NON-ANIMAL PROTEINS AS CLARIFYING AGENTS FOR RED WINES

María José NORIEGA-DOMÍNGUEZ[1], D. S.DURÁN[2], Paloma VÍRSEDA[1]
and María Remedios MARÍN-ARROYO[1]*

1: ALITEC-ENOL, Department of Food Technology, Universidad Pública de Navarra,
Campus Arrosadía, 31006 Pamplona, Navarra, Spain
2: Department of Food Engineering, Universidad de Pamplona, Ciudad Universitaria,
Pamplona, Colombia, Spain

Abstract

Aims: Dueto food security problems related to animal proteins and the growing demand of non-animal-based fining agents, interest in the use of gelatine alternatives for wine fining has increased in recent years. This work studies the use of proteins of non-animal origin for the clarification of Tempranillo red wines.

Methods and results: Proteins from different sources were tested: wheat (seven glutens), maize (one protein extract and one hydrolysed gluten), the yeast *Saccharomyces cerevisiae* (three protein extracts), and the alga *Spirulina platensis* (one protein extract). A preliminary physico-chemical characterisation of the proteins (solubility, isoelectric point, molecular weight) showed that some proteins presented very similar characteristics when belonging to the same source. Fining experiments, based on the principal technological parameters (turbidity of wine, volume and compactness of lees generated), were carried out on a laboratory scale, in both the presence and absence of bentonite as a co-adjuvant. Results obtained with hydrolysed maize gluten and yeast extracts showed that these proteins were particularly advantageous. The use of bentonite in combination with the proteins improved the natural sedimentation of floccules. The sensory analysis of the treated wines demonstrated favourable characteristics in all cases except from spirulina, which negatively affected sensory characteristics.

Conclusions: The effectiveness of non-animal proteins is comparable to that of the traditionally used gelatine, offering advantages due, mainly, to the lower amounts of lees generated and a greater compactness. These two parameters are of great importance for winemakers, as they are associated with wine losses.

Significance and impact of the study: The search for a substitute for gelatine as fining agent.

Keywords: Bentonite, fining, gelatine, non-animal proteins, red wine

Résumé

Objectifs : En raison des problèmes de sécurité alimentaire liés à l'utilisation des protéines d'origine animale et à la demande croissante des consommateurs, il y a un fort intérêt depuis quelques années pour trouver des alternatives à la gélatine pour le collage du vin. Ce travail étudie l'utilisation des protéines d'origine non animale pour le collage des vins rouges Tempranillo.

Méthodes et résultats : Les protéines d'origines différentes, telles que le gluten de blé, de maïs, les extraits de levure *Saccharomyces cerevisiae* et l'algue *Spirulina platensis* ont été testés. Une première caractérisation physico-chimique des protéines a montré que certaines présentent des caractéristiques très similaires quand elles appartiennent à la même famille. Des essais de collage en laboratoire, basés sur les paramètres technologiques principaux (turbidité, volume et compacité des lies générées), ont été réalisés en présence et en absence de bentonite en tant que co-adjuvant. Les résultats obtenus avec le gluten de maïs hydrolysé et les extraits de levure ont montré que ces protéines sont particulièrement avantageuses. L'emploi de la bentonite combiné avec les protéines améliore la sédimentation naturelle. L'analyse sensorielle des vins traités a montré des caractéristiques favorables dans tous les cas sauf pour la spiruline, qui a affecté négativement les caractéristiques sensorielles.

Conclusion : L'efficacité des protéines d'origine non animale est comparable à celle de la gélatine traditionnellement utilisée. Elles offrent des avantages dus principalement à la faible quantité de lies générées et à une plus grande compacité. Ces deux paramètres sont très importants pour les caves car ils sont synonymes de pertes de vin.

Impact scientifique de cette étude : Recherche d'un agent de collage d'origine non animale pour remplacer la gélatine.

Mots clés : Bentonite, collage, gélatine, protéines d'origine non animale, vin rouge

manuscript received the 18th February 2010 - revised manuscript received the 30th July 2010

*Corresponding author: remedios.marin@unavarra.es

©Vigne et Vin Publications Internationales (Bordeaux, France)

INTRODUCTION

Limpidity is a determining characteristic in the valuation of a wine and is a requirement to which consumers give much attention. During the clarification stage of the vinification process, the removal of suspended particles (dregs, yeasts, etc.) reduces turbidity to achieve limpidity. The term fining is used in winemaking to describe the deliberate addition of adsorbents to allow settling or precipitation of partially soluble components from the wine (Boulton *et al.*, 1996). The principal factors affecting the efficiency of the clarification process are the nature, dose and method of preparation of the fining agent, and the pH, temperature, type and age of the wine (Ribérau-Gayon *et al.*, 2006). In the fining of red wines, oenological gelatines have been normally used. In recent years, the use of gelatine has been affected by problems related to food security. Thus, demand for wines that have not been treated with fining agents of animal origin is increasing. In this way, the OIV (International Organization of Vine and Wine) has authorised, at the Vienna Assembly of July 2004, the use of some proteins of vegetal origin as clarifying agents of musts and wines; subsequently, the use of wheat gluten and pea protein was approved in the countries of the European Union in December 2005. Knowledge of the behaviour of gluten in the process of clarification is especially important because wheat glutens are considered to be allergenic due to the risk of celiac disease. Thus, the purpose of this work was to study the use of non-animal proteins as clarifying agents of Tempranillo red wines from the Navarre Appellation. The proteins under study were wheat glutens, which have been investigated in previous studies (Fischerleitner *et al.*, 2003; Lallement *et al.*, 2003, Lefebvre, 2001; Marchal *et al.*, 2002a; 2002b; 2003), yeast extracts from *Saccharomyces cerevisiae*, which have been investigated for colour correction in white wines (Bonilla *et al.*, 2001; López-Toledano *et al.*, 2004) and for fining of red wines (Charpentier *et al.*, 2006; Iturmendi *et al.*, 2005), and proteins from maize and the alga Spirulina platensis, which have never been studied before. After characterising the physico-chemical properties of the proteins, we evaluated their oenological behaviours compared to gelatine (turbidity, lees volume and compactness) followed by sensory analyses.

MATERIALS AND METHODS

1. Wine

Experiments were conducted with a Tempranillo red wine (Navarre Appellation) elaborated in a local winery by traditional methods. The wine arrived at the laboratory after completing malolactic fermentation and was stored at 14 ± 2 ºC until being used in experiments. The wine oenological characteristics are reported in table 1. Wine pH was determined using a Crisol Basic 20 pH meter, according to Regulation 1990/2676/EC. Alcohol content was determined according to the method proposed by Regulation 1990/2676/EC. Total acidity was determined by potentiometry evaluation according to Regulation 1990/2676/EC and expressed as tartaric acid. Volatile acidity was determined by the Mathieu method and expressed as grams of acetic acid per litre (García, 1976). The Total Polyphenol Index (TPI) was determined according to Ribéreau-Gayon (1970), measuring the wine absorbance at 280 nm with a Cintra 20 spectrophotometer (GBC Scientific Equipment Ltd.). This measurement is an estimation of total phenolic compounds. The protein content was determined according to Lowry's modified method using a kit for protein determination (Sigma Chemical Co., USA) and measuring the absorbance at 560 nm.

2. Fining agents

In the preliminary study, 13 proteins of non-animal origin were tested as fining agents: three yeast extracts supplied by Sigma: Y-1625, Y-4000 and Y-1001 (designated 1 to 3, respectively); two maize proteins supplied by Sigma: zein Z-3625 (designated 4) and hydrolysed gluten G-4138 (designated 5); protein extract from the alga *spirulina platensis* (Anova Diet) (designated 6); and seven wheat glutens (designated 7 to 13): gluten 7 was crude gluten (G-5004 Sigma), gluten 9 was a partially hydrolysed devital gluten (Roquette Laisa, Spain), and glutens 8 (Campo Betica, Spain), 10 (Roquette Laisa, Spain), 11 (Laboratorios Girona), 12 (Ferrer Alimentación) and 13 (ADM) were vital glutens. Gelatine (designated G) used for comparative purposes was pure atomised powder gelatine soluble in cold water (Vinigel AT, Agrovin, Spain). All tests were carried out in both the presence and absence of powdered bentonite Bengel (Agrovin, Spain), used as a co-adjuvant in the clarification treatments. Protein

Table 1 - Analytical characteristics of the Tempranillo test wine before fining.

pH	Alcohol content (% v/v)	Total acidity (g tartaric/l)	Volatile acidity (g acetic/l)	TPI (A_{280})	Turbidity (NTU)	Protein content (mg/l)
3.87	13.19	3.78	0.69	60.74	60.21	548.24

©Vigne et Vin Publications Internationales (Bordeaux, France)

solutions were prepared 2 h before being used for wine fining. For the clarifications performed with the combined treatment, bentonite was added after the protein solution. Table 2 shows the protein contents of the fining agents.

3. Fining experiments

Before performing the experiments, the wine was transferred to a plastic container for homogenisation. Fining tests were carried out in 1-L graduated Imhoff plastic sedimentation cones. Treatments were performed in duplicate, with 1 litre of wine each, during 48 h at 20 ± 2 °C. The experiments were performed in three stages. The first stage consisted of a preliminary study to shortlist the 13 original fining agents (proteins from non-animal origin) on the basis of their oenological behaviours (residual turbidity, volume and compactness of lees). All fining agents were added at doses of 12 g/hl (this value is the average recommended dose for gelatine). All tests were performed in both the presence and absence of bentonite as an adjuvant (dose: 30 g/hl). Bentonite was added immediately after the protein according to the manufacturer's technical data sheet. In the second stage, trials were conducted to compare the fining behaviours of the seven proteins selected in the first stage with those of gelatine. The selected clarifying agents were added at doses of 4, 8 or 12 g/hl (these values cover the range of use recommended for gelatine). In addition, the effect of bentonite was measured (dose: 30 g/hl). After clarification, the wine was filtered at atmospheric pressure, bottled and stored at 14 ± 2 °C for later use in the third stage of the study; the sensory evaluation.

4. Characterisation of protein fining agents

a) Solubility tests

Protein solubility was tested in water and in synthetic wine solutions (13% v/v of absolute ethanol (Merck, Darmstadt, Germany) and concentrations of 1, 2, 3 or 5 g/l of tartaric acid (Sigma Chemical Co., St Louis, MO, USA) in deionised water). The pH was adjusted to 3.5 with 1 m NaOH. The proteins were used at concentrations of 1.5, 3.0 and 5.0%.

b) Superficial charge density

The superficial charge of the particles was measured to determine the streaming potential (PE) using a PCD 03 pH equipment (Mütek, Germany). The PCD 03 pH was connected to a compact titrator (Crison, version S) that allows the addition of polyelectrolyte until the PE is zero. If the charge of the studied protein was positive, the anionic polyelectrolyte used for titration was 0.001 N sodium polyethylene sulfonate (PES-Na). If the charge was negative, the cationic polyelectrolyte used was 0.001 N Polydiallyldimethylammonium chloride (Poly-DADMAC). To determine the PE = 0 of all clarifying agents, 4 repetitions using each 20 ml of a synthetic wine solution (5 g of tartaric acid (Sigma Chemical Co., St Louis, MO, USA) and 130 ml of absolute ethanol (Merck, Darmstadt, Germany) in deionised water to a final volume of 1 litre and adjusted to pH 3.84 with 0.1 N NaOH) with the corresponding dose of clarifying agent (20 g/hl) were performed.

b) Isoelectric point

The isoelectric point was measured by the method suggested by Cosme *et al.* (2007) with the same PCD 03 pH equipment used to measure superficial charge density, as it has an incorporated electrode for pH measurement. The addition of an acid or base allows neutralisation of the charge until the PE is zero. If the studied protein had negative charge, 1 N hydrochloric acid (HCl) was used for titration; if the charge was positive, 0.1 N sodium hydroxide (NaOH) was used. Two repetitions with 15 ml of protein solution (3%) were carried out with the same synthetic wine used for superficial charge density determination, in agreement with the solubility tests.

Table 2 - Protein contents of fining agents.

Fining agent	Protein (%)	Fining agent	Protein (%)
Gelatine	100	7-Crude wheat gluten (G-5004)	80.0
1-Yeast extract (Y-1625)	65.6	8-Vital wheat gluten (Campo Betica)	82.0
2-Yeast extract (Y-4000)	53.0	9-Devital wheat gluten (Roquette L.)	85.3
3-Yeast extract (Y-1001)	67.0	10-Vital wheat gluten (Roquette L.)	85.0
4-Zein	100	11-Vital wheat gluten (Lab. Girona)	78.3
5-Maize hydrolysed gluten	67.0	12-Vital wheat gluten (Ferrer Aliment.)	75.0
6-Spirulina (Espirudiet)	65.0	13-Vital wheat gluten (ADM)	75.0

©Vigne et Vin Publications Internationales (Bordeaux, France)

c) Molecular mass

A vertical electrophoresis SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) method was used to measure the molecular weights of the proteins. Electrophoresis was carried out with the EC120 Mini Vertical Gel System (TDI, Spain). It consists of two different acrylamide gels; the stacking gel, where the samples are loaded, and the resolution or separating gel, which allows the identification of protein molecular weights. The gels (0.75 mm thickness) were run at a constant voltage of 150 V until the bromophenol blue tracker dye reached the bottom of the gel (usually 50-65 min). Standard proteins (400 µg of each) ranging from 6.5 to 200 kDa (BioRad Spain, Barcelona, Spain) were used as molecular weight markers. Fining agents and standard proteins were treated with 1 ml of sample buffer (0.5 m Tris pH 6.8; 0.2 m EDTA; 10% SDS; β-mercaptoethanol, glycerol) and 20 µl of sample protein were loaded into each well. After migration, the gel was stained with 0.04% Coomassie brilliant blue in a methanol/water/acetic acid solution (40:50:10) and destained in methanol/acetic acid/water (30:60:10). Finally, molecular mass analysis was carried out using the 1D Manager program (TDI, Spain) which allows the automatic measurement of the distance between a band and one of the reference markers and presents them in several types of regression equations. The molecular weights of the protein bands were calculated from the regression equation of MW vs. mobility (distance between one band and another).

5. Clarifying characteristics

a) Turbidity

Wine turbidity was determined in agreement with « Resolution OENO 4/2000 » before the clarifying trials and 48 h after fining. The samples were taken at the 200-ml mark of the cone, corresponding to the half height. Turbidity was measured with a turbidimeter Hach 2100 N with colour compensation, calibrated with the Gelex secondary turbidity standards kit (Formazin standards 20, 200, 1000 and 4000 NTU). The measurements were carried out in triplicate and expressed in terms of percentage (% residual turbidity = final turbidity x 100/initial turbidity).

b) Determination of lees volume and compactness

The lees volume was read directly from the graduations on the cones 48 h after the addition of the clarifier and was expressed in terms of percentage (% lees volume = lees volume x 100/initial wine volume). Lees compactness was determined by visual appraisal of the volume of lees that did not move when the cones were rotated a quarter turn to the left or right 4-6 times. Compactness was expressed as a percentage (% compactness = lees volume well-compacted x 100/total lees volume).

6. Sensory analysis

Sensory evaluation was conducted to investigate the effects of the different non-animal proteins on clarified wine sensory attributes, establishing differences between wines clarified with these proteins and wine clarified with gelatine. Sensory tests were carried out in the normalised test room of the Public University of Navarre. The room temperature was set to 20-22 ºC with a relative humidity of 60-67% and a light intensity of 400 lux. In each test, different aspects were monitored: order of presentation (balanced so that each sample appears in a given position an equal number of times), coding (randomised three-digit numbers), serving container and size (50 ml in a normalised glass according to UNE 87-022-92). The tasting panel was composed of twelve wine expert assessors. Independent tests were carried out for wines clarified with or without bentonite. Two kinds of tests were performed. In order to know whether significant differences existed between the wines clarified with non-animal proteins and the wine clarified with gelatine, a preliminary bilateral paired comparison test (UNE 87-005-92) was performed. A completely balanced-block design with repetition, where all assessors evaluated all samples (Cochran *et al.*, 1990) was used. Afterward, a descriptive assessment (multiple scale test UNE 87-020-93) was carried out. In this test, five sensory attributes (aspect, colour intensity, aroma intensity, astringency and global appreciation) were evaluated based on a 7-point scale, where 1 is the lowest and 7 the highest score. An incomplete balanced-block design with repetition was used.

7. Statistical analysis

Experimental data were analysed with the Statgraphics Plus statistical software for Windows, version 5.1 (Statistical Graphics Corporation, USA).

RESULTS AND DISCUSSION

1. Physico-chemical characteristics of the clarifying proteins

a) Solubility

In the solubility tests, complete solubility in water was obtained for gelatine, yeast extracts and spirulina. Wheat glutens and maize hydrolysed gluten presented partial solubility in tartaric acid solution (1 g/l) at a concentration of 3.0%, acquiring a suspension aspect that is in agreement with results presented by Marchal *et al.* (2002a), who observed that the glutens tested remained in suspension

©Vigne et Vin Publications Internationales (Bordeaux, France)

without particle sedimentation. Finally, zein at a concentration of 1.5% was completely soluble in 12% v/v hydro-alcoholic solution.

b) Superficial charge density and isoelectric point

Table 3 shows the superficial charge densities and isoelectric points of the proteins tested.

With respect to superficial charge density, gelatine reached a value of 0.073 meq/g, which is in the range of values of 0.02-1.2 meq/g mentioned by other authors (Cosme *et al.*, 2007). Glutens had higher charges that were associated with lower degrees of hydrolysis, whereas spirulina presented the lowest charge. Yeast extracts and zein did not reach high values. The isoelectric point (IP) is an important characteristic, as it determines the net charge of the protein at wine pH. The IP was measured at 20 ± 2 ºC in the most favourable solution for each protein, in accordance with the previous solubility tests. The results show that gelatine, zein, wheat glutens and spirulina presented positive charges, whereas the yeast extracts and hydrolysed maize gluten presented negative

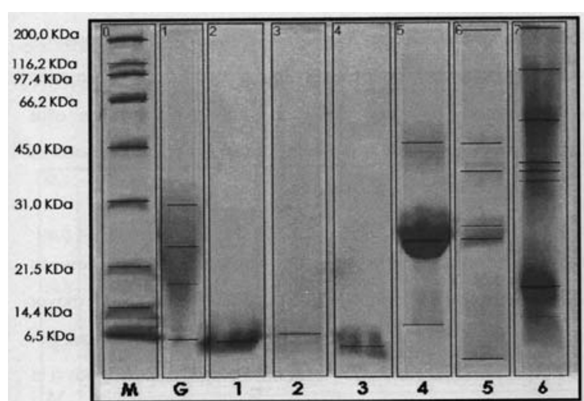

Figure 1 - Electropherogram of the gel 1. Analysis of the lanes.

§ The lanes identified are: M: reference marker; G: gelatine; 1, 2, and 3: yeast extracts; 4: zein; 5: spirulina; 6: hydrolysed maize gluten.

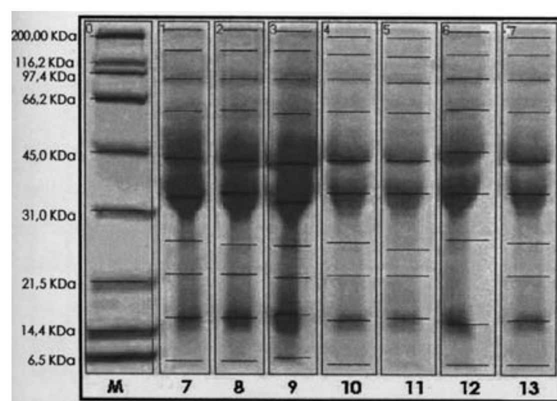

Figure 2 - Electropherogram of the gel 2. Analysis of the lanes.

§ The lanes identified are: M: reference marker; 7: crude wheat gluten; 8: vital wheat gluten; 9: devital wheat gluten; 10, 11, 12 and 13: vital wheat gluten.

Table 3 - Superficial charge densities and isoelectric points of proteins

(Test LSD: mean value ± standard deviation).

Proteins	Superficial charge density (eq/g).10^{-6}	Isoelectric point
Gelatine	(73.37 ± 0.72)	4.37 ± 0.00 *a*
1-Yeast extract	(122.40 ± 5.02)	1.85 ± 0.01 *b*
2-Yeast extract	(50.69 ± 2.76) *c*	2.47 ± 0.00 *c*
3-Yeast extract	(89.13 ± 4.76)	1.37 ± 0.02 *d*
4-Zein	(56.83 ± 1.85) *c*	5.95 ± 0.00 *e*
5-Maize hydrolised gluten	(212.90 ± 12.16)	1.85 ± 0.00 *b*
6-Spirulina	(22.13 ± 3.37)	2.84 ± 0.01 *f*
7-Crude wheat	(332.28 ± 6.35) *a*	6.29 ± 0.01 *g*
8-Vital wheat gluten	(340.48 ± 12.66) *a*	6.28 ± 0.00 *g*
9-Devital wheat gluten	(277.85 ± 11.87)	6.16 ± 0.01 *h.i*
10-Vital wheat gluten	(338.85 ± 14.78) *a*	6.16 ± 0.00 *h.i*
11-Vital wheat gluten	(335.85 ± 11.72) *a*	6.13 ± 0.04 *h*
12-Vital wheat gluten	(319.33 ± 12.48) *b*	6.20 ± 0.14 *i*
13-Vital wheat gluten	(318.48 ± 3.08) *b*	6.17 ± 0.02 *i*

§ Same letters in the same column indicate no significant differences at 95% level.

©Vigne et Vin Publications Internationales (Bordeaux, France)

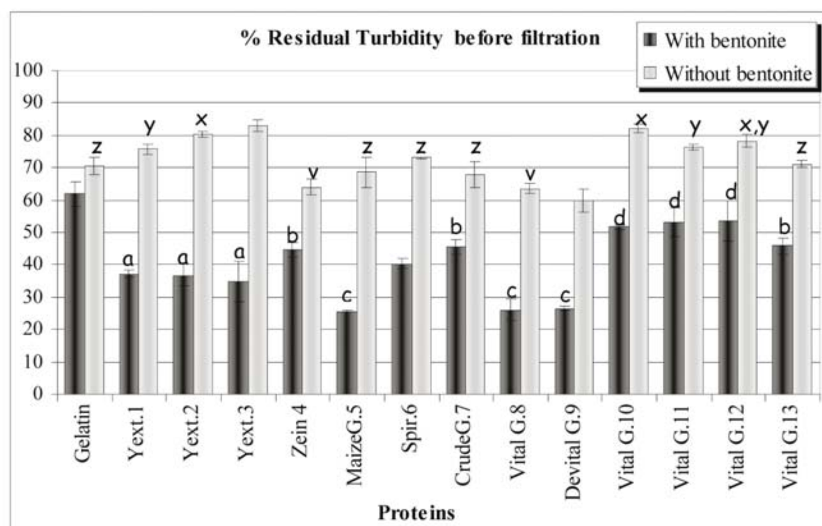

Figure 3 - Residual turbidity before filtration at 12 g/hl dose.
Same letter for the bars indicates no significant differences at 95% level (test LSD).

charges. Spirulina had the lowest value. The analysis of variance (table 3) shows statistically significant differences among the proteins. According to their isoelectric points, the proteins could be classified as follows: proteins with low IP (yeast extracts, hydrolysed maize gluten and spirulina), proteins with medium IP (gelatine) and proteins with high IP (wheat glutens and zein). Spirulina presented an isoelectric point close to wine pH, explaining its low superficial charge.

c) Molecular mass

The proteins were separated by SDS-PAGE and visualised by Coomassie staining. The digitised images of the electropherograms are shown in figures 1 and 2.

In the first lane (M) of both electropherograms, which corresponds to the molecular weight marker, nine bands ranging from 6.5 to 200 kDa were visualised. The molecular mass distribution differed among the fining proteins analysed but were similar within each group (yeast extracts, wheat glutens, etc.). In gel 1, with regard to gelatine, four bands of low molecular masses, ranging from 6.5 to 28.1 kDa, were observed. This result is in accordance with Marchal *et al.* (2000a), who indicated that gelatine is obtained by hydrolysis of porcine collagens. Hydrolysed maize gluten presented a large distribution of molecular masses, ranging from below 6.5 to 186.6 kDa. The yeast extracts presented a very narrow distribution in the low molecular mass range (below 6.5 kDa). The major proportion of zein was in band 2, which corresponds to a molecular mass of approximately 24 kDa. The molecular mass distribution of spirulina ranged from below 6.5 to 191.5 kDa with a major proportion in two bands at 21.0 and 23.4 kDa. In gel 2, the molecular mass distribution of wheat gluten is shown. The profiles of the seven gluten extracts (distribution and number of bands) were very similar. The 1D manager program identified approximately 10 protein bands, ranging from 6.5 to 200.0 kDa, with a major proportion in two bands at 45.0 and 31.0 kDa, with an important fraction at approximately 15 kDa. This result shows a classical profile of vital gluten, which also contains numerous minor proteins between 10.0 and 100.0 kDa (Marchal *et al.*, 2002a).

2. Selection of proteins with the best oenological behaviours

To select proteins with the best oenological behaviours, an initial selection process was carried out with regard to principal technological parameters. Later, the dose effect of the selected proteins on the clarification process was analysed. Finally, a sensory analysis of wines treated with the selected proteins at the appropriate doses was performed.

a) Preselection based on principal technological parameters

Clarification tests were conducted at doses of 12 g/hl of proteins in the presence or absence of bentonite at 30 g/hl.

In figure 3, the results of turbidity before filtration are shown.

All proteins used in combination with the adjuvant presented better turbidity reduction results than gelatine. Hydrolysed maize gluten 5, wheat vital gluten 8, devital gluten 9 and the yeast extracts reduced the initial turbidity of the wine by a greater percentage, between 27 and 37% more than gelatine. In the absence of adjuvant, hydrolysed

©Vigne et Vin Publications Internationales (Bordeaux, France)

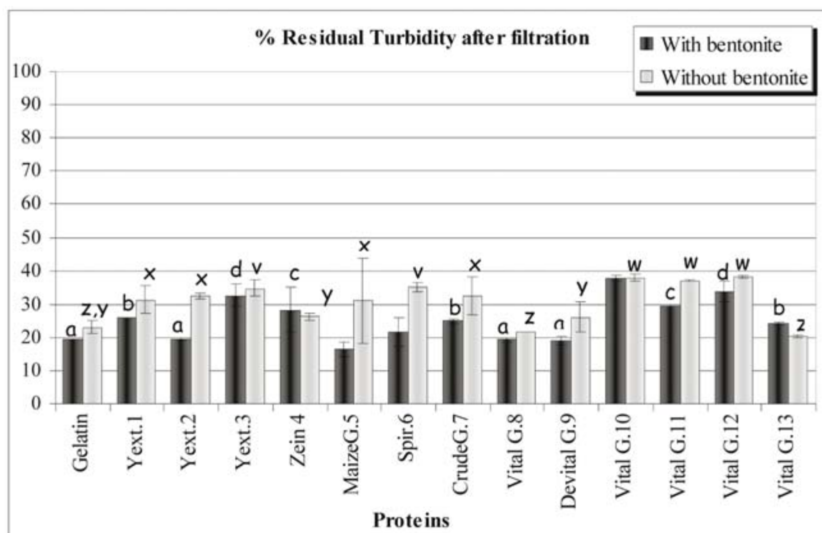

Figure 4 - Residual turbidity after filtration at 12 g/hl dose.
Same letter for the bars indicates no significant differences at 95% level (test LSD).

maize gluten 5, crude gluten 7, vital gluten 13 and spirulina presented fining efficiencies similar to that of gelatine. The highest wine turbidity reduction was found with wheat gluten vital 8 and zein, whereas the yeast extracts and wheat glutens 10, 11 and 12 presented the lowest turbidity reduction. The use of bentonite improved the clarifying efficiency of non-animal proteins compared to gelatine, especially in the case of the yeast extracts. These results show that proteins with high isoelectric points and high superficial charge densities were more efficient at reducing turbidity, and are in accordance with results obtained by Cosme *et al.* (2007) for traditional fining agents such as gelatine and egg albumin.

The results of residual turbidity after filtration through filter paper are shown in figure 4.

Proteins used in combination with bentonite presented residual turbidity values between 16 and 38%. In this case, hydrolysed maize gluten 5 had the best behaviour, whereas wheat glutens 8 (vital) and 9 (devital) and yeast extract 2 had efficiencies similar to that of gelatine. In the absence of bentonite, residual turbidity reached values similar to those previously reported, between 21 and 38%. Turbidities measured after filtration showed better efficiency results than before filtration. Differences were more pronounced in the absence of bentonite, with reductions of more than 50% in some cases. This suggests that the effect of bentonite on turbidity reduction is less important when measured after filtration. This observation could be due to the ability of bentonite in improving sedimentation. On the other hand, results from the analysis of variance (one-way ANOVA) showed statistically significant differences among the wines clarified with different proteins (95% significance level) before and after filtration (figures 3 and 4, respectively). Figure 3 (turbidity before filtration) highlights differences in relation to the origin of the proteins. Six homogeneous groups can be observed (gelatine, yeast, spirulina and three groups of glutens) when used in combination with bentonite, whereas in the absence of adjuvant, proteins were grouped in six homogeneous groups with no similarity with respect to origin. It is worth mentioning that before filtration, the turbidity reduction obtained with gelatine was similar in the presence or absence of bentonite; with non-animal proteins, however, the difference was more pronounced. This could be explained by the fact that gelatine produces floccules that have difficulty to settle and the filtration process improves its sedimentation.

Analysis of the influence of the type of fining protein on lees volume shows that, in combination with bentonite, yeast extracts, zein, maize gluten 5 and spirulina generated lower volumes than gelatine (figure 5).

In the absence of bentonite, a great difference is observed between the volume of lees generated with gelatine and the volumes generated with the tested proteins. Lees volumes generated by all non-animal proteins were very low compared to gelatine, particularly hydrolysed maize gluten and yeast extracts. The bentonite effect was less important in the case of clarification with gelatine, as the variation of lees volume was very small, whereas with non-animal proteins, the lees volume was reduced between 13 and 17 times more compared to gelatine. The analysis of variance showed significant differences among the tested proteins at the 95% significance level, identifying four homogeneous groups when tested in combination with bentonite: group 1: yeast extracts, hydrolysed maize gluten and spirulina; group

©Vigne et Vin Publications Internationales (Bordeaux, France)

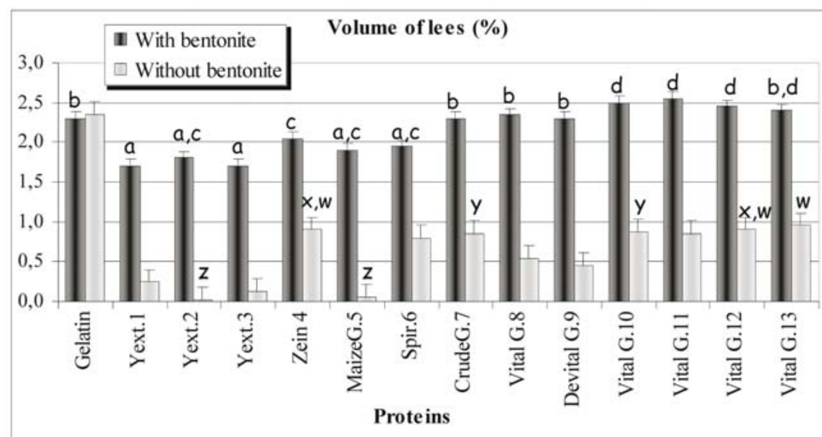

Figure 5 - Lees volume at 12 g/hl dose.
Same letter for the bars indicates no significant differences at 95% level (test LSD).

Table 4 - Compactness of lees (% ml).

Protein	Combination with bentonite	Absence of bentonite
G-Gelatine	26.1	0.0
1-Yeast extract	100	100
2-Yeast extract	100	100
3-Yeast extract	100	100
4-Zein	58.5	66.6
5-Maize hydrol. gluten	100	100
6-Spirulina	100	37.5
7-Crude wheat	65.2	100
8-Vital wheat gluten	42.5	0.0
9-Devital wheat gluten	43.4	100
10-Vital wheat gluten	72.0	68.6
11-Vital wheat gluten	39.2	35.3
12-Vital wheat gluten	51.0	44.4
13-Vital wheat gluten	47.9	42.1

2: yeast extract 2, hydrolysed maize gluten, spirulina and zein; group 3: gelatine, wheat glutens vital 8, devital 9, crude gluten 7 and vital 13; and group 4: wheat vital glutens 10, 11, 12 and 13. In this case, gelatine presented an efficiency similar to that of the wheat glutens. With regard to lees compactness, table 4 shows that all tested proteins presented more compact lees than gelatine. This could be due to the formation of small floccules, which do not leave free spaces for the wine. Yeast extracts and hydrolysed maize gluten presented the most compact lees. This result was not affected by the presence or absence of bentonite.

Taken together, the analysis of the technological parameters reveals that the proteins that showed the best results were spirulina (6) and the yeast extracts (1,2 and 3) when used in combination with bentonite, and hydrolysed maize gluten (5), vital wheat gluten (8) and devital wheat gluten (9) in the absence of bentonite.

b) Effect of selected protein doses on technological parameters

We tested doses of 4, 8 and 12 g/hl based on the gelatine dosage recommended for clarification of red wines, and the vegetal proteins doses used by Lefebvre *et al.* (1999, 2000, 2001) and Marchal *et al.* (2002a, 2003).

Table 5 shows the results of the analysis of variance of the residual turbidity data after 48 h of clarification and filtration at atmospheric pressure.

©Vigne et Vin Publications Internationales (Bordeaux, France)

Table 5 - Effect of protein doses on residual turbidity (% NTU). Analysis of variance (test LSD).

Protein	4 g/hl	8 g/hl	12 g/hl	*p-value*	F
		With bentonite			
Gelatine	11.7 ± 0.1	15.6 ± 0.1*E,F*	19.4 ± 0.1*H*	0.0000	7032.3
Yeast extract 1	19.4 ± 0.1	13.5 ± 0.0*D*	26.1 ± 0.1	0.0000	707.5
Yeast extract 2	13.8 ± 1.0*a,A*	13.7 ± 0.1*a,D,E*	19.5 ± 0.4*H*	0.0000	166.4
Yeast extract 3	15.9 ± 0.7*b,B*	14.3 ± 0.8*b,D,E*	32.6 ± 2.5	0.0000	353.3
Hydrol. maize gluten	13.7 ± 1.2*A*	18.5 ± 0.8*G*	16.9 ± 1.1	0.0000	31.8
Vital wheat gluten	17.7 ± 0.5*c,C*	22.7 ± 0.6	19.4 ± 0.6*c,H*	0.0085	6.7
Devital wheat gluten	17.8 ± 0.1*d,C*	18.2 ± 2.0*d,G*	19.1 ± 0.9*d,H*	0.2442	1.5
Spirulina	16.1± 0.3*B*	16.7 ± 0.1*F*	21.6 ± 0.7	0.0000	286.8
p-value	0.0000	0.0000	0.0000		
F	73.6	20.6	136.6		
		Absence of bentonite			
Gelatine	13.4 ± 0.1	19.1 ± 0.3*J,K,L*	23.0 ± 1.6*R*	0.0000	165.9
Yeast extract 1	21.5 ± 1.5*e,I*	21.0 ± 0.1*e,L,N,O*	31.3 ± 3.2*S*	0.0000	48.1
Yeast extract 2	21.4 ± 0.8*f,I*	21.8 ± 0.1*f,N,O*	32.6 ± 0.4*S*	0.0000	649.5
Yeast extract 3	24.7 ± 0.7*g*	25.2 ± 5.2*g,P,*	34.7 ± 1.9*S*	0.0001	18.0
Hydrol. maize gluten	16.4 ± 0.4*h*	17.8 ± 1.2*h,J,K*	31.0 ± 9.7*S*	0.0008	12.0
Vital wheat gluten	14.8 ± 0.6	17.1 ± 0.8*J*	21.6 ± 0.2*R*	0.0000	229.0
Devital wheat gluten	17.4 ± 0.3*i*	19.8 ± 3.2*i,K,L,N*	26.2 ± 4.5	0.0002	16.7
Spirulina	23.3 ± 0.4*j*	23.8 ± 0.3*j,P*	35.1 ± 1.0*S*	0.0000	693.2
p-value	0.0000	0.0000	0.0000		
F	200.9	9.3	10.3		

§ Mean ± standard deviation.
Same minuscule letters in the same row indicate no significant differences among dose at 95% level.
Same capital letters in the same column indicate no significant differences among proteins at 95% level.

Table 6 - Effect of protein doses on lees volume (% ml). Analysis of variance (test LSD).

Protein	4 g/hl	8 g/hl	12 g/hl	*p-value*	F
		With bentonite			
Gelatine	1.80 ± 0.00*A*	2.00 ± 0.00*C*	2.30 ± 0.00*E*	0.0000	0.00
Yeast extract 1	1.45 ± 0.00*B*	1.55 ± 0.01	1.70 ± 0.00*F*	0.0009	28.50
Yeast extract 2	1.50 ± 0.00*a,B*	1.45 ± 0.01*a*	1.80 ± 0.00*G*	0.0001	25.80
Yeast extract 3	1.50 ± 0.00*B*	1.65 ± 0.00*b,D*	1.70 ± 0.00*b,F*	0.0004	39.00
Hydrol. maize gluten	1.65 ± 0.01*c*	1.65 ± 0.00*c,D*	1.90 ± 0.00*G*	0.0004	37.50
Vital wheat gluten	1.90 ± 0.00*d,A*	1.95 ± 0.00*d,C*	2.35 ± 0.01*E*	0.0000	109.50
Devital wheat gluten	1.85 ± 0.01*e,A*	2.00 ± 0.00*e,C*	2.30 ± 0.20*E*	0.0096	11.12
Spirulina	1.50 ± 0.00*B*	1.75 ± 0.01	1.95 ± 0.01*G*	0.0000	91.50
p-value	0.0000	0.0000	0.0000		
F	28.95	72.00	40.76		
		Absence of bentonite			
Gelatine	0.95 ± 0.10	1.75 ± 0.10	2.35 ± 0.10	0.0000	592.00
Yeast extract 1	0.14 ± 0.00	0.18 ± 0.00	0.20 ± 0.00*L*	0.0000	0.00
Yeast extract 2	0.00 ± 0.00	0.02 ± 0.00	0.20 ± 0.00*L*	0.0000	0.00
Yeast extract 3	0.60 ± 0.00	0.10 ± 0.00*J*	0.13 ± 0.01*M*	0.0000	7077.00
Hydrol. maize gluten	0.45 ± 0.10*H*	0.08 ± 0.00*f,J*	0.10 ± 0.00*f,M*	0.0000	174.39
Vital wheat gluten	0.43 ± 0.03*H*	0.60 ± 0.00*K*	0.50 ± 0.00	0.0000	117.00
Devital wheat gluten	0.40 ± 0.00*g,H,I*	0.60 ± 0.00*K*	0.40 ± 0.01*g*	0.0004	39.00
Spirulina	0.35 ± 0.00*I*	0.55 ± 0.00*K*	0.80 ± 0.00	0.0000	0.00
p-value	0.0000	0.0000	0.0000		
F	349.91	3089.01	2742.57		

§ Mean ± standard deviation.
Same minuscule letters in the same row indicate no significant differences among dose at 95% level.
Same capital letters in the same column indicate no significant differences among proteins at 95% level.

©Vigne et Vin Publications Internationales (Bordeaux, France)

Table 7 - Descriptive test. Analysis of variance (test LSD. P ≤ 0.05).

Protein	Limpidity	Colour Intensity	Aroma Intensity	Astringency	Global Appreciation
			With bentonite		
Gelatine	4.1 ± 1.3	6.0 ± 1.3	4.4 ± 1.3	4.4 ± 1.5	3.3 ± 1.1
Yeast extract 1	3.7 ± 1.4	6.0 ± 1.2	4.6 ± 1.0	4.9 ± 1.6	3.7 ± 1.0
Yeast extract 2	4.4 ± 1.2	6.1 ± 0.9	4.3 ± 1.1	4.4 ± 1.3	3.9 ± 1.0
Yeast extract 3	4.1 ± 1.5	6.1 ± 1.1	4.2 ± 1.4	4.3 ± 1.5	4.1 ± 1.4
Spirulina	4.1 ± 1.3	5.9 ± 1.0	3.7 ± 1.6	4.4 ± 1.4	3.4 ± 1.2
p-value	0.7350	0.9882	0.4948	0.8365	0.2471
F	0.50	0.08	0.86	0.36	1.39
			Absence of bentonite		
Gelatine	5.5 ± 1.2	6.0 ± 1.3	5.3 ± 0.5	3.5 ± 1.2	4.0 ± 1.0
Maize hydrol.gluten	5.0 ± 1.6	6.0 ± 0.9	5.3 ± 0.7	4.1 ± 1.9	4.0 ± 0.9
Vital wheat gluten	4.7 ± 1.9	6.0 ± 0.9	4.5 ± 1.2	4.5 ± 1.3	4.8 ± 1.3
Devital wheat gluten	4.8 ± 1.0	5.7 ± 1.4	5.3 ± 0.9	4.1 ± 1.0	4.6 ± 1.4
p-value	0.7447	0.9719	0.2269	0.5506	0.4482
F	0.41	0.08	1.54	0.72	0.91

§ Mean ± standard deviation.

Statistically significant differences were observed among the proteins for the tested doses and among the doses for each protein tested, in both the presence and absence of bentonite, except for the devital wheat gluten with bentonite. At 4 and 8 g/hl, differences between gelatine and the other proteins became more pronounced in the presence of bentonite, whereas without bentonite, these differences were detected at 4 and 12 g/hl. There were no turbidity differences between yeast extracts 1 and 2 for the three tested doses when used without bentonite. It was observed that the protein clarifying efficiency depends on the quantity of fining agent used. Accordingly, for most of the proteins tested, an increase in residual turbidity was obtained with increasing doses. This effect was particularly pronounced in the cases of yeast extract 3 (with bentonite), and hydrolysed maize gluten and spirulina (without bentonite).

With respect to lees volume, the analysis of variance in table 6 showed that all fining agents generated higher volumes with increasing doses when used with bentonite. On the other hand, the volumes generated by non-animal proteins were always less than that generated by gelatine when proteins were used in the absence of bentonite; the greatest differences were found at doses of 8 and 12 g/hl. This result is in accordance with results found by Lefebvre *et al.* (1999, 2000, 2001). Statistically significant differences were observed among gelatine and the other proteins for the three tested doses, except for the vital and devital glutens in the presence of bentonite. It is worth mentioning that of the twenty-four tests carried out in the absence of bentonite, the 12 g/hl dose was not the most suitable for only four of them (yeast extract and hydrolysed maize gluten at 4 g/hl, and vital and devital wheat gluten at 8 g/hl).

3. Sensory analysis of wine treated with the selected proteins

The sensory analysis of red clarified wine was carried out establishing two groups according to the presence or absence of bentonite. Group 1: wine clarified with gelatine, spirulina and yeast extract; Group 2: wine clarified with gelatine, hydrolysed maize gluten and vital and devital wheat glutens.

a) Difference test (paired comparison)

Each sample group was analysed separately, using the wine clarified with gelatine as a reference. The results showed no significant differences (at 95% level) in the global quality attribute.

b) Descriptive test (multiple scale)

The scores of each attribute are shown in table 7.

The analysis of variance showed that statistically significant differences were not observed in all sensory attribute. With regard to colour, the best-valued attribute, all samples obtained similar scores. The assessors found that the wines clarified in the absence of adjuvant were more aromatic. Spirulina had the lowest score for this attribute. Of the assessors' comments collected at the end of the trial, four of them indicated that wine clarified with spirulina presented a strange aroma and flavour, similar

©Vigne et Vin Publications Internationales (Bordeaux, France)

to floral air-freshener and chlorophyll, respectively. For this reason, spirulina could not be appropriate for this use.

CONCLUSION

The three yeast extracts, spirulina, hydrolysed maize gluten, vital wheat gluten and partly hydrolysed wheat gluten (devital) had the best oenological behaviours (turbidity of wine, volume and compactness of lees generated by fining) compared to gelatine. With respect to the doses studied, increases in dose lead, in general, to increases in residual turbidity and lees volume. The non-animal origin proteins always generated a smaller volume of lees than gelatine, presenting greater difference when the proteins were used in the absence of bentonite. The sensory analysis proved that there were no significant differences between the wines treated with the selected proteins and those treated with gelatine, except for spirulina, where assessors noticed a strange taste and smell. In summary, the non-animal proteins that presented the best behaviours as clarifying agents in comparison with gelatine were the yeast extracts in the presence of bentonite, and hydrolysed maize gluten and vital and devital wheat glutens in the absence of the adjuvant. Bentonite was shown to be useful for improving sedimentation.

Acknowledgements: This work was made possible thanks to funding from the Government of Navarre. We would also like to express our gratitude to Roquette Laisa Spain, Laboratorios Girona, Campo Betica and Ferrer Alimentación for kindly providing the proteins extracts, to the winemaker Coop. Olitense (Olite, Navarra, Spain) for kindly providing the red wine and to the Navarre Sommeliers Club for the participation of their members in the sensory analysis.

BIBLIOGRAPHICAL REFERENCES

Bonilla F., Mayén M., Mérida J. and Medina M., 2001. Yeasts used as fining treatment to correct browning in white wines. *J. Agric. Food Chem.*, **49**, 1928-1933.

Boulton R., Singleton V., Bisson L. and Kunkee R., 1996. The fining and clarification of wines, 279-319. *In: Principles and practices of winemaking*. The Chapman and Hall Oenology Library, New York.

Charpentier C., Caillet M-M. and Feuillat M., 2006. Essais de collage de moûts blancs et de vins rouges avec un extrait protéique levurien : comparaison avec les colles traditionnelles. *Rev. Œnol.*, **120**, 47-50.

Cochran W. and Cox G. 1990. Diseños experimentales. Ed. Trillas, México.

Cosme F., Ricardo-Da-Silva J.M. and Laureano O., 2007. Protein fining agents: characterization and red wine fining assays. *It. J. Food Sci.*, **1**, 19, 39-56.

Fischerleitner E., Wendelin S. and Eder R., 2003. Comparative study of vegetable and animal proteins used as fining agents. *Bull. OIV*, **76**, 863-864, 31-52.

García Barceló J., 1976. Metodología de análisis de vinos y derivados. SEPSA.

Iturmendi N., Durán D. and Marín R., 2005. Caracterización de proteínas con fines de utilización en la clarificación de vinos. Influencia de las condiciones del medio. *Bull. OIV*, **79**, 891-892, 322-335.

Lallement A., Bournerias P.Y. and Marchal R., 2003. Use of vegetable proteins for clarification of musts and wines intended for secondary fermentation. *Rev. Œnol.*, **107**, 26-30.

Lefebvre S., Maury C., Poisaut P. and Gerland C., 1999. Le collage des vins : influence du poids molécualire des gélatines et premiers essais de colles d'origine végétale. *Rev. Œnol.*, **935**, 37-40.

Lefebvre S., Gerland C., Maury C. and Gazzola M., 2000. Nouvelles colles végétales : origines, propriétés et performances. *Rev. Fr. Œnol.*, **184**, 28-32.

Lefebvre S., 2001. Estudios de la clarificación con proteínas de origen vegetal. *Semana Vitivinícola*, 2882, 3846-3855.

López-Toledano A., Mayén M., Mérida J. and Medina M., 2004. Optimization of the operating conditions for color correction in white wines based on the use of yeast as fining agent. *J. Food Sci. C: Food Chemistry Toxicology*, **69**, 8, C599-C603.

Marchal R., Marchal-Delahaut L., Lallement A. and Jeandet Ph., 2002a. Wheat gluten used as a clarifying agent of red wines. *J. Agric. Food Chem.*, **50**, 177-184.

Marchal R., Marchal-Delahaut L., Michels F., Parmentier M., Lallement A. and Jeandet Ph., 2002b. Use of wheat gluten as clarifying agent of musts and white wines. *Am. J. Enol. Vitic.*, **53**, 4, 308-314.

Marchal R., Lallement A., Jeandet P. and Establet G., 2003. Clarification of Muscat musts using wheat proteins and the flotation technique. *J. Agric. Food Chemistry*, **51**, 2040-2048.

Ribéreau-Gayon P., 1970. Le dosage des composés phénoliques totaux dans les vins rouges. *Chimie analytique*, **52**, 6, 627-631.

Ribéreau-Gayon P., Glories Y., Maujean A. and Dubourdieu D., 2006. Clarification and stabilization treatments: Fining wine, 271-299. *In: Handbook of Enology, Volume 2, 2nd edition, The Chemistry of Wine: Stabilization and Treatments*. Ed. John Wiley & sons, Ltd., Chichester.

©Vigne et Vin Publications Internationales (Bordeaux, France)