

PROTEIN CHARACTERIZATION OF RODITIS GREEK GRAPE VARIETY AND SAUVIGNON BLANC AND CHANGES IN CERTAIN NITROGEN COMPOUNDS DURING ALCOHOLIC FERMENTATION

CARACTÉRISATION DE PROTÉINES DU CÉPAGE GREC « RODITIS » ET DU SAUVIGNON BLANC ET ÉVOLUTION DE CERTAINS COMPOSÉS AZOTÉS PENDANT LA FERMENTATION ALCOOLIQUE

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Abstract: Must and wine samples of the Greek grape variety Roditis and the French one Sauvignon blanc were analysed in order to obtain further knowledge of the protein profile of Roditis and to watch the evolution of grape proteins during the alcoholic fermentation of Roditis and Sauvignon blanc musts. For these purposes protein samples were isolated from must and wine samples by ammonium sulphate precipitation and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS - PAGE). Eleven and nine bands with molecular weights between 11,1 and 64,4 kDa were detected on the electrophoregrams of Roditis and Sauvignon blanc must and wine samples respectively, using Coomassie Brilliant Blue R-250 and silver staining methods. Two protein fractions of must and wine samples with molecular weights of 64,4 kDa and 34,4 kDa were identified as being glycoproteins in the profile of the Greek grape variety, according to the Periodic acid - silver staining, while only one must and wine fraction of 64,4 kDa had positively react with this stain, as far as it concerns Sauvignon blanc. None of the low molecular weight protein fractions found to be responsible for haze formation. A modified (Bradford) dye - binding procedure was used for the determination of musts and wines soluble proteins. Free amino nitrogen and the contents of neutral and acidic polysaccharides in the protein fractions after chromatography on Sephadex G - 25, were also analyzed.

Résumé: Des échantillons de moût et de vin issus du cépage grec « Roditis » et du Sauvignon blanc de l'année 2003 ont été analysés afin d'acquérir, tout d'abord, plus de connaissances sur le profil des protéines du cépage grec « Roditis » et, ensuite, d'observer l'évolution des protéines pendant la fermentation alcoolique. Dans ce but, nous avons isolé des protéines à partir des échantillons de moût et de vin par précipitation à l'aide d'ammonium sulfate. Puis nous les avons étudiés par électrophorèse sur gel de polyacrylamide dénaturant (SDS - PAGE). La meilleure précipitation de protéines a été obtenue par une saturation de 70 % $(\text{NH}_4)_2\text{SO}_4$. En utilisant la méthode de coloration au bleu de Coomassie, onze bandes de poids moléculaires de 11,1 kDa à 64,4 kDa ont été détectées dans les électrophorégrammes des échantillons du vin issus du cépage Roditis. Au milieu de la fermentation du moût de Roditis est apparue une nouvelle fraction de protéines de poids moléculaire 20,0 kDa. Cette fraction pourrait être le résultat de l'hydrolyse de protéines par l'action de la protéase extracellulaire des levures pendant la fermentation alcoolique. La méthode de coloration au nitrate d'argent c'est avérée incapable de détecter de nouvelles protéines pendant la fermentation alcoolique dans les électrophorégrammes de cette variété. Quant au cépage S. blanc, cinq bandes de poids moléculaires entre 14,5 et 64,4 kDa ont été détectées en utilisant la méthode de coloration au bleu de Coomassie. Par ailleurs et contrairement aux échantillons de Roditis, quatre nouvelles bandes sont apparues en employant la méthode de coloration au nitrate d'argent dans les électrophoregrammes des échantillons de Sauvignon blanc. Ce cépage est caractérisé par de basses concentrations de protéines et la méthode de coloration au nitrate d'argent montre une plus grande sensibilité à ces concentrations en protéines. L'apparition d'une nouvelle bande a été observée au milieu de la fermentation du cépage Roditis, probablement en raison de l'hydrolyse de protéines. Deux fractions de protéines de poids moléculaires de 64,4 et de 34,4 kDa ont été identifiées comme des glycoprotéines dans le profil du cépage Roditis, selon l'acide périodique - coloration argentée, alors que seulement une fraction de 64,4 kDa avait réagi positivement à cette coloration, dans le cas du Sauvignon blanc. La détection des bandes colorées en haut du gel, en utilisant la coloration par PA-Ag, est due aux grandes quantités de polysaccharides concentrés à cet endroit là. Aucune des fractions de protéines de bas poids moléculaire issues du moût ou du vin de Roditis et de Sauvignon blanc n'a été jugée responsable de la formation de trouble. L'absence de glycosylation de protéines de bas poids moléculaires pourrait expliquer la stabilité protéique détectée. Nous avons appliqué la méthode de Bradford pour la détermination des protéines solubles dans le moût et le vin. Les résultats obtenus par cette méthode sont similaires à ceux rapportés pour d'autres cépages européens. Plus spécifiquement, la concentration en protéines des échantillons de Roditis et de Sauvignon blanc, pendant la fermentation alcoolique, est passée respectivement de 82,7 à 93,5 mg/l et de 34,2 à 105,2 mg/l. Le dosage de l'azote aminé libre et des polysaccharides neutres et acides dans les fractions de protéines après la chromatographie sur Sephadex G - 25, n'a pas montré de différences remarquables en comparaison avec les résultats obtenus par d'autres chercheurs.

Key words : protein, white must, fermentation, nitrogen compounds, polysaccharides, sodium dodecyl sulphate polyacrylamide gel electrophoresis

Mots clés : protéine, moût blanc, fermentation, composés azotés, polysaccharides, SDS - PAGE

INTRODUCTION

Proteins present in musts and wines are responsible for many technological and biological phenomena. They have positive effects such as the stabilization of foam in sparkling wines thanks to their tensioactive properties (PUEYO *et al.*, 1995; ANDRÉS-LACUEVA *et al.*, 1996), the increased sensation of body of wines and the protection of wine against tartaric salt precipitation (LUBBERS *et al.*, 1993; GERBAUD *et al.*, 1997; MOINE - LEDOUX *et al.*, 1997), the reduction of haze formation in white wines due to the presence of yeast mannoproteins (WATERS *et al.*, 1994) and the interaction with aroma compounds (LUBBERS *et al.*, 1994). On the other hand, proteins may be responsible for the appearance of turbidity in bottled wine (LUGUERA *et al.*, 1997) due to coagulation and precipitation of soluble proteins as a result of unfavorable storage conditions (DORRESTEIN *et al.*, 1995). This lack of stability, which is more frequently encountered in white wines, can seriously damage the image of the product and therefore affect its acceptance by the consumer. Despite the fact that numerous investigations have been made on grape juice and wine proteins, their origin, structure and composition remains unclear (WATERS *et al.*, 1991; DORRESTEIN *et al.*, 1995; CANALS *et al.*, 1998).

Many authors have studied the proteins of white musts and wines and concluded that soluble proteins originate exclusively from grapes (RUIZ-LARREA *et al.*, 1998; FERREIRA *et al.*, 2000; DAMBROUCK *et al.*, 2003). According to GUMP and HUANG (1999) autolysis of yeast cells following fermentation is a secondary source of protein in wine. It is accepted that these proteins are responsible for haze formation in white wines although their exact nature is still subject to debate. Molecular size and isoelectric point (pI) of proteins have also been studied, resulting to the conclusion that the lower molecular mass and lower pI proteins are the major and most important fractions contributing to the protein clouding among wines (MESROB *et al.*, 1983; HSU and HEATHERBELL 1987a, 1987b). Investigation of DAMBROUCK *et al.* (2003) on wine proteins of a Chardonnay grape variety, using an immunotechnique, confirms that most of these proteins came from the grapes and many of them were glycoproteins, fractions also responsible for the irregular protein clouding among white wines (GUMP and HUANG, 1999).

There is limited information available for the nature and structure of nitrogenous fractions involved in Greek grape varieties and wines. The aim of this study is to increase the knowledge of protein profile of a white Greek variety, Roditis, in comparison with a French internationally known grape variety such as Sauvignon blanc. Both varieties were analysed for their must and wine pro-

tein fractions during alcoholic fermentation. Proteins of the samples have been isolated by ammonium sulfate precipitation and gel filtration and characterized using electrophoretic methods.

MATERIALS AND METHODS

I- METHOD OF VINIFICATION

Roditis, Greek grape variety, and Sauvignon blanc must and wine samples from the 2003 vintage were used in this study. Grapes were hand - harvested at common commercial maturity, pressed and SO₂ was added (4,5 g/hL and 4,0 g/hL for Roditis and Sauvignon blanc must, respectively). After skin contact at 10 - 12 °C for 5 hrs in presence of liquid CO₂, the grape juice was inoculated with 15 and 10 g/hL of a commercial dry *Saccharomyces cerevisiae* yeast for Roditis and Sauvignon blanc must, respectively. The composition of the 2003 Sauvignon blanc and Roditis must is shown in table I. Fermentation was carried out at about 16 °C for ten days for Sauvignon blanc and eighteen days for Roditis must.

Samples were collected at the beginning, middle and end of alcoholic fermentation and they were frozen and stored at -30 °C. Before analyses all samples were filtered.

II- ANALYTICAL DETERMINATIONS

1) Enological parameters

A modified (Bradford) dye - binding procedure was used for total soluble protein determination, using bovine serum albumin as a standard (BOYES *et al.*, 1997). Ethanol concentration was determined according to the official methods of E.U. and sugar content by °Brix measurement.

Free amino nitrogen analysis was carried out according to Modified Cd - Ninhydrin Method C (DOI *et al.*, 1981).

2) Isolation of soluble proteins

Must and wine samples were submitted to ammonium sulphate fractionated precipitation. 40 %, 70 % and 80 % (w/v) saturation of ammonium sulphate were tried. 70 % saturation found to be capable for precipitation of most of the proteins in must and wine samples. In order to achieve 70 % saturation, ammonium sulphate was added to the samples under continuous stirring at room temperature. The samples were maintained for one hour at 4 °C. The precipitate formed was collected by centrifugation at 12.000 x g at 4 °C for 15 min. The pellets were suspended in 5 mL of dialysis buffer A (acetic acid/sodium acetate 10 mM, pH 3,5) and submitted to dialysis against buffer A. Three changes of dialysis buffer, for three days,

were performed to ensure removal of salts present in the precipitates. After dialysis the samples were concentrated by lyophilization and resuspended in 1 mL dionized water. These must and wine samples were submitted to SDS-PAGE.

3) Polyacrylamide gel electrophoresis (SDS-PAGE)

Discontinuous SDS-PAGE was performed according to the method of LAEMMLI (1970). The stacking gel consisted of T= 5 % and C= 2 % and the separating gel of T= 12,5 % and C= 3 %. A vertical electrophoresis apparatus (Amersham Pharmacia Biotech, Hoefer Mighty Small, SE250 Dual Gel Caster) was used to run the gel at a constant voltage setting of 470 V until the bromophenol blue tracker dye reached the bottom of the gel (usually 40 min at room temperature). The dimensions of the gels were 10,0 x 8,0 x 0,075 cm. Samples were mixed in a proportion 2:1 with sample buffer, consisting of 1 mL 0,5 % bromophenol blue, 1 mL 2- mercaptoethanol, 2 mL glycerol and 6 mL stacking gel buffer (pH 6,8). Protein molecular standards (12,4 kDa - 66,0 kDa; Sigma) were included in each electrophoretic analysis. The MWs of unknown molecules were calculated from the linear equation of log MW vs mobility. Following electrophoresis, gels were stained at room temperature for 24 hours with 0,1% Coomassie Brilliant Blue R - 250. Densitometry of bands was carried out at 590 (A590) nm with densitometer (RFT Transidyne General Corp. Ann Arbor., Michigan, USA). Protein concentrations were calculated using analog to digital converter (Nelson Analytical Inc., Paramus NJ 07652, USA).

The PAGE gels were also stained with silver nitrate (WRAY *et al.*, 1981) in order to determine the proteins in very low concentrations.

4) Sephadex G - 25 chromatography

In order to remove free carbohydrates, must and wine samples were submitted to Sephadex G - 25 chromato-

Table I - Characteristics of must/wine samples of Roditis and Sauvignon blanc grape varieties used in study.

Caractéristiques des échantillons de moût/vin issus des cépages Roditis et de Sauvignon blanc utilisés dans la présente étude.

	Roditis	Sauvignon blanc
Initial Baumé	11,3	13,7
Total acidity (g/l H ₂ SO ₄)	3,4	3,0
Total SO ₂ (mg/l)	35,0	38,0
pH	3,42	3,56
NTU	227	297

graphy. Samples were concentrated to 1/10 of their original volume by rotary evaporation below 40 °C and centrifuged at 10.000 x g at 4 °C for 30 min. Ammonium sulphate (70 % saturation) was added to the supernatant, mixed well and the mixture was allowed to stand for one hour at 4 °C. The precipitates formed were collected by centrifugation at 12.000 x g at 4 °C for 15 min. The pellets were suspended in 2 mL 0,05 M Tris - HCl (pH 6,8) and applied to a column (220 x 9 mm) of Sephadex G-25 equilibrated with 0,05 M Tris - HCl (9,1 mL bed volume, 3,5 mL sample). The column was eluted with 0,05 M Tris - HCl at room temperature. The protein content of the fractions that were eluted was measured by ultraviolet absorption at 280 (A280) nm. The fractions with the greater absorption were the samples of must and wine for the determination of polysaccharides content of glycoproteins (YOKOTSUKA *et al.*, 1994).

5) Determination of glycoproteins

In an attempt to detect glycoproteins that can be present on a PAGE gel, the gels were stained with the PA-Ag staining method (periodic acid - silver staining) of OAKLEY *et al.* (1980). After SDS-PAGE electrophoresis, the gels were soaked in a solution of 25 % isopropyl alcohol and 10% acetic acid for one night at room temperature. They were then soaked in 7,5 % acetic acid for 30 min. Gels were put in 0,2 % aqueous periodic acid and stored for 1 hour at 4 °C. Then, they were washed for 3 hours in several changes of distilled water. Water was drained off and a freshly ammoniacal silver solution was added (in order to prepare this solution 1,4 mL of concentrated NH₄OH was added to 21 mL of 0,36 % NaOH. To this solution 4 mL of 19,4% AgNO₃ were added slowly while agitating vigorously. When the transient brown precipitate has cleared the solution was brought to 100 mL with distilled water). The gels were removed from the solution and washed for 2 min in glass-distilled water. Then, they were transferred to a freshly prepared solution containing 0,05 % citric acid and 0,019 % formaldehyde solution containing 10 % methanol. The reaction was stopped using commercially photography fixative.

6) Determination of neutral and acidic polysaccharides of glycoproteins

The contents of neutral and acidic polysaccharides of glycoproteins were measured in protein fractions after elution from Sephadex G-25 using the phenol - sulfuric method (DUBOIS *et al.*, 1956) and carbazole - sulfuric acid method (BITTER and EWINS 1961), respectively.

7) Heat stability test

The samples in the end of fermentation were subjected to heat stability test in order to detect the behavior of their protein fractions in such kind of denaturing

conditions. Using the method of MURPHEY *et al.* (1989) samples were stored at 50 ± 2 °C for 48 hours. The samples were allowed to stand at room temperature for another 24 hours and at 0 ± 2 °C for 48 hours. Then they were brought to room temperature, mixed thoroughly and examined for haze under strong light in comparison with the blank.

RESULTS AND DISCUSSION

I- PROTEIN CONTENT AND ITS EVOLUTION DURING FERMENTATION

Protein concentration of Roditis and Sauvignon blanc samples during alcoholic fermentation varied from 82,7 to 93,5 mg/L and 34,2 to 105,2 mg/L, respectively, (table II). These values are considerably higher than those reported in the musts of four white grape varieties by MORENO - ARRIBAS *et al.* (1996), while MURPHEY *et al.* (1989) and HSU and HEATHERBELL (1987) reported concentrations for Gewürztraminer juice and wine close to ours. In contrary, SANTORO (1995) determined higher concentrations in the must of red and white varieties. These differences may be due to a number of factors including grape variety, climate, conditions of vinification or to the method that is used in order to determine the protein concentration (DORRESTEIN *et al.*, 1995).

During fermentation, the protein content of must and wine samples underwent severe changes (table II). A decrease in protein content of Roditis and Sauvignon blanc samples was observed in the middle of fermenta-

tion (11.5 % and 67.5 %, respectively). According to MORENO - ARRIBAS *et al.* (1996), LUGUERA *et al.* (1997) and CANALS *et al.* (1998) the decrease in protein content is acceptable due to the insolubilization of protein nitrogen, as ethanol concentration increases during alcoholic fermentation. MURPHEY *et al.* (1989) and

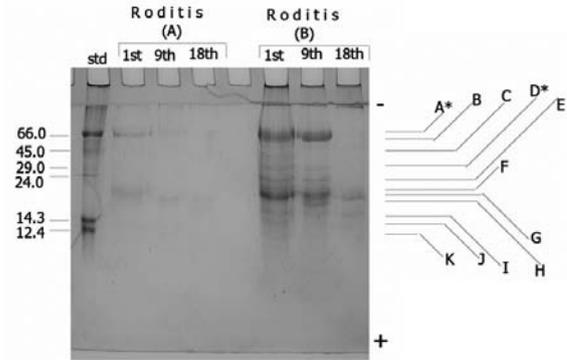


Figure 1 - SDS-PAGE of Roditis must/wine proteins, during 1st, 9th and 18th day of alcoholic fermentation, precipitated by (A) 40% and (B) 70% ammonium sulphate.

Bands are designated by letters A to K. Molecular masses (kDa) of standards are given on the left side of the gel. Bands A* and D* resemble the proteic fractions that reacted positively in PA - Ag staining. The gel was CBB R-250 stained.

SDS - PAGE des protéines de moût/vin du cépage Roditis pendant les 1^{er}, 9^e et 18^e jours de la fermentation alcoolique, précipitées par l'addition de sulfate d'ammonium de 40% (A) et 70% (B).

Les bandes sont indiquées par les lettres A à K. Les poids moléculaires (kDa) des protéines de référence sont données au coté gauche du gel. Les bandes A* et D* ont été identifiées comme glycoprotéines en utilisant la coloration par PA - Ag. Le gel a été coloré au CBB R - 250.

Table II - Enological parameters of must/wine samples of Roditis and Sauvignon grape varieties (means of three repetitions).

Paramètres œnologiques des échantillons de moût/vin issus des cépages Roditis et Sauvignon blanc (moyennes de trois répétitions).

Fermentation period	Soluble protein (mg/l) ⁽¹⁾	Must PH ⁽¹⁾	Initial Baumé	Free amino nitrogen (mg/l) ⁽¹⁾
Roditis				
1 st day	93,5	3,42	11,7	32,5
9 th day	82,7	2,68	2,5	31,5
18 th day	85,2	2,84	1,7	47,6
Sauvignon blanc				
1 st day	105,2	3,56	13,7	43,4
5 th day	34,2	2,68	3,9	27,6
10 th day	39,4	2,70	2,5	30,9

1: By the mean and the formal divergence was calculated the coefficient of variation (CV) less than 5 % in all cases.

DORRESTEIN *et al.* (1995) also reported that protein concentration correlates well with pH and increased linearly with pH. Protein retention in our fermenting musts was greatest at pH 3,42 and 3,56 (table II) for Roditis and Sauvignon blanc samples, respectively, and this supports the above reports.

In the end of fermentation, the protein concentration increased 3,0 % and 15,2 % for Roditis and Sauvignon blanc, respectively. Little increase to protein content may result from the increased autolysis of yeast cells due to cell growth activation by some components extracted from seeds and skins during skin contact (FUKUI and YOKOTSUKA, 2003).

II- SDS - PAGE

The must and wine proteic fractions obtained after 70 % ammonium sulphate fractionated precipitation and lyophilization were compared using the SDS - PAGE method. Figure 1 shows the electrophoretic pattern of Roditis samples after staining with Coomassie Brilliant Blue R - 250. Eleven bands (A - K) were detected during alcoholic fermentation with MWs ranging from 64,4 to 11,1 kDa (table III). Two very intense bands (A) and (G) of 64,4 and 22,2 kDa were observed in the beginning of fermentation and constituted about 24,3 and 29,8 % of the whole protein mixture, respectively (table III).

During alcoholic fermentation, protein changes detected in our samples were quantitative rather than qualita-

tive. Indeed, in the middle of fermentation, Roditis sample showed a decrease in the intensity of the bands (A) and (G) ranging to 13,3 and 30,2%, respectively, (table IV). In this phase, a new protein fraction (H) was detected as shown in figure 1, with MW 20,0 kDa (table III). This protein could be suspected as being a result of protein hydrolysis by the action of extracellular yeast protease during alcoholic fermentation (FERREIRA *et al.*, 2000). DAMBROUCK *et al.* (2003) reported that, during alcoholic fermentation of a Chardonnay must, the protein profile of the wine originating from the same must was almost similar and no changes were observed in the electrophoretic mobility of proteins.

The end of fermentation is characterized by important changes (figure 1). Only four bands (A), (B), (G), and (I) can be detected (table 3), in comparison to ten bands of the first day of fermentation. The intensity of these four bands diminished about 93,9, 51,7, 77,5 and 72,3 % respectively (table IV). This reduction indicates, according to previous data, that during fermentation some proteins become insoluble and precipitate, whilst others are hydrolyzed and can not be detected in the protein profile (LUGUERA *et al.*, 1997; FERREIRA *et al.*, 2000).

Figures 2a and 2b show the electrophoregramm of Sauvignon blanc must during fermentation, after Coomassie Brilliant Blue R - 250 staining and silver staining method, respectively. A comparison between these two staining methods shows the increased sensitivity of

Table III - Relative proportions of individual protein bands separated by SDS - PAGE of must/wine proteins of Roditis grape variety precipitated by ammonium sulphate.

Proportions relatives de bandes protéiques individuelles séparées par la SDS - PAGE de protéines de moût/vin du cépage grec Roditis précipitées par le sulfate d'ammonium.

Proteins fraction band (KDa)	Rm ⁽²⁾	1 st day	9 th day	18 th day
A (64,4)	0,20	24,3	31,2	13,2
B (50,2)	0,27	2,25	2,0	9,8
C (44,2)	0,30	2,3	- ⁽¹⁾	-
D (34,4)	0,37	7,7	4,65	-
E (28,5)	0,42	8,3	8,7	-
F (25,2)	0,45	8,34	3,26	-
G (22,2)	0,48	29,8	30,7	60,0
H (20,0)	0,52	-	12,4	-
I (16,2)	0,57	6,9	3,6	17,0
J (14,3)	0,60	7,4	3,4	-
K (11,1)	0,67	2,71	-	-

(1): Presence of protein band: - indicates no band detected.

(2): Relative mobility: migration distance of protein bands.

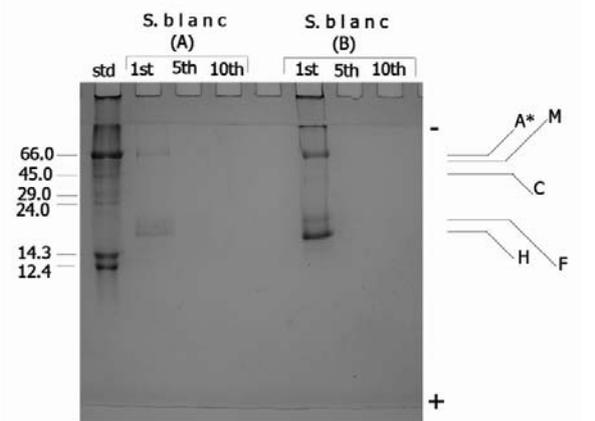


Figure 2a - SDS-PAGE of Sauvignon blanc must/wine proteins during 1st, 5th and 10th day of alcoholic fermentation, precipitated by (A) 40 % and (B)

70 % ammonium sulphate. The gel was CBB R - 250 stained. Molecular masses (kDa) of standards are given on the left side of the gel.

Band A* resemble the proteic fraction that reacted positively in PA - Ag staining.

SDS - PAGE des protéines de moût/vin de Sauvignon blanc pendant les 1^{er}, 5^e et 10^e jours de la fermentation alcoolique, précipitées par l'addition de sulfate d'ammonium de 40% (A) et 70% (B). Le gel a été coloré au CBB R - 250.

Les poids moléculaires (kDa) des protéines de référence sont donnés au coté gauche du gel. La bande A* a été identifiée comme glycoprotéine en utilisant la coloration par PA - Ag.

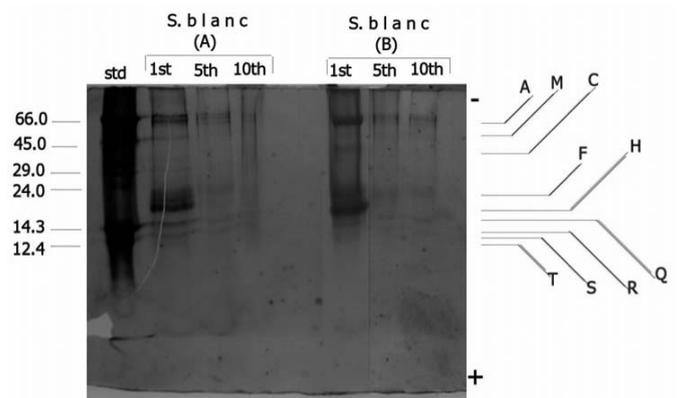


Figure 2b - SDS-PAGE of Sauvignon blanc must/wine proteins during 1st, 5th and 10th day of alcoholic fermentation, precipitated by (A) 40% and (B) 70% ammonium sulphate. The gel was silver stained.

Molecular masses (kDa) of standards are given on the left side of the gel.

SDS - PAGE des protéines de moût/vin de Sauvignon blanc pendant les 1^{er}, 5^e et 10^e jours de la fermentation alcoolique, précipitées par l'addition de sulfate d'ammonium de 40% (A) et 70% (B).

Le gel a été coloré au nitrate d'argent.

Les poids moléculaires (kDa) des protéines de référence sont données au coté gauche du gel.

the silver staining procedure in the case of low protein concentration. According to Kwon (2004) this variety (S. blanc) is characterized by low concentrations of proteins. Nine bands were detected with MW from 64,4 to 14,5 kDa (table V), four of which (A), (C), (F) and (H) were also found in Roditis samples. This observed similarity of protein profiles is acceptable, since musts from grapes of different varieties grown in the same year, under similar climate conditions, in the same type of soil and under the same vinification procedure can contain an identical set of polypeptides (DORRESTEIN *et al.*, 1995; FERREIRA *et al.*, 2000). The appearance of a new band (Q) 17,7 kDa was determined in the middle of fermentation probably as a result of protein hydrolysis. The protein pattern at the end of fermentation seems to resemble that of the middle, whilst a small decrease can be detected in the intensity of the proteins, which is due to their origination from the grape berry (DAMBROUCK *et al.*, 2003).

III- GLYCOPROTEINS

The PA - Ag staining revealed the presence of proteins associated with sugars. Many authors have demonstrated the glycosylate nature of wine proteins (MARCHAL *et al.*, 1996; YOKOTSUKA and SINGLETON 1997; DAMBROUCK *et al.*, 2003). The

Roditis samples studied contained two proteins that positively reacted to the PA - Ag staining. These PA - Ag positive proteins, one large colored band (A) with MW 64,4 kDa and another very slight colored band (D) 34,4 kDa, appeared at the same Rm as the bands stained with CBB R - 250. The colored area of 64,4 kDa was also observed in the middle of fermentation with the same intensity. During the last day of fermentation at the electrophoretic pattern of Roditis sample none of the above glycoprotein fractions were detected. A similar profile, with an intensively colored area at 64,4 kDa was also observed the first day of fermentation of Sauvignon blanc must. These results seems to be normal, since proteins in Chardonnay must with MW ranging from 25,0 to 60,0 kDa are shown to be essentially glycoproteins (DAMBROUCK *et al.*, 2003). No PA - Ag colored bands were observed during fermentation of Sauvignon blanc samples, while, according to YOKOTSUKA and SINGLETON (1997) during fermentation the proportion of sugars gradually decreases, perhaps as a result of modification or degradation of the sugar portions of glycoproteins by enzymes. They also observed that the protein fraction of glycoproteins was not degraded during vinification procedure maybe because of no protease or peptidase activity to glycoprotein fractions.

Table IV - Reduction of the density (A 590) of individual protein band during alcoholic fermentation of the must of Roditis grape variety (expressed in % of the first day of alcoholic fermentation).**Réduction de la densité (A 590) de la bande protéique individuelle pendant la fermentation alcoolique de moût du cépage Roditis (exprimé en % du premier jour de la fermentation alcoolique)**

Protein fraction band (kDa)	Roditis fermentation period	
	9 th day	18 th day
A (64,4)	13.3	93,9
B (50,2)	39.7	51,7
C (44,2)	- ⁽¹⁾	-
D (34,4)	59	-
E (28,5)	29.2	-
F (25,2)	73.5	-
G (22,2)	30.2	77,5
H (20,0)	100	-
I (16,2)	63.8	72,3
J (14,3)	68.9	-
K (11,1)	-	-

(1): Presence of protein band: - indicates no band detected.

Using the PA - Ag staining, it was of great interest the detection of colored bands at the upper of the gel. This indicates that large amounts of polysaccharide material were concentrated there. According to SAULNIER and BRILLOUET (1989) and DAMBROUCK *et al.* (2003) the appearance of an intensively colored area at the upper part of the separating gel is due to the presence of yeast mannoproteins released during alcoholic fermentation.

IV- NEUTRAL AND ACIDIC POLYSACCHARIDES COMBINATED TO PROTEINS

The contents of neutral and acidic polysaccharides in the protein fractions after precipitation with ammonium sulphate and gel chromatography are shown in table VI.

The content of acidic polysaccharides of Roditis samples was less than that of neutral polysaccharides during fermentation, result that was also observed by FUKUI and YOKOTSUKA (2003). During alcoholic fermentation, the quantities of neutral and acidic polysaccharides determined in the glycoprotein fractions changed markedly. From the beginning until the middle of fermentation an important decrease of total polysaccharides can be detected. YOKOTSUKA and SINGLETON (1997) found that during vinification process, sugar-splitting enzymes cleave the sugar chains attached to poly-

peptide chains resulting to the decrease of the sugar content of the proteins.

Neutral polysaccharides of Sauvignon blanc samples show a remarkable increase during fermentation, by contrast with neutral polysaccharides of Roditis (table VI). Previous studies by FUKUI and YOKOTSUKA (2003) have also reported such differences of neutral and acidic polysaccharides of glycoproteins during vinification process.

V- FREE AMINO NITROGEN

The values for amino nitrogen of Roditis and Sauvignon blanc must and wine samples are shown in table II. There are great differences in the concentrations of amino acids between these varieties. Studies of BOU-LOUMPASI *et al.* (2002) and SOUFLEROS *et al.* (2003) are in agreement with OUGH *et al.* (1991) reporting that the grape cultivar, the maturity of the grape and vineyard fertilization, the region of cultivation and the winemaking conditions are functions that deal with the different amounts and different amino acid profiles between varieties.

During the first stage and until the middle of fermentation, there was a decrease in amino nitrogen to 3,0 and 36,4 % for Roditis and Sauvignon blanc samples, respectively (table II). MORENO-ARRIBAS *et al.* (1996)

Table V - Estimated molecular masses of must/wine proteins of Sauvignon blanc grape variety, precipitated by 70% ammonium sulphate and separated by SDS-PAGE. The gel was silver stained.

Poids moléculaires estimés de protéines de moût/vin du cépage Sauvignon blanc, précipitées par le sulfate d'ammonium de 70% (v/v) et séparées par la SDS - PAGE. Le gel a été coloré au nitrate d'argent.

Protein fraction band (kDa)	Sauvignon blanc fermentation period		
	1 st day	5 th day	10 th day
A (64,4)	+ ⁽¹⁾	+	+
M (53,4)	+	+	+
C (44,2)	+	-	-
F (25,2)	+	+	+
H (20,0)	+	-	-
Q (17,7)	-	+	+
R (16,5)	+	+	+
S (15,5)	+	-	-
T (14,5)	+	-	-

(1): Presence of protein band: - indicates no band detected; + indicates presence of band.

Table VI - Contents of acidic (uronic acids) and neutral polysaccharides in the precipitates obtained by 70% (v/v) ammonium sulphate addition and Sephadex G-25 chromatography of Roditis and Sauvignon blanc must/wine during alcoholic fermentation.

Contenu de polysaccharides acides et neutres dans les précipités obtenus par l'addition de sulfate d'ammonium de 70% (v/v) et la chromatographie de Sephadex G-25 de moût/vin de Roditis et du Sauvignon blanc pendant la fermentation alcoolique

Fermentation period	Neutral polysaccharides (µg/mg sample) ⁽¹⁾	Acidic polysaccharides (µg/mg sample) ⁽¹⁾	Total Polysaccharides (µg/mg sample) ⁽¹⁾
	Roditis		
1 st day	73,5	14,5	88,0
9 th day	94,7	11,3	106,0
18 th day	62,3	13,9	76,2
	Sauvignon blanc		
1 st day	20,9	27,7	48,6
5 th day	51,8	50,5	102,3
10 th day	50,6	67,0	117,6

(1): Presence of protein band: - indicates no band detected; + indicates presence of band.

and USSEGLIO-TOMASSET and BOSIA (1990) found that at the beginning of fermentation peptides and amino acids are assimilated by the yeasts since they act as a source of nitrogen for them. The last day of vinification process it was reported an increase in the concentration of amino acids for the two studied varieties. MORENO-ARRIBAS *et al.* (1991) and KOSIR *et al.* (2001) found that except for the assimilation of amino acids during fermentation there is also a diffuse of these components into the fermenting must. Hydrolysis of proteins is responsible for the formation of peptides, which secondarily degrade to amino acids, increasing, that way, the concentration of free amino nitrogen in wine. This not remarkable increase (table II), may also result from the autolysis of the yeast cells.

VI- PROTEIN STABILITY

Heat stability was determined according to the heat test of MURPHEY *et al.* (1989) to the wine samples of the last day of fermentation for each variety. No haze formation was detected in our samples.

Protein fractions between 12,6 and 30,0 kDa could be considered as fractions responsible for protein clouding (HSU and HEATHERBELL 1987a, 1987b; MURPHEY *et al.*, 1989). Despite that fractions of low MW (G) 22,2 kDa and (I) 16,2 kDa of Roditis sample and (F) 25,2 kDa, (Q) 17,7 kDa and (R) 16,5 kDa of Sauvignon blanc, were detected at the electrophoretic profile of each variety, it was not possible to observe haze formation.

YOKOTSUKA *et al.* (1994) reported that the degree of proteins' glycosylation could affect interactions with phenolic components and therefore affect formation of protein turbidity. In Sauvignon blanc and Roditis samples, lack of glycosylation in low MW proteins is estimated to influence the protein stability (RUIZ - LARREA *et al.*, 1998).

CONCLUSIONS

The concentrations of soluble proteins in Roditis and Sauvignon blanc samples during alcoholic fermentation are in agreement with previously published studies. The loss of protein content that is determined from the beginning to the middle of fermentation, may result from the precipitation of proteins due to augmentation of ethanol concentration, the changes in must pH and the hydrolysis, which takes place due to protease activity. The observed increases of soluble proteins at the end of fermentation for both varieties could be a function of skin contact treatment.

Eleven protein bands, after CBB R - 250 staining, and nine bands, after silver staining, were detected in the electrophoretic pattern of Roditis and Sauvignon blanc

samples, respectively. Their MWs ranged from 11,1 to 64,4 kDa. Only one new proteic fraction was determined in the profile of each cultivar, showing that hydrolysis of the proteins may be responsible for the decrease in high MW fractions and the increase in low MW protein fractions. The electrophoretic patterns on polyacrylamide gels were almost similar at the different stages of fermentation for each variety but the relative concentrations of the bands differed. Hydrolysis and ethanol concentration of the protein solution could be considered as factors responsible for the reduction of the gel band intensity.

The PA - Ag staining procedure suggested that, from all the fractions of Roditis and Sauvignon blanc must and wine electrophoretic profiles, only two and one protein bands, respectively, were glycoproteins. Their intensity during alcoholic fermentation diminished as a result of glycanase activity (sugar - splitting enzymes).

The contents of neutral and acidic polysaccharides in the protein fractions changed significantly during fermentation. Concentration of neutral was higher than that of acidic polysaccharides of Roditis sample, whilst Sauvignon blanc must and wine polysaccharides showed the opposite changes.

From the beginning to the middle of fermentation, there was determined a decrease in free amino nitrogen as a result of yeast assimilation. The increase of amino nitrogen content at the last stage of fermentation may result from peptides' hydrolysis.

According to other studies, protein fractions with MWs between 12,6 to 30,0 kDa are responsible for protein clouding. Significantly, protein fractions with MWs 12,6 and 28,0 kDa are considered to be responsible for protein turbidity. In Roditis and Sauvignon blanc samples the proteins smaller than 30,0 kDa could belong to the group of troublesome proteins. Nevertheless, no haze formation was detected in our samples since none of the above 12,6 and 28,0 kDa protein fractions was determined. The degree of glycosylation of proteins of low MWs could affect interactions with phenolic components and, as a result, affect positively the formation of protein turbidity. The presence of glycoproteins of high MWs in our samples (64,4 and 34,4 kDa) are estimated to be related to the inhibitory action of protein turbidity in Roditis and Sauvignon blanc must.

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