease due to the loss of some wine stabilizing compounds during the wine fractionation process.

UF membranes were able to concentrate the wine proteins of the retentate fractions in a similar way for both wines up until a mean volume concentration ratio (Cheryan, 1998) between 58 and 60 was reached, increasing the protein concentration of CV wine from 31.4 mg/L to 96.4 mg/L (table 1) and that of CBV wine from 40.5 mg/L to 90.2 mg/L (table 2). Maximum protein concentrations were achieved with the 10-30 kDa NMWCO retentate fractions. According to Sauvage *et al.* (2010), the majority of the proteins of Sauvignon blanc wine are ranging from 12 to 24 kDa.

The use of UF membranes allowed the concentration of thermally unstable compounds in the 10-30 kDa retentate fractions, increasing heat induced haze by a factor

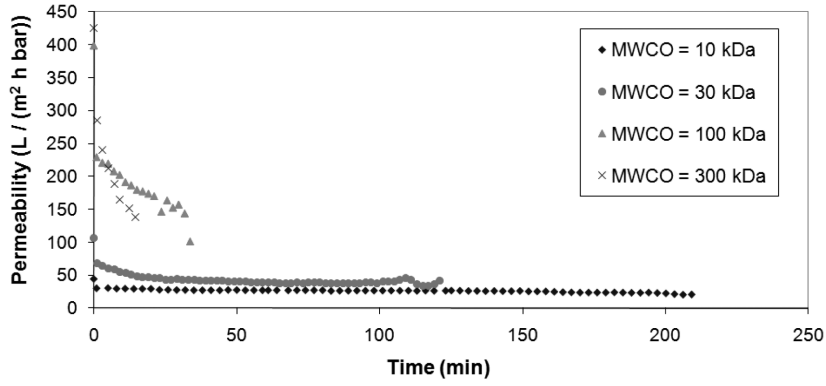


Figure 2 - Evolution of permeability during the fractionation of Curicó valley (CV) wine using membranes with molecular weight cut-off (MWCO) of 300 kDa, 100 kDa, 30 kDa and 10 kDa.

Table 2 - Membrane performance during protein fractionation of Casablanca valley wine.

Membrane (MWCO)		Protein concentration in feed C_0 (mg/L)	Protein concentration in permeate C_p (mg/L)	Protein concentration in retentate C_r (mg/L)	Volume of feed V_0 (L)	Volume of permeate V_p (L)	Volume of retentate V_r (L)	Loss (%)	Haze[a)] (ΔNTU)	Rejection R	Observed Sieving coefficient S_0
10 kDa	Init	30.8			29.7						
	Final		17.0	90.2		29.3	0.57	39.9	374	0.81	0.55
30 kDa	Init	33.8			30.7						
	Final		30.8	80.6		30.1	0.65	5.5	111	0.62	0.91
100 kDa	Init	37.3			31.4						
	Final		33.8	50.9		31.0	0.41	8.6	14	0.34	0.91
300 kDa	Init	40.5			32.6						
	Final		37.3	37.5		31.5	0.59	9.3	45	0.01	0.92

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of 11.3 for CV wine (table 1) and 6.4 for CBV wine (table 2). Moreover, UF membranes with a 100 and 300 kDa NMWCO allowed the separation of heat unstable compounds from heat stabilizing species, reducing heat induced haze by a factor 6.4 for CV wine (table 1) and 4.1 for CBV wine (table 2). Therefore, the fractionation of white wine by UF allowed us to get at least two different fractions: a highly heat unstable retentate fraction of 10-30 kDa and a 100-300 kDa retentate fraction with improved thermal stability.

The strong increase of protein rejection and the improved protein yield found after using UF membranes with a small molecular weight cut-off is related to their pore size (tables 1 and 2). However, the higher rejection of proteins from CV wine by UF membranes compared to CBV wine may be explained by stronger denaturation and aggregation characteristics of the macromolecular species in CV wine that increases particle size. This agrees with the higher loss of proteins reported before (De Bruijn *et al*., 2011). Recently we found that the vast majority of the wine proteins from CV wine are heat unstable, while CBV wine contains a higher amount of heat stable proteins (De Bruijn *et al*., 2011).

A better transmission - expressed by the observed sieving coefficient – through membranes with a higher molecular weight cut-off can be observed for the CBV wine proteins compared to the transmission of CV wine proteins (tables 1 and 2). Increased instability of proteins from CV wine during membrane filtration or during wine storage may explain the lower transmission of these species.

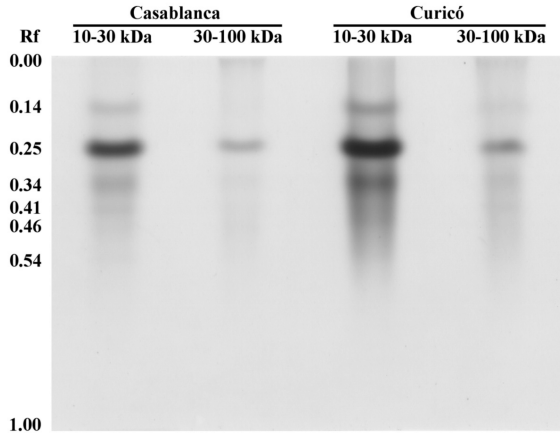


Figure 3 - Native PAGE after Coomassie Blue staining: retentate fractions of 10-30 kDa and 30-100 kDa of Curicó valley (CV) and Casablanca valley (CBV) wines.

2. Size exclusion chromatography

Several peaks were observed after size exclusion chromatography (SEC), indicating the presence of macromolecular species in the range of 28-131 kDa for CV wine and 26-311 kDa for CBV wine (table 3). At least 87% of the macromolecular compounds found in the 10-30 kDa fractions of both wines had a molecular weight in the range between 43 kDa and 131 kDa (table 3). This is due to the formation of aggregates after ultrafiltration and may explain the wide range of molecular weights reported for wine proteins (Flanzy, 2003). As stated before, most of the wine proteins are unstable, causing an increased rejection and loss of proteins with the advance of fractionation. Moreover, the presence of low molecular weight compounds within the high molecular weight fractions is due to the lack of washing after UF.

3. Native PAGE

Native electrophoresis gel of CV and CBV wine fractions showed a maximum of five bands (Rf: 0.14, 0.25, 0.34, 0.46 and 0.54) after Coomassie Blue staining, with a better resolution and higher concentration of proteins in the 10-30 kDa fractions (figure 3). This relatively small number of protein bands found in this gel agrees with the maximum number of peaks found after SEC. Similar protein profile for natural precipitate of wine haze after native PAGE and Coomassie Blue R-250 staining was found by Esteruelas *et al*. (2009), detecting seven bands in the 20-30 kDa fraction.

After periodic acid Schiff staining of the native electrophoresis gel, glycoproteins were detected in the 30-100 kDa and 100-300 kDa fractions of CV wine; however, these compounds were almost absent in the 10-30 kDa fraction (figure 4). On the other hand, more intense

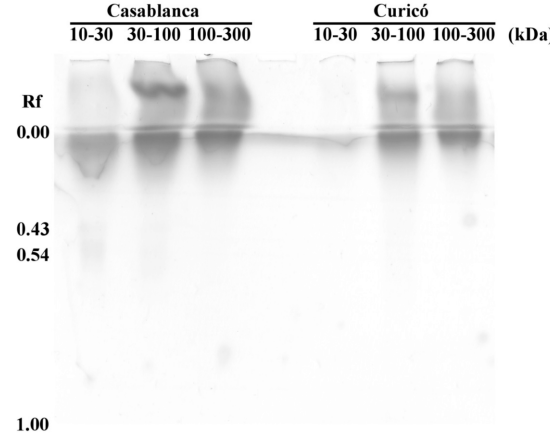


Figure 4 - Native PAGE after Periodic Acid Schiff staining: retentate fractions of 10-30 kDa, 30-100 kDa and 100-300 kDa of Curicó valley (CV) and Casablanca valley (CBV) wines.

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Table 3 - SEC analyses after fractionation of Curicó valley (CV) and Casablanca valley (CBV) wines.

CV wine 10-30 kDa		CV wine 30-100 kDa		CV wine 100-300 kDa		CBV wine 10-30 kDa		CBV wine 30-100 kDa		CBV wine 100-300 kDa	
MW (kDa)	Area (%)	MW (kDa)	Area (%)	MW (kDa)	Area (%)	MW (kDa)	Area (%)	MW (kDa)	Area (%)	MW (kDa)	Area (%)
								311	14.9		
131	66.6	131	25.9			119	14.9	128	16.4	130	5.6
61.3	20.3	62.2	59.0	62.2	94.1	71.6	30.4	77.7	38.0	77.1	47.6
						43.1	45.0	43.5	25.9	43.5	42.7
27.8	13.1	27.7	15.1	27.7	5.9	25.5	9.7	28.0	4.8	28.2	4.1

glycoprotein bands could be observed for CBV wine, and these could still be detected in the 10-30 kDa fraction (figure 4). Higher concentration of glycoproteins in CBV wine may explain the lower loss of proteins during the fractionation of this wine. Moreover, the high molecular weight of glycoproteins impairs the molecular mobility and the migration of these macromolecules through the separation gel (figure 4). Glycoproteins have been detected in the high and intermediate molecular weight fractions of the precipitate of natural wine haze (Esteruelas *et al.*, 2009). Wine mannoproteins cover a broad range of molecular weights from 53.4 to 560 kDa (Gonçalves *et al.*, 2002). Hence the separation of the main fraction of glycoproteins should be expected during the first stages of the fractionation process.

4. SDS-PAGE

Non reducing SDS-PAGE gel of CV and CBV wine fractions showed a maximum of twelve bands (figure 5). After fractionation using membranes with a NMWCO of 10 kDa and 30 kDa, 81% of the protein fraction was indeed within the 10-30 kDa range, taking into account a measurement error of more or less 10% in the determination of molecular weight by SDS-PAGE (table 4). However, some higher molecular weight species in the range between 35 kDa and 76 kDa were still present in this fraction (figure 5). Esteruelas *et al.* (2009) reported two major bands of 18-26 kDa and 41 kDa for natural wine haze after SDS-PAGE and Coomassie Blue R-250 staining.

A 90% rejection of dextran at 22 kDa for the PLC GC membrane with a mean pore size of 2.6 nm and 90% rejection of dextran at 157 kDa for the PLC HK membrane with a mean pore size of 8.2 nm was calculated by Zydney and Xenopoulos (2007) from stirred cell sieving profiles. These large differences between effective MWCO and nominal MWCO may further explain the passage of some of the larger compounds. On the other hand, the molecular weight of proteins may still increase after membrane filtration even under non reducing SDS-PAGE conditions by the formation of disulfide bridges between the units.

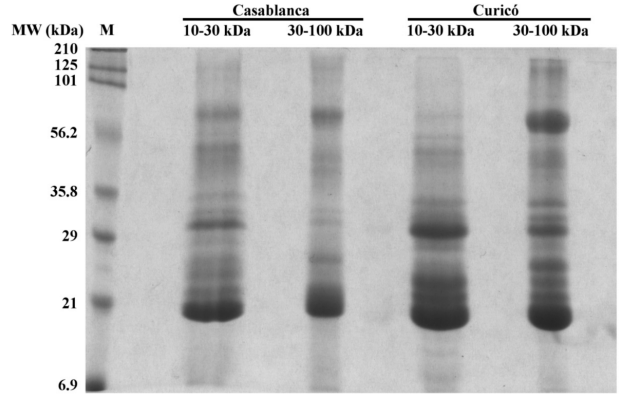


Figure 5 - SDS-PAGE after Coomassie Blue staining: retentate fractions of 10-30 kDa and 30-100 kDa of Curicó valley (CV) and Casablanca valley (CBV) wines.

After fractionation by using membranes with a NMWCO of 30 and 100 kDa, the 30-100 kDa fraction consisted of a considerable amount (26%) of species within this range together with a huge amount of smaller species (< 30 kDa) able to permeate through the membrane (table 4). These smaller species may be present in wine in an aggregated state, preventing their passage through the membrane. Indeed the stability of most of the low molecular weight proteins was low or very low (table 4). Only the stable wine protein of 76 kDa from CBV wine and the unstable wine protein of 28 kDa from CV and CBV wines could be observed after both SDS-PAGE and SEC (tables 3 and 4). Thus, this indicates that most of the species detected in the SDS-PAGE gel but absent from the size exclusion chromatograms are participating in haze formation, which agrees with previously found stability characteristics. Moreover, SDS is able to break down non covalent linkages between protein aggregates and thus helps to detect individual wine proteins that are part of wine haze.

Finally, UF seems to be an appropriate method for wine fractionation because it is able to separate wine proteins into a 10-30 kDa fraction that has been enriched with highly heat unstable proteins and a 30-100 kDa

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Table 4 - Densitometry scanning of SDS-PAGE gel after fractionation of Curicó valley (CV) and Casablanca valley (CBV) wines.

CV wine 10-30 kDa			CV wine 30-100 kDa			CBV wine 10-30 kDa			CBV wine 30-100 kDa		
MW (kDa)	Area (%)	Stability[a]	MW (kDa)	Area (%)	Stability[a]	MW (kDa)	Area (%)	Stability[a]	MW (kDa)	Area (%)	Stability[a]
74.7	0.6	+	70.4	17.4	+	76.2	4.9	+	75.9	11.6	+
64.5	0.9	?									
57.5	3.8	--	57.7	5.1	--	59.6	4.3	?			
52.7	4.2	-	52.2	0.8	-	54.2	3.5	+	55.0	4.7	+
						48.8	2.5	--	49.4	4.7	--
39.1	5.8	--	38.9	2.5	--	41.8	1.3	?	44.6	1.8	?
35.1	4.8	-	35.0	3.1	-	36.7	1.6	--	37.0	1.0	--
31.2	18.8	--	31.5	7.6	--	32.9	10.0	--	33.7	2.3	--
27.9	4.6	-	28.0	0.7	-	29.2	1.3	?			
			24.0	8.8	?	25.2	3.7	-	25.6	12.7	-
21.3	19.1	--	21.4	8.1	--	22.6	9.9	--			
19.0	13.0	-	19.1	8.5	-	19.9	8.9	-	20.4	11.2	-
16.0	24.5	--	16.0	37.4	--	16.7	48.1	-	17.4	50.0	-

a: +: high; -: low; --: very low

fraction that has an increased amount of highly stable proteins (table 4). Highly heat unstable proteins constituted the majority of proteins in the fractions of CV wine, while these proteins constituted only a minor percentage in the fractions of CBV wine (table 4).

CONCLUSIONS

UF is a useful method to separate wine macromolecules and to study the protein stability of white wine. SDS is a reagent that helps to detect individual wine proteins that take part in wine haze. The wine proteins did not produce significant membrane fouling. The loss of wine proteins by membranes during fractionation was related to the loss of glycoproteins. Wine glycoproteins are high molecular weight species in the range between 30 and 300 kDa. The fractionation of white wine by UF allowed the separation of wine proteins into a highly heat unstable retentate fraction of 10-30 kDa and a retentate fraction of 100-300 kDa with improved thermal stability. The increased instability of the wine proteins explained the lower transmission and higher rejection of these species by UF membranes.

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