BENTONITE FINING DURING DIFFERENT WHITE WINEMAKING STAGES: EFFECT ON THE CHEMICAL AND SENSORY PROPERTIES OF THE WINE

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BENTONITE FINING DURING DIFFERENT WHITE WINEMAKING STAGES: EFFECT ON THE CHEMICAL AND SENSORY PROPERTIES OF THE WINE

Thesis submitted by

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Certify: That the doctoral thesis entitled "Bentonite fining during different white winemaking stages: effect on the chemical and sensory properties of the wine" by Eugenio Lira Miranda to obtain the degree of doctor from Universitat Rovira i Virgili has been carried out under our supervision at the Department of Chemical Engineering of the Universitat Rovira i Virgili.

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Abstract

Protein stability in white wines is a requisite of quality that needs to be ensured by oenologists on bottled wine when it reaches consumers. This stability is affected by the tendency of some proteins to precipitate, which hazes the wine and affects its limpidity. To prevent this from occurring, at the end of the alcoholic fermentation the wine is usually treated with bentonite to remove proteins and stabilize the wine. However, since this treatment is non-selective, it also removes molecules associated with sensorial characteristics such as aromatic compounds or affects the foam quality in sparkling wines. In addition, the treatment is discontinuous and it has several drawbacks in terms of handling.

The protein content in each wine depends on several factors, the most important of which are the characteristics of the grape variety, the vintage and pre- and post-fermentative treatments. This means that every year oenologists have to find the best way of achieving a stable product that is qualitatively attractive and which needs to be manipulated as little as possible.

This study focuses on how the addition of bentonite at different stages of the alcoholic fermentation can achieve protein stability and/or reduce the amount of bentonite required to reach the stability. It also focuses on the effect that this practice has on alcoholic fermentation, the chemical composition of the resulting wines, the protein content, the aromatic profile and the foam quality. The protein profile from the initial grape must through to the finished wine is also analyzed to determine the type and content of proteins at all the different stages and how this affects the amount of bentonite required to stabilize the wine.

This study was carried out on both a pilot and an industrial scale to determine the reproducibility of this practice. The results have been validated so that it can be used in the oenological industry. Several varieties have been studied, all from different geographical areas and three consecutive vintages, to give an overall idea of the effects of the proposed treatment.

It has been established the advantage of using bentonite during alcoholic fermentation. In particular, if it is used in moderate doses and from the middle of the fermentation the resulting wines will be stable and the amount of bentonite used reduced.

At the same time it can be seen that those wines that have been stabilized by treatment during fermentation are the ones that received the higher sensorial evaluations. Reproducibility between scales was confirmed in our studies for almost every parameter evaluated. We were also able to identify and correlate the protein fractions with the bentonite dose required to stabilize the different white wines.

This study has improved a technique that is commonly used by wineries. The results have been validated on an industrial scale, help to obtain better quality wines and are respectful of their origin. We expect to be able to continue working to adjust the technique of protein stabilization in white wines.

Resumen

La estabilidad proteica en vinos blancos es un requisito de calidad que debe ser asegurado por los enólogos en el vino envasado una vez que llegue al consumidor. Esta estabilidad se relaciona con la tendencia de ciertas proteínas a precipitar generando turbidez y afectando su limpidez. Para evitar lo anterior se realiza habitualmente un tratamiento con bentonita al final de la fermentación alcohólica, que remueve estas proteínas y estabiliza el vino. Sin embargo, al ser un tratamiento no selectivo elimina consigo moléculas asociadas a características sensoriales como compuestos aromáticos o afecta la calidad de la espuma en vinos base para espumantes. Además es un tratamiento discontinuo cuya aplicación tiene ciertos inconvenientes del punto de vista de su manipulación.

El contenido de proteínas en cada vino depende de diversos factores, siendo los más relevantes las características de cada variedad de uva, de la vendimia y los tratamientos pre y post fermentativos. Lo anterior implica que los enólogos deben buscar cada año la mejor manera de combinar la elaboración de un producto estable, cualitativamente atractivo y que implique la menor manipulación posible.

En esta tesis se ha estudiado el efecto de dosificar bentonita en diferentes etapas de la fermentación alcohólica con el fin de obtener vinos estables y/o reducir la dosis total de bentonita necesaria para su estabilización. De manera paralela se ha estudiado el efecto sobre la fermentación alcohólica que puede implicar esta práctica, la composición química de los vinos finales, el contenido de proteínas, el perfil aromático y la calidad de la espuma. También se ha analizado el perfil proteico desde el mosto hasta el vino final con el fin de conocer el tipo y contenido de proteínas y relacionarlo con los requerimientos de bentonita para estabilizarse.

Este trabajo se ha llevado a cabo en escala piloto e industrial, para conocer la reproductibilidad de esta práctica, validando así los resultados de manera de poder aplicarse en la industria enológica. Además se ha trabajado con diferentes variedades, en diferentes áreas geográficas y en tres vendimias consecutivas, para obtener una idea más global de los efectos del tratamiento aquí propuesto.

Se ha podido establecer la ventaja que implica el uso de bentonita durante la fermentación alcohólica, especialmente en dosis moderadas y a partir de la mitad de la fermentación, para obtener vinos estables y reducir la dosis total de bentonita a emplear. A su vez se ha podido ver como aquellos vinos que han resultado estables con el tratamiento durante fermentación son mejor evaluados sensorialmente. La reproductibilidad entre escalas también ha sido confirmada en nuestros estudios en casi todos los parámetros evaluados. Por último se ha podido identificar y correlacionar las fracciones proteicas con la dosis de bentonita requerida para estabilizar diferentes vinos blancos.

Este trabajo permite mejorar una técnica habitualmente utilizada por las bodegas, validando los resultados a escala industrial y siendo un aporte para la obtención de vinos de mejor calidad y respetuosos de su origen. Se espera poder seguir trabajando para ajustar la técnica de estabilización proteica de vinos blancos.

Resum

La estabilitat proteica en vins blancs es un requisit de qualitat que deu ser assegurat pels enòlegs en el vins embotellats una vegada arriben al consumidor. Aquesta estabilitat es relaciona amb la tendència de certes proteïnes a precipitar, generant terbolesa i afectant la seva limpidesa. Per evitar aquest fenomen es realitza habitualment un tractament amb bentonita al final de la fermentació alcohòlica, que elimina aquestes proteïnes i estabilitza el vi. No obstant, al ser un tractament no selectiu elimina també molècules associades a característiques sensorials com compostos aromàtics o afecta la qualitat de la espuma en vins base per a l'elaboració de cava. A més a més és un tractament discontinu, que la seva aplicació presenta certs inconvenients des del punt de vista de la seva manipulació.

El contingut de proteïnes en cada vi depèn de diversos factors, com les característiques de cada varietat de raïm, de la verema i los tractaments pre i post fermentatius. És per això que els enòlegs deuen cercar cada any la millor forma de combinar l'elaboració d'un producte estable, qualitativament atractiu i que impliqui la menor manipulació possible.

En aquesta tesi s'ha estudiat l'efecte de dosificar bentonita en diferents etapes de la fermentació alcohòlica amb l'objectiu d'obtenir vins estables i/o reduir la dosis total de bentonita necessària per a la seva estabilització. Paral•lelament s'ha estudiat l'efecte sobre la fermentació alcohòlica que pot implicar aquesta pràctica, la composició química dels vins finals, el contingut de proteïnes, el perfil aromàtic i la qualitat de l'espuma. També s'ha analitzat el perfil proteic des del most fins el vi final amb la fi de conèixer el tipus i contingut de proteïnes, i relacionar-lo amb els requeriments de bentonita necessaris per a estabilitzar-lo.

Aquest treball s'ha realitzat a escala pilot i industrial, a fi de conèixer la reproductibilitat d'aquesta tècnica, validant així els resultats de forma que siguin aplicables a la indústria enològica. A més a més s'ha treballat amb diferents varietats de raïm, en diferents àrees geogràfiques i en tres veremes consecutives, pera obtenir una idea més global dels efectes del tractament proposat.

S'ha pogut establir l'avantatge que implica l'ús de la bentonita durant la fermentació alcohòlica, especialment en dosis moderades i a partir de la meitat de la fermentació, per a obtenir vins estables i reduir la dosis total de bentonita a emprar.

Tanmateix se ha pogut veure como aquests vins que han resultat estables amb el tractament amb bentonita durant la fermentació són millor avaluats sensorialment. La reproductibilitat entre escales també ha esta confirmada en aquest estudi en gairebé tots els paràmetres avaluats. També s'ha pogut identificar i correlacionar les fraccions proteiques amb la dosis de bentonita requerida per a estabilitzar diferents vins blancs.

Aquest treball permet millorar una tècnica habitualment utilitzada en els cellers, validant els resultats a escala industrial i contribuint en l'obtenció de vins de millor qualitat i respectuosos del seu origen. Hom espera poder seguir treballant per a ajustar la tècnica d'estabilització proteica de vins blancs.

Contents

Chapter 1

INTRODUCTION

Sensorial evaluation of white wines by consumers is the final step of a long process. This process involves different techniques, variables and treatments that have an influence on the final result. Integrity of the final product and its characteristics should be assured by wine producers. Tasting, visual and aromatic properties, together with the global impression should be kept in the best possible way until the wine get to the consumer's tables. If a bottle of white wine loses its limpidity and clarity shortly after been bottled, the evaluation of the product will be negative. On the other hand, if a wine is in perfect visual conditions, but the organoleptic characteristics are not good, neither will be appreciated by the consumer. Both aspects are closely related to the wine proteins, which are present from the grapes to the final product. The proteins are one of the main factors affecting stability of white wine during storage (Bayly & Berg, 1967; Hsu & Heatherbell, 1987).

Wine proteins have different functions and are associated to different characteristics. A part of them are associated with molecules that are part of the aromatic profile or involved in the foam quality. However, there is also a portion of them that is unstable and precipitates hazing the bottled wine. Though wine proteins are usually found at very low concentrations, some of them may precipitate due to lack of stability (Waters et al., 1991). Protein stability does not correlate well with total protein concentration because individual proteins behave differently (Bayly & Berg, 1967; Hsu & Heatherbell, 1987). Other factors may also have a role in protein precipitation (Siebert, 1999;

Waters et al., 1995), such as ethanol content (Achaerandio et al., 2001). Each variety and each wine has a different proportion of protein, depending on different elements such as the characteristics of the vintage, the pre and post fermentative treatments and the variety of grape used (Waters et al., 2005).

It is known that to avoid risk of haze in bottled wine a treatment with bentonite is often done, removing unstable proteins and protecting the wine. However, this is a non selective treatment, making that some of the proteins associated with the good characteristics previously described are removed as well. This fact makes that oenologist should take care when applying bentonite for wine protein stabilization.

Bentonite is a fine montmorillonite clay consisting of aluminium silicate anions ($Al_2O_3.SiO_2$) (H_2O)n neutralised by cations such as sodium, calcium, potassium and magnesium. Bentonite's microscopic structure consists of many plates which position permit to swell considerably when adsorbing water (Rankine, 1963). Bentonite, cation exchanger clay, is an inorganic fining agent that removes wine proteins by electrostatic adsorption (Pocock et al., 2011). At the pH of wine, these surfaces have a negative charge, which is responsible for the binding of proteins with a net positive charge, thus adsorbing and removing them from the wine. Bentonite is still extensively used because of its established efficacy as well as its low cost and simple batch process that does not require any specialized equipment or knowledge, however, has some drawbacks such a significant wine volume losses because of poor settling (Waters et al., 2005), cost associated with waste disposal, occupational health and safety issues, interference with common membrane-based winemaking technologies (Waters et al., 2005; Salazar et al., 2007), is not a specific adsorbent and may reduce both undesirable and desirable compounds such as aroma, flavour and anthocyanin compounds (Miller et al., 1985; Voilley et al., 1990). Bentonite

dose above 0.8 g/L affects the wine's organoleptic properties (Lubbers et al. 1993; Ribéreau-Gayon et al., 2000). In spite of all above drawbacks bentonite is still the most effective agent in wine protein stabilization according to recent work published by Chagas et al. (2012). Bentonite is the commercial designation of an expansive clay material mainly composed by montmorillonite (Maujean, 1993; OIV Resolution, 2003). In addition to montmorillonite, bentonite could contain quartz, chalcedony, feldspars, calcite, dolomite, analcime, and pyrite, among others, as accessory minerals.

Besides, it is equally important to consider the effect of bentonite on the aromas of the final wine. The volatile compounds of wine responsible for its aroma could be originated during fermentation, derived from the grape (as free aroma compounds and aroma precursors), or generated during wine aging. With regard to the fermentative compounds, mainly esters, higher alcohols and organic acids are the majority compounds generated during alcoholic fermentation. In particular, esters (ethyl esters and acetates) are responsible for the fruity aroma notes of wines. In the case of organic acids, their sensory effects on wines at low concentrations are related with the aroma freshness. However, although higher alcohols are the most quantitative aroma generated during alcoholic fermentation, their sensory effects are not usually associated with positive notes, and can modify the perception of other aroma notes (Etievant, 1991). Conversely, fining agents can perform unpredictably and may result in over fining, excessive lees production and loss in wine quality (Sanborn et al., 2010). Fining agents have been shown to reduce the concentration of total flavonoids and aromatic compounds such as ethyl esters, acetates, and alcohols in various wine typologies (Moio et al., 2004). Most of fermentative aromatic compounds are indirectly removed by bentonite via deproteinisation, and only a few odour-active molecules are directly removed through adsorption on bentonite (Armada

& Falqué, 2007; Lambri et al. 2010). There is not a lot of bibliography about the effects of bentonite fining on the aroma compounds, but in general the studies are focused on the effects of bentonite fining on fermentative and varietal aroma compounds in finished wines (Lambri et al., 2010; Sanborn et al., 2010). Moreover, very few works have studied the effects on aroma compounds of the use of bentonite in musts (Lambri et al., 2012) and none considering the addition during fermentation. In this sense, the bentonite fining may affect both the production and the losses of fermentative compounds.

An aspect to be considered in the protein stabilizing treatment is also the effect on foamability when these treatments are performed on base wines destined for *cava* (sparkling wine). The foamability of sparkling wines is related to the grape variety, vintage and vinification techniques (Andrés-Lacueva et al., 1996). The foam maximum height (HM), persistence (HS) and persistence time (TS) are the parameters evaluated using the Mosalux method (Maujean et al., 1990). Achieving stable wines and foam quality is a challenge for the industry. Some authors have studied the influence of polysaccharides and nitrogen compounds, reporting a direct relationship between their content and foamability (López-Barajas et al., 2001). Salazar et al. (2010) indicated that bentonite treatment significantly reduces maximum height and persistence of the foam, with some exceptions depending on the dose. Vanrell et al. (2007) also pointed out that removing proteins using bentonite can seriously affect the foaming ability of base wine for cava.

Keeping the dry wine in contact with their lees seems to be a good alternative to achieve, if not total, major stability, reducing the dose of bentonite needed before bottling. The definition of wine lees given by EEC regulation No. 337/79 states that ‘‘wine lees is the residue that forms at the bottom of recipients

containing wine, after fermentation, during storage or after authorized treatments, as well as the residue obtained following the filtration or centrifugation of this product". The effect of keeping the wine on lees on protein stability seems to be due to the presence of yeast mannoproteins, which can be released during fermentation or by autolysis during wine ageing (Pérez-Serradilla & Luque de Castro, 2008). As a matter of fact, mannoproteins have been shown to protect wines from protein precipitation (Moine-Ledoux & Dubourdieu, 1998; Waters et al., 1993; Waters et al., 1994). In this context, Dupin et al. (2000) proposed that the mechanism of haze protection may be a competition between mannoproteins and wine proteins for unknown wine component(s), the latter being required for the formation of large insoluble aggregates of denatured proteins. Therefore, as the presence of mannoproteins decreases the available amount of these unknown components, the size of the haze particles decreases and thus visible turbidity declines. Thus, the combined effect of the presence of bentonite in the lees and the action of the mannoproteins released by the yeast could improve the protein stability of wine.

In general, the published studies have been carried out at laboratory or pilot scales, where the main goals were to know the moment of application of the treatments, dosing, treatment effectiveness and the effects on the wine quality. However, these results have not been extrapolated to the industrial winemaking. Thus, Aguera & Sablayrolles (2005) have described pilot-scale fermentations with sensory profiles that are well adapted to industrial fermentations. Malherbe (2003) compared fermentations conducted on 1: 100 L and 100: 10.000 L scales and reported similar kinetics in both scales. However, Vila (1998) showed that the production of aromatic compounds during fermentation was different in laboratory and pilot scales. Casalta et al. (2010) found that the hydrodynamic conditions affecting the production of aromatic compounds are similar to those

affecting the production of CO_2, so the size of the fermentation tank and operating conditions generate significant kinetic differences on the production of the volatile compounds. The differences were small and from a practical point of view the fermentations in laboratory reproduced pilot-scale and, to a lesser extent, industrial-scale fermentations.

Relationship between grape varieties, protein content and protein stability have been briefly described up to now, and generally in studies carried out in laboratory or pilot scale or as a secondary aspect in other works.

Salazar et al. (2006) described in studies with Macabeo wine that the characteristics of this variety show a low protein content, and a low level of protein instability. In another work a variety usually described as very unstable such as Sauvignon Blanc has been characterized by Esteruelas et al. (2009) in assays about protein haze. Other varieties used in different experiments about the protein content and the stability treatments are Chambave Muscat (Lambri et al., 2012), Semillon (Pocock et al., 2011), Chardonnay (Sanborn et al., 2010), Gewürztraminer (Sanborn et al., 2010), Parellada (Ferrando et al., 1998) and Manzoni Bianco (Vincenzi et al., 2010), among others. For other varieties such as Pinot Gris, Viognier or Sauvignon Gris there is no information available about protein stability, bentonite treatment or protein profile.

The main objective of this work was to study the effect of the addition of bentonite during the alcoholic fermentation on the protein instability of white wines and the total bentonite dose needed to reach that stability. The effect of fermentation scale, grape varieties and vintages on this treatment was also studied. Finally, it was analyzed the relationship between the stabilizing treatment with bentonite and the sensorial properties of the obtained wines.

Chapter 2

MATERIALS AND METHODS

2.1. WINE SAMPLES

The experimental work of this thesis about the dosage of bentonite at different stages of vinification has been carried out at two different scales (pilot and industrial). The study has been applied to different grape varieties as detailed below.

2.1.1. Study with Macabeo grape variety.

Macabeo grape variety was studied during 2010, 2011 and 2012 vintages. Assays were done at pilot and industrial scale according to the procedure described below.

The pilot scale experiments were carried out during two consecutive years (2010, 2011) at the experimental cellar of Mas dels Frares (Constantí, Tarragona) of Universitat Rovira i Virgili. The grape must used for these assays was the same one used for the industrial scale and for each treatment. Experiments were done by triplicate for each treatment and the control in 100 L tanks filled up to 90 L.

The experiments at industrial scale were done during three consecutive years in Cooperativa Agrícola de Vila-rodona (CEVIPE) (Tarragona, Spain) using grapes of a same origin, harvested in consecutive days and following the winemaking practices of the winery. The tanks used for 2010 and 2011 had a capacity of 50.000 L, meanwhile for 2012 the tanks were of 100.000 L.

Once finished the alcoholic fermentation of the industrial scale wines, a representative sample of each tank was moved to plastic drums of 33 L, to continue with the process in the experimental cellar of Mas dels Frares together with the other wines obtained at pilot scale. This was done by triplicate for each treatment studied.

Information about the types of bentonites and the dose used for Macabeo wines each year are detailed in Table 2.1.

2.1.2. Study with Pinot Gris grape variety.

Pinot Gris grape variety was studied during vintage 2012 (March – April) in Piedra Negra – François Lurton cellar in the Uco Valley (Mendoza, Argentina).

Experiments were done at industrial scale in 25.000 L tanks using the usual protocol of the winery except for the treatment with bentonite. In this case one of the usual practices carried out in the winery is concerning to the colour removal of the must by activated carbon prior to alcoholic fermentation, usual enological practice for this variety.

Type and dose of bentonite used is showed in Table 2.1.

2.1.3. Study with Sauvignon Blanc grape variety.

With the Sauvignon Blanc variety the experiment was carried out at industrial scale in winery Misiones de Rengo in the Cachapoal Valley (Sixth region, Chile) during the 2012 vintage (March – April)

The grapes used came from the same vineyard located in Maule Valley and the vinification process was the usual used in the cellar, except for the bentonite treatment. 50.000 L tanks were used.

Information about type and dose of bentonite used is showed in Table 2.1.

2.1.4. Study with Albariño grape variety.

The research work was done during vintage 2012 (September – October) in Estación Experimental de Viticultura y Enoloxía de Ribadumia (EVEGA – Ingacal, Pontevedra, España).

Grapes were harvested and immediately pressed, obtaining a must that was divided for the different treatments and the control, one part was clarified with bentonite and the rest with enzymes, both at low temperature.

Study was done at pilot scale with 30 L tank by duplicate for each treatment and for the control. The usual procedure for this grape variety of the cellar was used to carry out the alcoholic fermentation, except for bentonite treatment.

Bentonite used for the experiment and its dose is showed in Table 2.1.

2.1.5. Study with other grape varieties.

A first study was carried out with different white wines from the Central Valley of Chile, obtained when alcoholic fermentation was finished and with no stabilizing treatment used during its vinification process.

The objective was to know the final protein content and protein stability at the end of alcoholic fermentation, determine bentonite dose necessary in case they were unstable and study the protein profile to determine the protein fraction related with instability.

Samples were obtained from the winery following the sampling protocol. The grape varieties studied were Chardonnay, Sauvignon Blanc, Sauvignon Gris, Semillon, Viognier and Riesling. The initial juices and the final wines were analytically characterized.

To make a deeper study of the protein profile and its relationship with wine stability of Pinot Gris grape variety, fourteen samples of wines were obtained from different wineries and productive zones of Italy.

Samples were obtained from each winery at the end of alcoholic fermentation following the sampling protocol. For each wine a complete chemical analysis was done, together with the protein content by the Bradford method, heat stability test, determination of bentonite stabilizing dose in case it was necessary and macromolecular profile.

For the six Chilean wines and for the Italian Pinot Gris wines the bentonite used was liquid sodium bentonite Volclay (KWK) to determine the stabilizing dose when it was necessary.

Table 2.1. Bentonites used for each variety and assay.

Grape Variety	Vintage Year	Fermentation Scale	Bentonite		
			Brand	Dose (g/hL)	Type
Macabeo	2010	Pilot Industrial	Bentogran AEB Group	25	Sodium bentonite, granular.
	2011	Pilot Industrial	Microcol Alpha Laffort	5	Sodium bentonite, granular.
	2012	Pilot Industrial	Volclay KWK	5	Sodium bentonite, liquid.
Pinot Gris	2012	Industrial	La Elcha Minera	30	Sodium bentonite, granular.
Sauvignon Blanc	2012	Industrial	Volclay KWK	10	Sodium bentonite, granular.
Albariño	2012	Pilot	Microcol Alpha Laffort	40	Sodium bentonite, granular.

2.2. EXPERIMENTAL PROCEDURE FOR THE STUDY OF BENTONITE DOSAGE DURING ALCOHOLIC FERMENTATION

The experimental part of this study was done by the evaluation of four treatments against a control at pilot scale (by duplicate or triplicate) and at industrial scale, as described below:

- Treatment in grape must

- Clarification of must with bentonite, addition to the free run juice
- After racking, the must is fermented to dryness.
- Three samples were taken:
    1. Must before addition of bentonite, just released from the press.
    2. 24 hours later, with the must clarified before fermentation.
    3. End of fermentation, dry wine.
- If the wine was unstable, it was determined the necessary dose to stabilize the wine and it was added to the wine, so a new sample was taken.

- Treatment at the beginning of fermentation

- Clarification of the must with enzymes and/or cold temperature, indicating the product used.
- Addition of bentonite at the beginning of fermentation when density drops 10 points of its initial value.
- Four samples were taken:
    1. Initial clarified must
    2. Before bentonite addition
    3. 24 after bentonite addition
    4. End of fermentation, dry wine.

- If the wine was unstable, it was determined the necessary dose to stabilize the wine and it was added to the wine, so a new sample was taken.

- Treatment at the middle of fermentation

- Clarification of the must with enzymes and/or cold temperature, indicating the product used.
- Addition of bentonite at the middle of fermentation when density dropped to around 1040 – 1050.
- Four samples were taken:
    1. Initial clarified must
    2. Before bentonite addition
    3. 24 after bentonite addition
    4. End of fermentation, dry wine.
- If the wine was unstable, it was determined the necessary dose to stabilize the wine and it was added to the wine, so a new sample was taken.

- Treatment at the end of fermentation

- Clarification of the must with enzymes and/or cold temperature, indicating the product used.
- Addition of bentonite at the end of fermentation when density was around 1010.
- Four samples were taken:
    1. Initial clarified must
    2. Before bentonite addition.
    3. 24 after bentonite addition
    4. End of fermentation, dry wine.
- If the wine was unstable, it was determined the necessary dose to stabilize the wine and it was added to the wine, so a new sample was taken.

- Control

- Clarification of the must with enzymes and/or cold temperature, indicating the product used.
- Control wine, that won't receive any bentonite during the whole fermentation process.
- It will only be treated if after finishing the alcoholic fermentation it is still not stable, in which case the stabilizing bentonite dose needs to be determined.
- Two samples were taken:
    1. Initial clarified must.
    2. End of fermentation, dry wine.
- If the wine was unstable, it was determined the necessary dose to stabilize the wine and it was added to the wine, so a new sample was taken.

General instructions

- The must used in each case is obtained from initial free run juice or obtained at low pressures, lower than 1 bar, avoiding the must of the end of pressing. The must for each of the four trials and the control should be obtained under similar conditions and with similar characteristics.
- All the usual applications and products of the protocol that the winery or centre uses will remain, indicating brand product, dose, time and form of application.
- Inoculation dose, acidity correction, additions and fermentation temperatures are performed according to the protocol of the winery or centre.
- The bentonite dose for the must clarification and the treatments during fermentation are the same for each of the four treatments.
- A chemical analysis is done for the initial grape must (including at least pH, initial density, ºBrix and probable alcoholic degree) and on the final dry

wines (including at least alcoholic degree, total acidity, volatile acidity, pH, residual sugar), stability test and stability bentonite dose in case the wine is not stable.

- The protein content will be measured to each one of the samples obtained, in which case they will be frozen to keep them in good conditions till the moment the analyze will be run.
- Fermentation temperature should be kept between 15 – 20 ºC.
- All samples taken in the different stages of the vinification process should be frozen and properly labeled for further analyzes at the laboratory.

Protocol for sampling and storage of samples

1. The container used for sampling should be correctly labeled with date of sampling, tank number and treatment to which it corresponds.
2. A first aliquot of must or wine should be put inside the container for its setting and then eliminated.
3. Container should be filled up to ¾ parts of its capacity and properly sealed.
4. Once obtained the sample should be immediately frozen for its further analyzes. If freezing is not possible, it should be kept at low temperatures and with addition of 40 mg/L of potassium metabisulfite
5. Number and kind of samples are specified in the experimental procedure for each treatment.

2.3. ANALYTICAL METHODS

All analyzes were performed at the laboratories of Universitat Rovira i Virgili, Pontificia Universidad Católica de Valparaíso, Universitá di Padova and Estación Experimental de Viticultura y Enoloxía de Galicia, as well as the laboratories of the wineries involved in these studies.

2.3.1. Protein content by Bradford method

Total protein concentration was measured by Bradford's method using Coomassie brilliant blue reagent in which under acidic conditions the red form of the dye is converted into its bluer form upon binding to the protein, this change being assayed at 595 nm on a spectrophotometer (Cecil CE2021, England) after 5 min of incubation (Bradford, 1976). The (bound) form of the dye has an absorption spectrum maximum historically held to be at 595 nm. The cationic (unbound) forms are green or red. The binding of the dye to the protein stabilizes the blue anionic form. The increase of absorbance at 595 nm is proportional to the amount of bound dye, and thus to the concentration of protein present in the sample. The protein content was expressed as mg/L of bovine serum albumin (Sigma, cat. no. A-3803). Unlike other protein assays, the Bradford protein assay is less susceptible to interference by various chemicals that may be present in protein samples. All analyzes were done by triplicate.

2.3.2. Protein recovery by KDS

As described by Vincenzi et al. (2005) sodium- dodecyl sulphate (SDS) (Bio-Rad, Milano, Italy) from a 10% stock solution was added to wine to final concentrations of 0.1%. Samples were gently mixed then heated in a boiling water bath for 5 min. Potassium chloride (KCl) (1M) was then added to reach a final concentration of 200 mM. Samples were gently mixed for a further 2 hours, and KDS-protein pellets were recovered by centrifugation at 14,000 g for 15 min at 4°C. Pellets were washed three times with 1 M KCl before protein quantification with BCA Assay kit (Pierce) (Vincenzi et al., 2005).

2.3.3. Electrophoretic analyses

SDS-PAGE was performed according to Laemmli (1970). The protein recovered from 1.0 mL of wine by the procedures described above was solubilised with 200 µL of 62.5 mM Tris-HCl buffer pH 6.8, containing 5% (w/v) 2-mercaptoethanol, 1.3% (w/v) SDS, and 10% (w/v) glycerol. Samples were then heated at 100°C for 5 min and 30 µL was loaded onto SDS-14% PAGE. Two BSA standards (2 and 4 µg) were solubilised and electrophoresed in parallel. SDS-PAGE was carried out in a Mini Protean III apparatus (Bio-Rad) at 18 mA until the tracking dye bromophenol blue ran off the gel. Gels were stained with 0.05% (w/v) Coomassie Brilliant Blue R-250, 5% (w/v) TCA, 17% (v/v) methanol, and 6% (v/v) acetic acid, and destained in 7% (v/v) acetic acid.

2.3.4. Chemical analysis of musts and wines

The musts and wines were chemically analyzed following the official methodology of analysis proposed by the International Organization of Vine and Wine (OIV). The chemical properties were determined by an infrared technique using WineScan FT120 Basic (Foss, Denmark). Titrable and volatile acidity and pH were determined using Fourier Transform Infrared (FTIR) technology with a Foss and Flexible Foss Integrator Software platform, a liquid flow system and a 0.4 mm calcium fluoride cuvette (Foss, Foss Electric España, S.A.) to generate the FTIR spectra. The calibration provided with the equipment enabled us to analyze the pH and volatile acidity immediately, following the resolution OIV/OENO 390/2010. The samples were automatically thermostated at 20ºC in the spectrometer before analysis. The IR spectrum was scanned between 2,000 nm and 10,000 nm (NIR and MIR). The spectra were obtained in triplicate and averaged for each sample.

2.3.5. Aroma analysis

Volatile fermentative compounds (esters, organic acids and isoamyl alcohols) were extracted and concentrated by Stir Bar Sorptive Extraction with subsequent separation by gas chromatography with detection by mass spectroscopy (SBSE-GC/MS) (Coelho et al., 2009), which gave a quantitative analysis of the aromatic compounds of the stable wine samples. For the theoretical estimation of the sensory importance of each compound, the odor activities values [OAV] = (Concentration of compound)/(odor threshold) (expressed in units of aroma (u.a.)) were calculated by using the odor thresholds published in the bibliography (Escudero et al., 2004; Campo et al., 2006).

2.3.6. Foam quality

The foam quality of sparkling base wines was measured using the Mosalux method described by Maujean et al. (1990). The foam parameters measured in this study corresponded to the maximum height reached by the foam (HM) and the height of the foam at stability (HS). HM represented foamability and HS persistence (that is, the wine's ability to produce stable foam). The time needed for foam to collapse (TS) was not measured because it cannot be done precisely. Before the foam parameters were measured, the samples were degassed using a magnetic stirrer for 15 min and centrifuged at 4,000 g at 4 °C for 5 min. A glass cylinder placed on a glass frit was filled with 100 mL of sparkling base wine. Carbon dioxide was injected into the glass cylinder through the glass frit with a constant gas flow of 115 mL/min at a constant pressure of 200 kPa. Foam height was measured in millimetres and controlled by photoelectric cells (infra-red beams). Each sample was analyzed in triplicate.

2.3.7. Fast protein liquid chromatography (FPLC)

Samples of 200 mL of each must, wine or sample obtained during fermentation were centrifuged at 4000 x g for five minutes at 4°C. Aliquots of 45 mL of the supernatant were immediately dialyzed for 72 hours in three dialysis tubes (SIGMA, dialysis tubing-cellulose membrane; D-9652) to remove salts and other low molecular compounds. The dialyzed samples were lyophilized and conserved at -20°C. The lyophilized samples were resuspended in freshly obtained pure deionized water (15×10^6 W/cm) until the protein concentration was about 0.5 mg/mL expressed as bovine serum albumin (BSA). The samples were centrifuged 12,000 × g for two minutes at 4°C and frozen again. On the

day of the analysis they were lyophilized again and resuspended in 0.6 ml of 0.3 M ammonium acetate solution (pH 6.80) to obtain a protein concentration of 0.25 µg/µl. The samples were centrifuged (5 min at 12,000g, 4 ºC) and the supernatant was used directly for FPLC analysis (Canals et al., 1998). Analyses were carried out with a Superdex 75 PC 3.2/30 column on a fast protein liquid chromatography system (Smart System, Pharmacia, Uppsala, Sweden). The samples (50 µl) were injected and eluted with a 0.3 M ammonium acetate solution (pH 6.80) with a flow rate of 40 µl/min. The column eluents were continuously monitored at 280 nm using a µPeak Monitor (Pharmacia, Uppsala, Sweden).

2.3.8. Heat stability test

A wine sample of 20 mL was filtered through a cellulose nitrate membrane with a pore size of 0.45 µm (Whatman, cat. no. 7184009, England) and heated for 2 h at 80 ºC in a bath equipped with a digital control immersion thermostat (Digiterm 100 model). It was then incubated for 2 h at 4 ºC. Finally, the turbidity was measured by nephelometry (Turbiquant 1000 IR turbidimeter) and expressed in nephelometric turbidity units (NTU). The difference in turbidity between the initial wine and the wine after the thermal test was proportional to the protein instability. The wines were considered stable if this difference did not exceed 2 NTU (Moine-Ledoux & Dubourdieu, 1999). All analyzes were carried out in triplicate.

2.3.9. Wine sensory analysis

A test of preference with a semi professional tasting panel was performed. They were asked first to sort according to its appreciation in the nose and then in the mouth. The results were analyzed using the test of Friedman performing a global comparison of each series and then a comparison of pairs to determine the differences found by the tasters.

Chapter 3

RESULTS AND DISCUSSION

3.1 LOW UNSTABLE WINES: MACABEO VINTAGES 2010, 2011 AND 2012.

This assay was done during three consecutive vintages with the grape variety Macabeo, which is usually used for young wines and especially in Catalunya as cava base wine.

3.1.1 Macabeo vintage 2010

Chemical analysis of wines

The final characteristics of the wines obtained are shown in Table 3.1. The total amount of residual sugar was less of 1.5 g/L in all cases and the final ethanol concentration was between 9.0-9.5 % (v/v), the pH was in a range of 3.0-3.2, titrable acidity was between 4.0-4.4 g tartaric acid/L, and the volatile acidity was between 0.15-0.30 g/L. All fermentation kinetics showed the same behaviour throughout the process (data not shown).

As shown in Table 3.1, the chemical characteristics of the final wines obtained are quite similar in the context of winemaking. This shows that none of the treatments generated a disadvantage during the alcoholic fermentation of wine obtained on both the pilot and industrial scale.

Table 3.1. Chemical analysis of dry wines on industrial and pilot scale

Dosing time	Scale	Titrable acidity (g tartaric acid/L)	pH	Alcohol content (% vol)	Residual sugars (g/L)	Volatile acidity (g acetic acid/L)
Must	Industrial	4.42 ± 0.18 a, a	3.04 ± 0.12 a, a	9.43 ± 0.37 a, a	0.88 ± 0.05 a, c	0.32 ± 0.02 a, a
	Pilot	4.08 ± 0.08 b, b	3.14 ± 0.01 a, a	9.36 ± 0.70 a, a	0.93 ± 0.05 a, a	0.21 ± 0.02 b, a
Start of fermentation	Industrial	4.28 ± 0.18 a, ab	3.15 ± 0.13 a, a	9.04 ± 0.35 a, a	1.26 ± 0.07 a, a	0.24 ± 0.02 a, b
	Pilot	4.39 ± 0.02 a, a	3.10 ± 0.02 a, a	9.10 ± 0.12 a, b	0.98 ± 0.18 b, a	0.19 ± 0.01 b, ab
Middle of fermentation	Industrial	4.17 ± 0.17 a, ab	3.12 ± 0.12 a, a	9.42 ± 0.37 a, a	1.07 ± 0.06 a, b	0.24 ± 0.02 a, b
	Pilot	4.17 ± 0.07 a, b	3.15 ± 0.03 a, a	9.37 ± 0.07 a, a	1.04 ± 0.17 a, a	0.19 ± 0.02 b, ab
End of fermentation	Industrial	4.03 ± 0.17 a, b	3.20 ± 0.13 a, a	9.26 ± 0.36 a, a	1.11 ± 0.06 a, b	0.23 ± 0.01 a, b
	Pilot	4.11 ± 0.03 a, b	3.15 ± 0.02 a, a	9.23 ± 0.11 a, a	1.01 ± 0.12 a, a	0.17 ± 0.01 b, b
Control	Industrial	4.18 ± 0.17 a, ab	3.17 ± 0.13 a, a	9.38 ± 0.37 a, a	1.01 ± 0.05 a, b	0.20 ± 0.01 a, c
	Pilot	4.16 ± 0.12 a, b	3.16 ± 0.05 a, a	9.25 ± 0.12 a, a	1.09 ± 0.17 a, a	0.16 ± 0.02 b, b

Average ± Standard deviation

Different first letters indicate a significant difference ($P \leq 0.05$) for the same wine treatment on a different scale. Different second letters indicate a significant difference ($P \leq 0.05$) for the wine treatment on the same scale

Protein content, wine stability and macromolecular profile

Table 3.2 (a, b) shows the total protein content determined by Bradford and KDS methods for initial musts and dry wines in the two scales studied. Reducing the protein content is a complex phenomenon, as the protein content of grapes decreases during fermentation (Moreno-Arribas *et al.*, 1996, Canals *et al.*, 1998) and proteins are also generated by the action of yeasts. On the industrial scale and according to the Bradford method, the decrease in the protein content of the wines obtained after treatment with bentonite during fermentation was similar in the three treatments (70-80%). On the pilot scale, however, the percentage reduction of protein was greater when bentonite was added at the beginning of the fermentation (practically 70%). For the other two cases (middle and end of fermentation) the reduction in the protein content was similar (50%). At both pilot and industrial scale the treatment of the must was the one with the lower efficacy in protein reduction (about 30%). This can be due to the lower contact time of bentonite with the must, or to the absence of ethanol, which can increase the bentonite efficacy increasing the distance between montmorillonite layers (Achaerandio *et al.*, 2001). In addition smaller nitrogen compounds (ammonium ion, aminoacids, etc.) present in the must can compete with proteins on the bentonite surface, decreasing their removal from the must (Somers & Ziemelis, 1973).

In general, protein content is reduced less at pilot scale than at industrial scale, perhaps because at pilot scale the tanks were smaller (lower height) so the bentonite needs less time to settle at the bottom and the contact time between bentonite and the proteins in the wine is shorter. This is confirmed by the lower differences between pilot and industrial scale when the bentonite is added early during the fermentation, in these cases the active mixing caused by the yeast activity could help in maintaining the bentonite in suspension.

For the two methods of protein determination, the level of protein removal showed a similar behaviour in both pilot and industrial scale. However the concentration of proteins detected with the KDS method is higher, confirming that Bradford underestimate the protein concentration in wine as previously reported (Waters et al., 1991).

The wines treated with bentonite during fermentation were all protein stable, both at industrial and pilot scale, while the wine obtained from grape juice treated with bentonite was unstable at pilot scale and stable at industrial scale, although with small differences between the two scales (close ΔNTU= 2). Finally the control wines were unstable on both scales. These results show that the stability of the wines obtained has a similar behaviour for both scales with this type of bentonite treatment. Unstable wines were treated with an additional dose of 5 g/hL of bentonite. However, this minimal additional treatment did not affect the final concentration of proteins in the wines once they were stabilized.

Table 3.2a. Protein content by Bradford method. * Unstable wines at the end of the fermentation

Dosing time	Scale	Dry wine (mg BSA/L)	Stable wine (mg BSA/L) (bentonite treated)	Protein decrease dry wine (mg BSA/L) (%)a
Must clarification	Industrial	8.4 ± 0.7 a, a	8.4 ± 0.7 a, a	3.4 (29)
	Pilot	8.0 ± 0.6* a, a	7.5 ± 0.5 a, a	3.8 (32)
Start of fermentation	Industrial	2.5 ± 0.3 a, b	2.5 ± 0.3 a, b	9.2 (78)
	Pilot	3.7 ± 1.6 a, d	3.7 ± 1.6 a, d	8.1 (69)
Middle of fermentation	Industrial	3.5 ± 0.7 b, b	3.5 ± 0.7 b, b	8.3 (71)
	Pilot	5.8 ± 0.3 a, c	5.8 ± 0.3 a, c	5.9 (50)
End of fermentation	Industrial	2.4 ± 0.4 b, b	2.4 ± 0.4 b, b	9.3 (79)
	Pilot	6.2 ± 0.7 a, bc	6.2 ± 0.7 a, bc	5.6 (47)
Control	Industrial	8.7 ± 0.5* a, a	8.9 ± 0.5 a, a	3.0 (26)
	Pilot	7.4 ± 0.9* a, ab	7.8 ± 1.0 a, ab	4.4 (37)

Average ± Standard deviation. Initial musts value: 11.8 ± 2.7 mg BSA/L. a Decrease percentage respect initial musts value.

* Unstable wines at the end of the fermentation.

Different first letters indicate a significant difference ($P \leq 0.05$) for the same wine treatment on a different scale. Different second letters indicate a significant difference ($P \leq 0.05$) for the wine treatment on the same scale.

Table 3.2b. Protein content by KDS method. * Unstable wines at the end of the fermentation

Dosing time	Scale	Dry wine (mg BSA/L)	Stable wine (mg BSA/L (bentonite treated)	Protein decrease dry wine (mg BSA/L) (%)a
Must clarification	Industrial	13.0 ± 1.6 a, a	13.0 ± 1.6 a, a	2.4 (16)
	Pilot	12.7 ± 1.1* a, ab	11.6 ± 0.4 a, b	2.7 (18)
Start of fermentation	Industrial	6.4 ± 0.1 a, b	6.4 ± 0.1 a, b	9.0 (59)
	Pilot	5.9 ± 1.0 a, c	5.9 ± 1.0 a, c	9.5 (62)
Middle of fermentation	Industrial	6.0 ± 1.0 b, b	6.0 ± 1.0 b, b	9.4 (61)
	Pilot	11.3 ± 2.4a, b	11.3 ± 2.4 a, b	4.1 (27)
End of fermentation	Industrial	11.8 ± 0.4* b, a	11.8 ± 0.4 b, a	3.6 (23)
	Pilot	14.1 ± 0.3* a, a	14.1 ± 0.3 a, a	1.3 (8)
Control	Industrial	12.5 ± 0.9* a, a	11.6 ± 0.4 a, a	2.9 (19)
	Pilot	12.1 ± 0.8* a, b	13.6 ± 2.6 a, ab	3.3 (21)

Average ± Standard deviation. Initial musts value: 15.4 ± 1.9 mg BSA/L. a Decrease percentage respect initial musts value.

* Unstable wines at the end of the fermentation.

Different first letters indicate a significant difference ($P \leq 0.05$) for the same wine treatment on a different scale. Different second letters indicate a significant difference ($P \leq 0.05$) for the wine treatment on the same scale.

The protein profile of musts and wines was analyzed by electrophoresis in the different stages of this study. Figure 3.1 shows that Macabeo grape juice presents four types of macromolecules: the band with the highest molecular weight is the invertase, followed by a putative protein tentatively identified as β-glucanase, chitinase, and finally different isoforms of Thaumatin-like proteins (TLP).

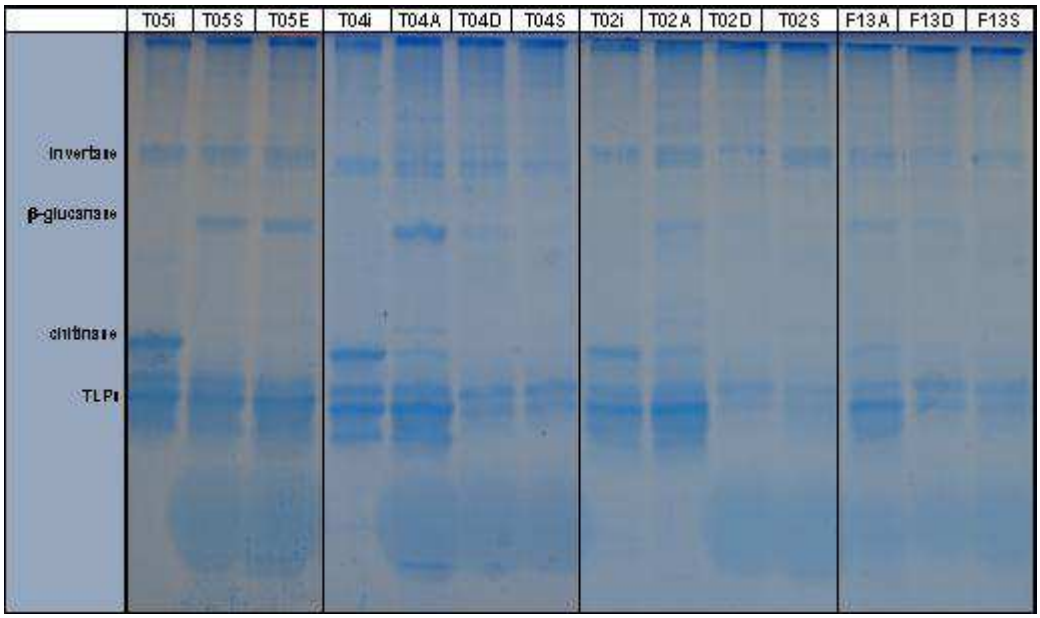

Figure 3.1. SDS-PAGE analysis (Coomassie staining) of the wines (lanes T05i, T04i and T02i initial must sample; lanes T05S, T04S, T02S and F13S dry wine / end of fermentation; lane T05E bentonite-treated stable wine; lanes T04A, T02A and F13A prior to bentonite addition during fermentation; lanes T04D, T02D and F13D 24 hours after bentonite addition during fermentation). T lanes are from the industrial scale, F lanes are from the pilot scale.

The putative β-glucanases appear during fermentation as can be observed in Figure 3.1, which compares lanes T05i and T05S of the control wine,

corresponding to the initial must and the dry wine, respectively. Probably this protein was extracted from the residual particles of grape by effect of the yeast activity, alternatively it can be a different protein released from the yeast during the fermentation. A MS analysis is needed to confirm one of the two hypotheses. The chitinases decreased during fermentation as can be observed in Figure 1 for wines obtained after all the different treatments and the control. Similar results were obtained by Ferreira et al. (2000). Thaumatin-like proteins, on the contrary, decreased noticeably only in bentonite treated wines, in particular when bentonite was added during fermentation. These proteins are known to be involved in protein haze (Vincenzi et al., 2011). In the case of the control wine, which was unstable after alcoholic fermentation, the intensity of the TLP signal decreased slightly since on the industrial scale only a small dose of bentonite was used to stabilize the wine (see lanes T05S and T05E). Treatment with bentonite significantly decreased the intensity of all the signals at 24 h of treatment. This effect can be observed by comparing lane T04A with T04D (before and after bentonite treatment, respectively), and was greater at the end of the fermentation (see lane T04S). Electrophoresis revealed the similarity of the industrial- and pilot-scale profiles. The intensity and behaviour of signals during the vinification (lanes T02 I-A-D-S and F13 A-D-S) were observed to be similar on both scales. Lanes T05S and T05E correspond to the unstable and stable wines of the control treatment. The protein profiles for both wines are quite similar and confirmed the protein content data previously reported. In this case the stabilizing dose was very low and, therefore, the removal of unstable protein was low as well.

The above results show that the industrial- and pilot-scale control wines and the pilot-scale wine obtained from the grape juice clarified with bentonite were unstable once the fermentation had finished. In these cases the final protein

concentrations were higher, in the range from 7.5 to 9 mg/L of BSA, and the putative β-glucanases were still present. On the other hand, the protein profiles of wines treated during fermentation varied. The β-glucanases almost totally disappeared and total protein content was lower (and, in fact, tended to decrease the earlier the bentonite was added).

Aroma fermentative compounds

Table 3.3 (a, b) presents the concentrations of the aroma compounds analyzed in this study. In addition, in the same table are also presented the odour activity values [OAV] of each compound and the total OAV for each chemical family with sensory importance (considering only the compounds with OAV > 1 u.a). As can be seen, the results indicate that the addition of bentonite not only affect the wine aroma by adsorption of compounds (Waters et al., 2005) but also the production of these compounds during fermentation. In this sense, our results showed higher influence on fermentative aroma compounds of the addition of bentonite than other studies in which the fining with bentonite were carried out in final wines (Sanborn et al., 2010).

The calculation of OAV have shown that most of the aroma compounds studied was present in concentrations higher than their odour thresholds, so them will contribute directly to the wine aroma. With regard to the total aroma intensity of wines estimated by the total OAV, this ranged between 970 and 1279 u.a for pilot scale, and between 1073 and 1172 u.a for industrial scale. Although important differences between scales were not observed (differences lower than 17 %) which are consistent with the results obtained by Casalta et al. (2010), the total estimated odour impact of wines for the different treatments was less dispersed in the case of industrial winemaking.

Table 3.3a Fermentative aroma compounds in pilot scale winemaking.

Compound (odour threshold (mg/L))	Must Clarification	Beginning of fermentation	Middle of fermentation	End of fermentation	Control
Ethyl acetate (12.3)	84.03 [6.83]	132.8 [10.80]	49.82 [4.05]	66.46 [5.40]	71.34 [5.80]
Isoamyl acetate (0.03)	12.33 [411.0]	9.41 [313.7]	11.07 [369.0]	12.22 [407.3]	11.23 [374.2]
n-Hexyl acetate (1.5)	1.46 [<1 u.a.]	0.93 [<1 u.a.]	1.34 [<1 u.a.]	1.39 [<1 u.a.]	1.30 [<1 u.a.]
Isoamyl alcohol (30)	77.93 [2.6]	59.99 [2]	62.87 [2.1]	67.07 [2.24]	84.56 [2.82]
Ethyl butyrate (0.02)	0.03 [1.50]	0.03 [1.50]	0.03 [1.50]	0.03 [1.50]	0.03 [1.50]
Ethyl hexanoate (0.014)	0.55 [39.29]	0.54 [38.57]	0.59 [42.14]	0.60 [42.86]	0.56 [39.64]
Ethyl octanoate (0.005)	3.83 [766.0]	2.92 [584.0]	3.04 [608.0]	3.98 [796.0]	3.21 [642.0]
Ethyl decanoate (0.2)	1.05 [5.25]	0.88 [4.40]	0.92 [4.60]	0.97 [4.85]	0.99 [4.95]
Isoamyl octanoate (0.125)	0.03 [<1 u.a.]	0.03 [<1 u.a.]	0.03 [<1 u.a.]	0.03 [<1 u.a.]	0.04 [<1 u.a.]
Octanoic acid (0.5)	9.09 [18.18]	4.98 [9.96]	7.60 [15.2]	7.56 [15.12]	9.36 [18.71]
Dodecanoic acid (1)	4.44 [4.44]	5.39 [5.39]	2.29 [2.29]	3.39 [3.39]	4.03 [4.03]
OAV (Ethyl esters)	812.0	628.5	656.2	845.2	688.1
OAV (Acetates)	417.8	324.5	373.1	412.7	380.0
Ratio OAV Ethyl/Acetates	1.94	1.94	1.76	2.05	1.81
OAV (Acids)	22.62	15.35	17.49	18.51	22.74
OAV Total	1254	970	1049	1279	1093

Concentration expressed in mg/L. Odour activity values indicate in brackets.

Table 3.3b. Fermentative aroma compounds in industrial scale winemaking

Compound (odour threshold (mg/L))	Must Clarification	Beginning of fermentation	Middle of fermentation	End of fermentation	Control
Ethyl acetate (12.3)	129.6 [10.54]	135.9 [11.05]	145.3 [11.81]	159.9 [13.00]	150.9 [12.27]
Isoamyl acetate (0.03)	12.85 [428.3]	12.19 [406.3]	11.28 [376.0]	11.77 [392.3]	11.48 [382.7]
n-Hexyl acetate (1.5)	1.33 [<1 u.a.]	1.19 [<1 u.a.]	1.30 [<1 u.a.]	1.23 [<1 u.a.]	1.28 [<1 u.a.]
Isoamyl alcohol (30)	66.48 [2.22]	60.58 [2.02]	63.67 [2.12]	61.68 [2.06]	61.70 [2.06]
Ethyl butyrate (0.02)	0.03 [1.50]	0.04 [2.00]	0.03 [1.50]	0.03 [1.50]	0.04[1.75]
Ethyl hexanoate (0.014)	0.79 [56.43]	0.57 [40.71]	0.57 [40.71]	0.55 [39.29]	0.53 [37.86]
Ethyl octanoate (0.005)	2.78 [556.0]	3.48 [696.0]	3.53 [706.0]	3.54 [708.0]	3.51 [702.0]
Ethyl decanoate (0.2)	0.61 [3.05]	0.33 [1.65]	0.37 [1.85]	0.42 [2.10]	0.53 [2.65]
Isoamyl octanoate (0.125)	0.00 [<1 u.a.]	0.02 [<1 u.a.]	0.02 [<1 u.a.]	0.04 [<1 u.a.]	0.03 [<1 u.a.]
Octanoic acid (0.5)	6.04 [12.08]	2.96 [5.92]	3.02 [6.04]	3.76 [7.52]	4.74 [9.48]
Dodecanoic acid (1)	3.39 [3.39]	0.33 [0.33]	2.32 [2.32]	6.61 [6.61]	1.37 [1.37]
OAV (Ethyl esters)	617.0	740.4	750.1	750.9	744.3
OAV (Acetates)	438.9	417.4	387.8	405.3	394.9
Ratio OAV Ethyl/Acetates	1.41	1.77	1.93	1.85	1.88
OAV (Acids)	15.47	6.25	8.36	14.13	10.85
OAV Total	1073	1166	1148	1172	1152

Concentration expressed in mg/L. Odour activity values indicate in brackets.

In general for pilot scale, the OAVs for ethyl esters and acetates were higher for wines treated with bentonite at the end of fermentation and in the case of must clarification, and lower in the case of addition of bentonite during winemaking (beginning and half). However, in the case of industrial winemaking with must clarification, the tendency for ethyl esters and acetates was opposite, being the differences among the other treatments less important. The sensory effect of esters could be evaluated by the using of OAV ratio of ethyl esters and acetates that may indicate the tendency to express notes of tropical fruit (low ratio) or tree fruit (high ratio) (Ferreira et al., 1995). Thus, in the case of pilot scale, the ratio was similar to control wine for all treatments with bentonite except for the addition at the end of fermentation (13% higher than the control value). However, in the case of industrial scale, the higher difference was observed for the addition of bentonite to the must (25% lower than the control value).

Other chemical group with sensory importance is the fatty acids family, which contributes to the freshness of wine and to the equilibrium of the fruity aroma notes (Etievant, 1991). The OAVs for fatty acids in pilot scale winemaking for bentonite treated wines were in general lower than in the control wine. However, for industrial winemaking, the behaviour was quite different, being the must clarification and the addition of bentonite at the end of fermentation higher than control and the additions during the fermentation lower than control.

Foam quality

Figure 3.2 shows the results of the foamability (HM) and foam persistence (HS) of wines obtained from the different treatments. Foamability and persistence were higher on the industrial scale than on the pilot scale, indicating that the treatments performed on the pilot scale had a more aggressive effect on the

foaming properties, irrespective of the lower removal of proteins in this case as reported in Table 3.2.

The foamability (HM) on an industrial scale is greater in wines treated in the middle and at the end of fermentation, while on a pilot scale it is greater only in wines treated in the middle of fermentation, as shown in Figure 3.2a. The treatments tended to have a similar effect on the two scales studied (industrial and pilot). The only exception was the effect of adding bentonite at the end of the fermentation on the pilot scale, which led to an HM value of 128 mm, lower than the same treatment on the industrial scale.

Industrial scale wines treated during alcoholic fermentation have lower values of foam persistence (HS) than the control and the must clarified with bentonite, while all the treatments presented the same level of persistence on the pilot scale (see Figure 3.2b). As in the pilot-scale study by Salazar et al. (2010), the addition of bentonite to the final wines led to similar persistence in all wines regardless of the dose applied. This may be due to the reduction of the 60 kDa protein fraction (putative invertase) that took place in this study (see Figure 3.1). As a matter of fact, the content of invertase has been correlated with the foaming properties of wines (Dambrouck *et al.*, 2005), even though other researcher suggested that invertase is not a good model for wine foamability (Puff *et al.*, 2001). In the study by Vanrell et al. (2007), the addition of bentonite to facilitate the riddling process seriously affected the foam quality, although the champanisation process does not seem to be so important. This addition caused a statistically significant decrease in foamability (HM) and foam persistence (HS), and removed nearly all the proteins from fractions of molecular weight of 60 kDa (invertase) and 20-30 kDa (chitinase and TLP), reducing the total soluble protein concentration by more than 80%.

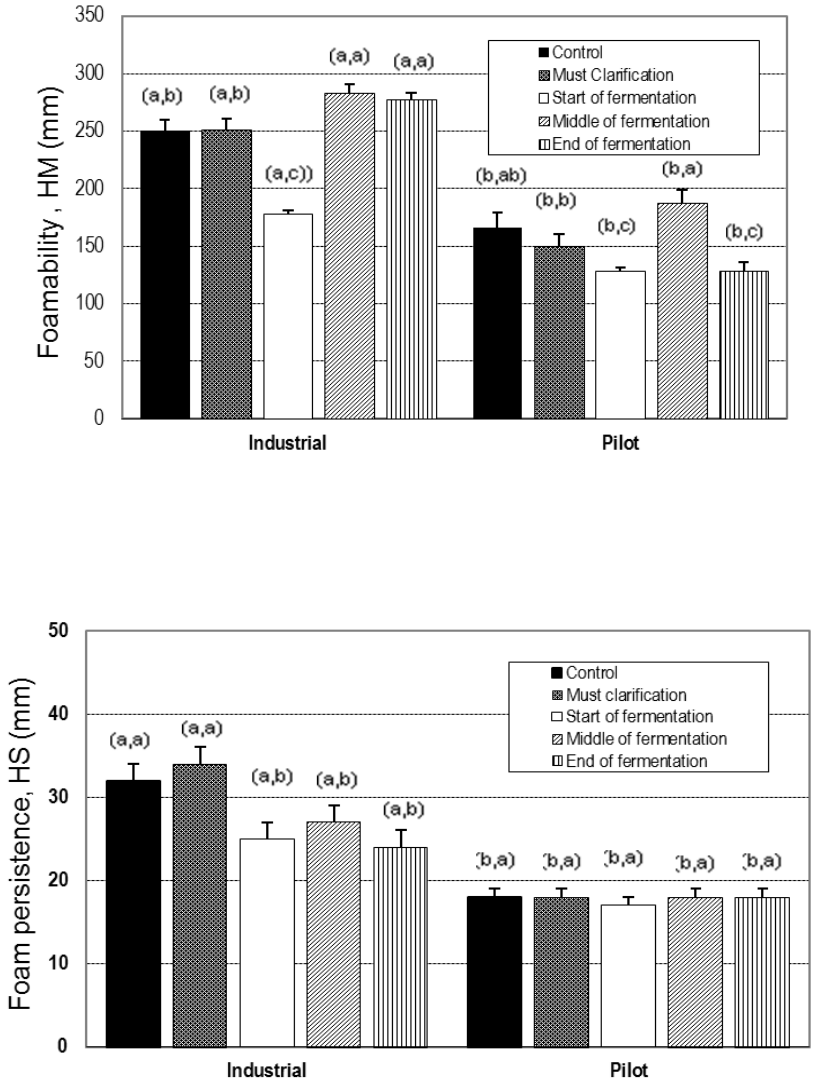

Figure 3.2. Foamability (a) and foam persistence (b) of sparkling base wine on industrial and pilot scale. Different first letters in each bar indicate a significant difference ($P \leq 0.05$) for the same wine treatment on a different scale. Different second letters in each bar indicate a significant difference ($P \leq 0.05$) for the wine treatment on the same scale.

3.1.2 Macabeo vintage 2011

Alcoholic fermentation

Alcoholic fermentation was carried out following the traditional procedure of Cooperativa Vilarodona for industrial scale and following the same protocol at pilot scale at Mas dels Frares.

The mean values of the analytical characteristics of the musts used in this study are as follows: density 1074 ± 1 kg/m^3, titrable acidity 3.85 ± 0.22 g tartaric acid/L, pH 3.38 ± 0.01, total SO_2 35 ± 9 mg/L, free SO_2 11 ± 3 mg/L, gluconic acid 0.31 ± 0.05 g/L, and expected alcoholic content 10.3 ± 0.1 % v/v.

Figure 3.3 (a, b) shows the fermentation kinetics of all experiments. No scale effect was observed. These results are in agreement with Aguera and Sablayrolles (2005) who described that pilot scale fermentations in 100 L tanks are similar to industrial fermentations. Regarding the effect of the treatment with bentonite it cannot be observed a significant effect, since it could be affected by the presence of solids in suspension (Ferrando et al., 1998; Casalta et al., 2010). However all initial musts have been clarified with static decantation, and the level of suspended solids is similar. The small variations observed are mainly due to the fluctuations of the fermentation temperature.

The fermentation times were around 15 days in all cases, and the fermentation temperatures were maintained in the range of 15 – 21°C (as shown in Figure 3.3).

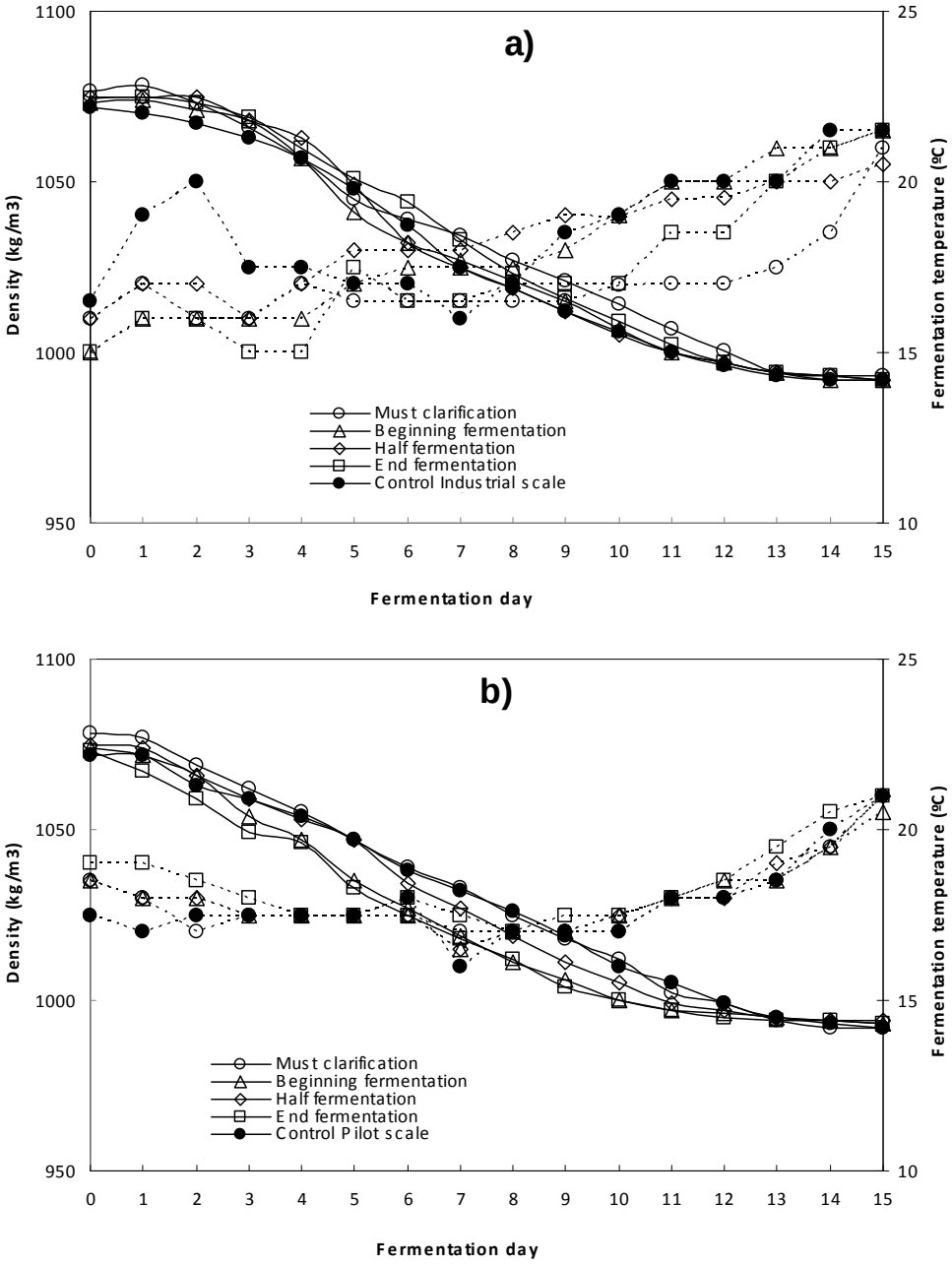

Figure 3.3. Evolution of must density and fermentation temperature during alcoholic fermentation: a) industrial scale, b) pilot scale. 5 g/hL of bentonite were added at the winemaking steps indicated in the figure.

The analytical parameters of the dry wines are shown in Table 3.4. The mean values of the wines were: alcohol content of 10.44 ± 0.38% v/v in pilot scale and 10.68 ± 0.34% v/v in industrial scale, titrable acidity of 4.5 ± 0.5 g tartaric acid/L in pilot scale and 4.0 ± 0.4 g tartaric acid /L in industrial scale, volatile acidity of 0.43 ± 0.12 g acetic acid/L in pilot scale and 0.24 ± 0.06 g/L in industrial scale, residual sugar content of 2.5 ± 0.1 g/L in pilot scale and 2.2 ± 0.2 g/L in industrial scale and a pH of 3.17 ± 0.06 in pilot scale and 3.13 ± 0.09 in industrial scale. The differences found for the total acidities of the wines produced at the pilot and those produced at the industrial scale, are probably due to different rates of transfer of the cold into the whole mass, leading to different extents of potassium bitartrate precipitation. Instead, a possibility for the higher volatile acidity values detected for the wines produced at the pilot scale would be due to their lower volume/surface ratio, resulting in an increased contact with the air oxygen and therefore a higher acetic acid production.

Table 3.4. Chemical analysis of the wines produced with addition of 5 g/hL of bentonite at different times of winemaking. Results obtained at the industrial and pilot scale are compared.

Wine (dosing time)	Scale	Alcohol content (% v/v)	Volatile Acidity (g acetic acid/L)	Titrable acidity (g tartaric acid/L)	pH	Residual Sugar (g/L)
Must	Industrial	11.25 ± 0.01 a, a	0.26 ± 0.02 b, b	3.81 ± 0.02 b, b	3.13 ± 0.02 a, b	2.39 ± 0.05 b, a
	Pilot	11.05 ± 0.06 b, a	0.35 ± 0.02 a, b	4.94 ± 0.03 a, c	3.15 ± 0.01 a, b	2.67 ± 0.14 a, a
Start of fermentation	Industrial	10.62 ± 0.02 a, b	0.22 ± 0.02 b, bc	3.49 ± 0.02 b, c	3.28 ± 0.02 a, a	2.05 ± 0.15 b, c
	Pilot	10.33 ± 0.05 b, c	0.61 ± 0.04 a, a	4.09 ± 0.01 a, d	3.31 ± 0.03 a, a	2.41 ± 0.05 a, bc
Middle of fermentation	Industrial	10.57 ± 0.02 a, c	0.18 ± 0.03 b, c	4.38 ± 0.02 b, a	3.05 ± 0.03 b, c	2.27 ± 0.04 b, b
	Pilot	10.43 ± 0.03 b, b	0.52 ± 0.05 a, a	5.17 ± 0.05 a, a	3.13 ± 0.04 a, b	2.44 ± 0.08 a, bc
End of fermentation	Industrial	10.62 ± 0.02 a, b	0.33 ± 0.02 a, a	4.38 ± 0.04 b, a	3.05 ± 0.00 b, c	2.44 ± 0.06 a, a
	Pilot	10.40 ± 0.02 b, bc	0.33 ± 0.03 a, b	5.09 ± 0.01 a, b	3.15 ± 0.02 a, b	2.35 ± 0.03 b, c
Control	Industrial	10.32 ± 0.01 a, d	0.21 ± 0.01 b, c	3.78 ± 0.01 b, b	3.12 ± 0.01 a, b	2.08 ± 0.02 b, c
	Pilot	10.00 ± 0.03 b, d	0.36 ± 0.06 a, b	5.13 ± 0.10 a, ab	3.11 ± 0.03 a, b	2.55 ± 0.12 a, ab

Average ± Standard deviation

Different first letters indicate a significant difference ($P \leq 0.05$) for the same wine treatment on a different scale. Different second letters indicate a significant difference ($P \leq 0.05$) for the wine treatment on the same scale

Protein concentration and wine stability

The results of the total protein concentration measured in wines at the end of fermentation, after one and three months of ageing on lees and after 7 months after being bottled are shown in Table 3.5.

At both the industrial and pilot scales, the wines treated with bentonite during fermentation had lower total protein concentration as compared to both the control wines and wines obtained from the must clarified with bentonite, that were also the most unstable, as determined by the heat test (see Table 3.5). All wines were slightly unstable, in spite of the low protein concentration typical of the Macabeo wines, whose usual destination is sparkling wine production (Salazar et al., 2006). After fermentation, the protein concentrations of the wines diminished with the time in all cases, with a reduction in the first month of around 5% and 13 % in the wines treated during the fermentation and the wines obtained with control and clarified must, respectively. However the protein concentration was similar in all cases, ranging from about 34 to about 39 mg/L. All wines resulted stable (ΔNTU <2, mean of all wines 1.3 ± 0.3) after one month without any additional treatment even in the case of control wines, where no bentonite was applied throughout the process. This decrease has been also observed during the post-fermentation period by Vincenzi et al. (2011), for Manzoni Bianco wine. This fact may be partially explained by protein insolubilisation and precipitation, although the activity of proteolytic enzymes released from yeast after the end of the fermentation cannot be excluded.

Table 3.5. Evolution of the protein concentration (mg/L of BSA) during time, of the experiments of wines produced with addition of 5 g/hL of bentonite at different stages of winemaking (symbol * indicates unstable wine). Results obtained at the industrial and pilot scale are compared.

Wine (dosing time)	Scale	Fermentation End (mg BSA/L) (Day 0)	Turbidity ΔNTU	1 Month Later (mg BSA/L) (Day 30)	3 Months Later (mg BSA/L) (Day 90)	7 months after bottled (mg BSA/L)
Must	Pilot	40.2 ± 0.2* b, b	6.5± 2.0 b, ab	34.4 ± 0.3 b, b	32.2 ± 0.5 b, bc	30.2 ± 0.2 a, a
	Industrial	42.4 ± 0.3* a, b	9.5± 1.6 a, a	36.8 ± 0.2 a, b	37.0 ± 0.4 a, a	29.7 ± 0.0 b, c
Start of fermentation	Pilot	36.5 ± 0.5* b, c	2.4± 0.0 b, c	35.3 ± 0.6 b, ab	33.1 ± 0.1 b, a	29.5 ± 0.2 b, b
	Industrial	39.5 ± 0.5* a, c	4.8± 1.9 a, b	38.6 ± 0.7 a, a	37.2 ± 0.6 a, a	31.6 ± 0.1 a, a
Middle of fermentation	Pilot	36.6 ± 0.5* a, c	6.1± 2.1 a, ab	34.5 ± 0.3 a, b	30.7 ± 1.2 b, c	30.3 ± 0.1 b, a
	Industrial	36.6 ± 0.4* a, d	2.3± 0.1 b, c	34.7 ± 0.3 a, c	35.6 ± 0.7 a, b	31.1 ± 0.2 a, b
End of fermentation	Pilot	37.0 ± 0.7* a, c	3.8± 0.9 a, b	35.9 ± 1.0 b, a	32.4 ± 0.1 a, b	30.2 ± 0.3 a, a
	Industrial	37.1 ± 0.5* a, d	3.7± 0.0 b, b	34.5 ± 0.2 a, c	31.3 ± 0.3 b, c	29.0 ± 0.1 b, d
Control	Pilot	40.9 ± 0.2* b, a	5.9± 0.3 b, a	35.0 ± 0.6 b, ab	31.4 ± 0.4 b, c	29.5 ± 0.3 a, b
	Industrial	44.1 ± 0.0* a, a	8.9± 0.1 a, a	38.2 ± 0.2 a, a	36.2 ± 0.9 a, ab	28.9 ± 0.0 b, d

Average ± Standard deviation

Different first letters indicate a significant difference ($P \leq 0.05$) for the same wine treatment on a different scale. Different second letters indicate a significant difference ($P \leq 0.05$) for the wine treatment on the same scale

The wine ageing on lees produces an enrichment of mannoproteins, contributing to a major stability and probably to maintenance of the wine quality after bottling (Rowe et al., 2010). As a matter of fact, all the wines showed a further slight improvement of protein stability after three months of ageing on lees since the ΔNTU values were minor (ΔNTU mean = 1.1 ± 0.2 <2) compared to one month ageing, as determined by the heat test. A slight decrease in protein content and complete protein stability (ΔNTU=0) was also observed in all wines after seven months from bottling.

3.1.3 Macabeo vintage 2012

Alcoholic fermentation

The vinification was carried out in Cooperativa de Vila-rodona (CEVIPE) in 100.000 L stainless steel tanks filled up to their fermentation level. The four treatments and the control came from grapes of a same origin harvested in consecutive days. Table 3.6 shows the chemical analysis of the five initial musts. The differences among certain values are due to the heterogeneity of the vineyard and the increase of ripeness during the harvest and the filling of the tanks. However these values don't have a meaningful effect on this study.

The usual protocol of the winery was followed except for the treatment with bentonite. Fermentations followed a similar behaviour in each tank lasting for 11 ± 2 day, under a fermentation temperature that moved between 15 – 20 ºC. The evolution of density through the fermentation is shown in Figure 3.4 for each tank, as well as the temperature evolution. Alcoholic fermentation were similar for each treatment except for the fermentation of the must clarified with bentonite, that was slower during the whole process, meanwhile the tank treated with bentonite at the end of fermentation was as slow as the control tank during the first three days, but followed a similar fermentation profile as the other treatments from the fourth day until the end.

Table 3.6. Chemical analysis of grape must used at industrial scale of Macabeo grape variety.

Must (dosing time)	Density (kg/m3)	Potential alcoholic degree (% v/v)	Titrable acidity (g tartaric acid/L)	pH	Gluconic acid (g acetic acid/L)
Must	1076 ± 2 a	10.5 ± 0.2 b	5.05 ± 0.10 b	3.20 ± 0.04 c	0.11 ± 0.01 c
Start of fermentation	1080 ± 2 a	11.1 ± 0.2 a	4.90 ± 0.10 b	3.25 ± 0.04 bc	0.19 ± 0.02 ab
Middle of fermentation	1078 ± 2 a	10.9 ± 0.2 ab	4.90 ± 0.10 b	3.30 ± 0.04 b	0.21 ± 0.03 a
End of fermentation	1076 ± 2 a	10.5 ± 0.2 b	5.36 ± 0.11 a	3.33 ± 0.04 b	0.15 ± 0.02 b
Control	1079 ± 2 a	11.0 ± 0.2 a	4,59 ± 0.09 c	3.44 ± 0.04 a	0.16 ± 0.02 ab

Average ± Standard deviation
Different first letters indicate a significant difference ($P \leq 0.05$) for the same wine treatment on a different scale. Different second letters indicate a significant difference ($P \leq 0.05$) for the wine treatment on the same scale

Table 3.7. Chemical analysis of Macabeo wines obtained after bentonite treatment at industrial scale.

Wine (dosing time)	Alcohol content (% v/v)	Volatile Acidity (g acetic acid/L)	Titrable acidity (g tartaric acid/L)	pH	Residual Sugar (g acetic acid/L)
Must	10.2 ± 0.2 ab	0.10 ± 0.00 b	5.58 ± 0.11 a	3.22 ± 0.03 a	2.09 ± 0.02
Start of fermentation	10.6 ± 0.2 a	0.10 ± 0.00 b	5.17 ± 0.10 c	3.23 ± 0.03 a	2.07 ± 0.02
Middle of fermentation	9.8 ± 0.2 b	0.10 ± 0.00 b	5.14 ± 0.10 c	3.22 ± 0.03 a	2.34 ± 0.03
End of fermentation	10.4 ± 0.2 a	0.03 ± 0.01 c	5.29 ± 0.11 bc	3.23 ± 0.03 a	2.07 ± 0.02
Control	10.7 ± 0.3 a	0.22 ± 0.01 a	5.46 ± 0.11 ab	3.23 ± 0.03 a	2.35 ± 0.03

Average ± Standard deviation
Different first letters indicate a significant difference ($P \leq 0.05$) for the same wine treatment on a different scale. Different second letters indicate a significant difference ($P \leq 0.05$) for the wine treatment on the same scale

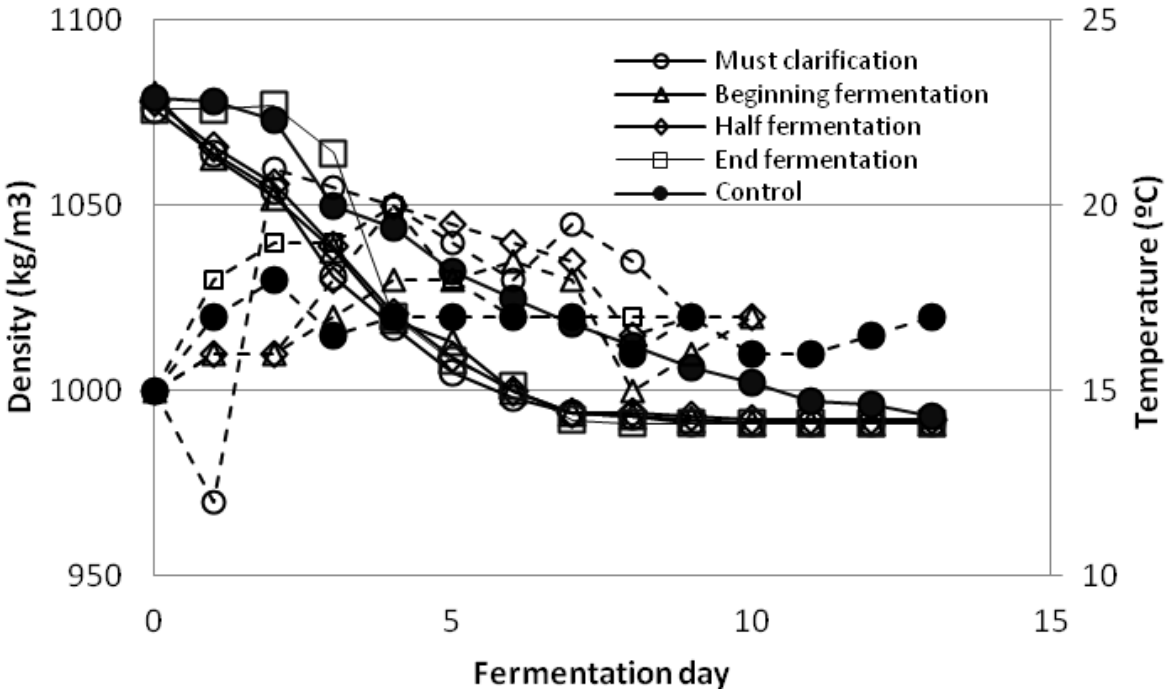

Figure 3.4. Evolution of density and temperature during alcoholic fermentation for four different treatments with 5 g/hL of bentonite and a control.

Additions, corrections and oenological practices were the same for each tank following the winery criteria until the end of the alcoholic fermentation. When wines were dry (≤ 2 g/L of residual sugar) a 33 L sample was obtained from each tank and maintained in plastic deposit, with sulphur and kept in cold atmosphere to follow the rest of the study. After checking protein stability and measured the bentonite stabilizing dose in each case, each deposit was treated to obtain stable wines. Correction of free sulphur was done before bottling. During the whole process samples were obtained following the sampling protocol for later analyzes in the laboratory.

Values of the chemical analyze of each wine are shown in Table 3.7, and correspond to stable wines just before bottling. Wine final values are similar between them, even though some differences could be appreciate in some cases due to the sampling of such a great fermentation volume.

Protein content evolution and stability

Following the experimental procedure different samples were obtained through the assay, determining the protein content by the Bradford method. Table 3.8 shows the total protein content of wines during the different steps of the applied treatments. In first place it can be observed how the total protein content does not differed much between the initial must and the dry wine, this is probably because the decrease of proteins coming from the grapes is balanced by the generation of proteins due to the yeast action (Moine-Ledoux & Dubourdieu 1999; Dupin et al., 2000; Dizy & Bisson, 2000).

Table 3.8. Protein content by Bradford method, stability, bentonite stabilizing dose and total bentonite dose applied

Dosing Time	Sample	Protein Content (mg BSA/L)	Stability	Stabilizing Bentonite Dose (g/hL)	Total Bentonite Treatment (g/hL)
Must	Free run juice	36,9 ± 0,2	Unstable	19	24
	Clarified must (w/bentonite)	37,1 ± 0,8			
	Dry Wine	40,9 ± 0,7			
	Stable Wine	36,9 ± 0,2			
Start of fermentation	Clarified must (w/enzymes)	34,9 ± 0,4	Unstable	13	18
	Pre-treatment	36,2 ± 0,2			
	24 hours Post-treatment	34,5 ± 0,2			
	Dry Wine	43,1 ± 0,2			
	Stable Wine	39,3 ± 0,1			
Middle of fermentation	Clarified must (w/enzymes)	36,1 ± 0,9	Unstable	10	15
	Pre-treatment	37,2 ± 0,1			
	24 hours Post-treatment	34,3 ± 0,5			
	Dry Wine	42,3 ± 0,4			
	Stable Wine	39,0 ± 0,2			
End of fermentation	Clarified must (w/enzymes)	32,8 ± 0,2	Stable	-	5
	Pre-treatment	35,2 ± 0,1			
	24 hours Post-treatment	30,1 ± 0,2			
	Dry Wine	33,5 ± 0,2			
	Stable Wine	29,6 ± 0,2			
Control	Clarified must (w/enzymes)	37,7 ± 0,8	Unstable	26	26
	Dry Wine	41,7 ± 0,4			
	Stable Wine	39,2 ± 0,3			

Once finished the alcoholic fermentation the stability of each wine was checked using the thermal stability test. Out from the five wines, four were unstable and one stable. The stable wine is the one obtained from the dosage of bentonite at the end of fermentation, being for this wine the base bentonite dose of 5 g/hL enough to reach protein stability. By the other hand in the unstable wines it could be observed that the control and the one obtained from treating the must prior to the fermentation needed a similar total bentonite dose for their stabilization of 26 and 24 g/hL, respectively. Meanwhile wines treated at the beginning and half of the fermentation needed a lower total bentonite dose of 18 and 15 g/hL, respectively. It could be appreciated that the tendency is that as later the dosage during fermentation the lower the bentonite dose needed to reach a stable wine.

Unstable wines had a protein content slightly over 40 mg BSA/L when finishing the alcoholic fermentation, meanwhile the stable wine had a content of 33.5 mg BSA/L. Using bentonite to clarify the must did not modified the protein content, presumably due to the volume of must treated in this assay (100.000 L), the treatment did not have enough contact time. However, in wines treated during fermentation the protein content decrease between 2 – 5 mg BSA/L in only 24 hours, being even more in the wine treated at the end of fermentation. This greater elimination may be due to the higher alcohol content, being the removal of proteins by bentonite more effective (Achaerandio et al., 2001). In addition, as being at the end of the fermentation, the generation of proteins by yeasts is smaller, and does not compensate for their loss by the action of bentonite, as can occur in treatments at the beginning and half of fermentation.

In unstable wines, the bentonite stabilizing treatment decrease the protein content between 2 – 4 mg BSA/L, leaving the final content of these wines in a range of 36 – 39 mg BSA/L. The wine treated at the end of fermentation (stable) was kept until the bottling with its lees, that include the added bentonite, making a combined action of bentonite and mannoproteins, decreasing the protein content to 29.6 ± 0.2 mg BSA/L. These results are coherent with studies carried out in previous years (see 3.1.2), where ageing with lees diminished the protein concentration and improves even more the stability (Vincenzi et al., 2011). These results together with earlier results in Macabeo 2010 (see 3.1.1) indicated that as later in the alcoholic fermentation the bentonite dosage is done, more efficient is the protein removal and better the protein stability. Finally highlight that the low levels of protein removed in wines with the stabilizing treatment is due to the usual low protein content of this grape variety, as pointed by Salazar et al., (2006), which, however, does not mean that these wines are stable and that the bentonite treatment could be skipped.

Protein profile evolution

The analysis of the protein profile for each sample obtained for each treatment from the initial must up to the stable wines is shown in Table 3.9.

Table 3.9. Protein profile of musts and wines treated with bentonite at different vinification stages, by FPLC.

Dosing Time	Sample	F1 Protein Content (mg BSA/L)	F2 Protein Content (mg BSA/L)	F3 Protein Content (mg BSA/L)
Must	Free run juice	0.0 ± 0.0	25.0 ± 3.2	60.8 ± 0.5
	Clarified must (w/bentonite)	0.0 ± 0.0	3.6 ± 0.2	17.3 ± 0.6
	Dry Wine	0.9 ± 0.0	0.5 ± 0.0	19.2 ± 0.9
	Stable Wine	0.4 ± 0.1	0.7 ± 0.2	10.4 ± 0.5
Start of fermentation	Clarified must (w/enzymes)	0.0 ± 0.0	4.7 ± 0.5	19.4 ± 0.7
	Pre-treatment	0.0 ± 0.0	9.4 ± 0.7	21.2 ± 2.1
	24 hours Post-treatment	0.3 ± 0.1	7.2 ± 0.3	18.5 ± 0.8
	Dry Wine	0.9 ± 0.0	17.1 ± 0.6	12.7 ± 0.0
	Stable Wine	0.4 ± 0.1	8.6 ± 0.2	5.0 ± 0.4
Middle of fermentation	Clarified must (w/enzymes)	0.0 ± 0.0	4.8 ± 0.1	17.0 ± 0.2
	Pre-treatment	0.4 ± 0.0	5.9 ± 0.5	23.0 ± 2.2
	24 hours Post-treatment	0.4 ± 0.0	4.6 ± 0.3	19.0 ± 1.5
	Dry Wine	0.0 ± 0.0	0.5 ± 0.0	19.4 ± 0.2
	Stable Wine	0.0 ± 0.0	0.8 ± 0.4	11.7 ± 1.6
End of fermentation	Clarified must (w/enzymes)	0.0 ± 0.0	4.5 ± 0.2	16.7 ± 0.1
	Pre-treatment	0.4 ± 0.0	6.9 ± 0.0	32.9 ± 2.4
	24 hours Post-treatment	0.5 ± 0.0	6.0 ± 0.4	29.9 ± 0.1
	Dry Wine	0.2 ± 0.0	0.8 ± 0.1	25.6 ± 0.3
	Stable Wine	1.5 ± 0.3	0.8 ± 0.1	17.1 ± 1.8
Control	Clarified must (w/enzymes)	0.0 ± 0.0	5.2 ±0.1	18.1 ± 1.3
	Dry Wine	0.5 ± 0.0	0.4 ± 0.0	23.1 ± 2.0
	Stable Wine	2.2 ± 0.1	0.9 ± 0.1	10.4 ± 0.2

It is possible to distinguish the three fractions usually associated with the protein profile of grape musts and wines. Intensity of signals change along the vinification process and depending on the treatment received in each case.

Fractions F1, F2 and F3, which correspond to a molecular weight of >100, 60 – 40 and 20 – 30 kDa, respectively (Canals et al., 1998), are associated to invertases, β-glucanases, chitinases and TLP (Sauvage et al., 2010).

Concentration of fraction F1 is small at the beginning, with a tendency to appear during fermentation (Dambrouck et al., 2005), but with the bentonite treatment it decrease slightly, except for wines treated at the end of fermentation and control, where its concentration increase after the stabilization.

F2 shows high concentration levels (around 25 mg BSA/L) in the free run juice, but after the clarification its concentration decrease to values between 3 – 5 mg/L. This fraction presents a tendency to increase in the first part of the fermentation, decrease later and keep on decreasing in a more clear way when bentonite is added and it stay acting in the medium.

F3 appears in higher concentrations, modifies more with the bentonite treatments and seems to be related with a major role in the protein stability of these wines. In the free run juice its concentration is around 60 mg BSA/L decreasing notoriously to less than 20 mg BSA/L with the clarifying treatment, either with bentonite or enzymes. Once the alcoholic fermentation is running its concentration increase, as it can be appreciated in the pre-treatment samples and in the dry wine sample of the control wine. This is in agreement with the observation of Vincenzi *et al. (*2011) in which the fraction corresponding to the chitinases and TLP increases during fermentation and stabilizes at the end of

fermentation. When applying bentonite in any of the stages this fraction decreases similarly to the other fractions. The diminution of this fraction between dry wines and stable wines is high in comparison with the other two fractions (see Table 3.9). This is coherent with results obtained by Sauvage *et al.* (2010) considering this fraction between 20 – 30 kDa as the one with a closer relationship with protein stability in white wine.

Sensorial evaluation

In the preference test, the panel decided that there were differences between the five wines at nose but not at palate. However, in a comparative analysis between pairs of wines, it could be appreciated that the control wine was the one most preferred among tasters, followed by the wine treated at the end of fermentation and the wine obtained from the must clarified with bentonite. At nose the wine that obtained the worst preference was the one treated at middle fermentation.

In the mouth the only wine which differed from the rest was that treated at the beginning of fermentation being the less preferred by the tasters. While for the other wines there are no differences, with no clear preference among wines.

From this study it can be concluded that the later the stabilizing treatment with bentonite is, the better is the sensory evaluation. Although the grape juice clarified with bentonite received a dose at the beginning of the process, the bentonite stabilizing treatment is not performed until the wine end the fermentation. Wines treated at end of fermentation, clarified with bentonite and control are preferred by the panel.

3.2 HIGH UNSTABLE WINES: SAUVIGNON BLANC, PINOT GRIS AND ALBARIÑO VINTAGE 2012.

In these assays the work was done with the grape varieties Pinot Gris, Sauvignon Blanc and Albariño during the 2012 vintage. These varieties are usually used for white wine winemaking and in general have high protein instability problems, needing often a high bentonite dose to reach the protein stability.

3.2.1 Pinot Gris, Argentina 2012

Alcoholic fermentation

Alcoholic fermentation was carried out following the traditional procedure of Piedra Negra cellar (Mendoza, Argentina), except for treatment with bentonite (dose of 30 g/HL).

In Figure 3.5 are shown the density and temperature curves for each Pinot Gris tank. Fermentation began with the grape must at low temperatures (6 - 8 ° C), because after the clarification of the grape juice, this value was not adjusted to the fermentation temperature (15 ºC). Fermentations followed a similar tendency in the different tanks lasting for 23 days.

As shown on the fermentation evolution graphic no significant differences were appreciated in density among the different treatments and control tanks. In addition there were no important differences in the vinification or in the final result of none of the tanks.

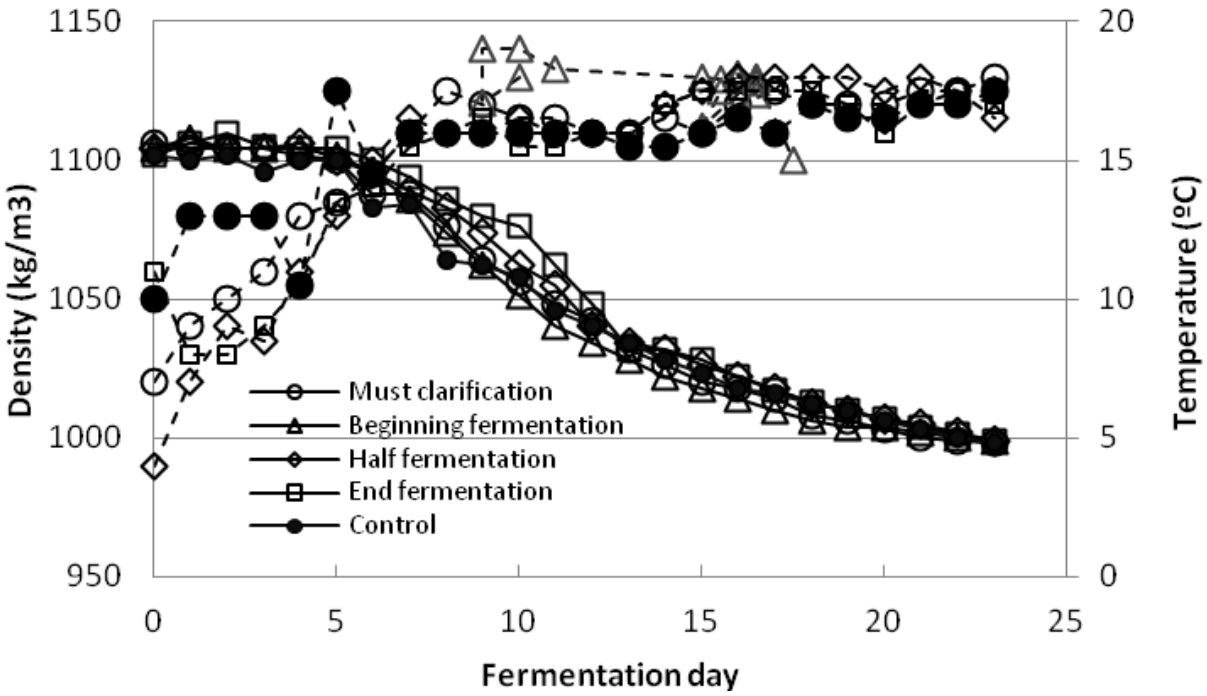

Figure 3.5. Evolution of density and temperature during alcoholic fermentation for four different treatments with 30 g/hL of bentonite and a control.

Chemical analyzes of initial musts and dry wines after alcoholic fermentation are shown in Tables 3.10 and 3.11, respectively.

The final wines chemical properties were similar for all of them, even though some differences could be appreciate in some cases due to sampling in large fermentation volume tanks and/or variability in the initial must, which has a common grape origin but was harvested during different days making their ripeness and sanitary conditions subjected to variability.

Protein content evolution and stability

The protein content for each of the samples obtained throughout the fermentation of the different treatment tanks of Pinot Gris are shown in Table 3.12. In first place it can be observed that in the must clarification treatment with bentonite, the total protein decrease goes from 79.9 to 33.9 mg/L. This important decrease is due to the effect of bentonite and activated carbon added for discoloration of must prior to vinification. Salazar et al. (2007) observed a synergistic effect of treatment with bentonite and activated carbon on filterability of wines as a consequence of a major protein removal associated with the reduction of polyphenols by activated carbon. If we compare with the other treatments (in which the protein content of the free run juice was not determined) in which the musts were clarified by the traditional procedure of the winery (enzymes and discoloration) a slightly higher total protein content is obtained, except for the control, being anyway the values after clarification in the same range.

Table 3.10. Chemical analysis of Pinot Gris must used at industrial scale.

Must (dosing time)	Density (kg/m3)	Sugar content (g/L)	Potential alcoholic degree (% v/v)	Titrable Acidity (g tartaric acid/L)	pH
Must	1106 ± 2 a	249,0 ± 4,7 b	14,8 ± 0,1 a	3,86 ± 0,27 a	3,47 ± 0,16 bc
Start of fermentation	1104 ± 1 a	256,8 ± 4,8 ab	14,9 ± 0,4 a	3,94 ± 0,27 a	3,38 ± 0,15 c
Middle of fermentation	1104 ± 2 a	259,4 ± 4,9 a	15,0 ± 0,5 a	3,93 ± 0,27 a	3,43 ± 0,15 bc
End of fermentation	1102 ± 2 a	251,5 ± 4,7 ab	14,6 ± 0,5 a	3,07 ± 0,21 b	3,80 ± 0,17 a
Control	1102 ± 2 a	249,0 ± 4,7 b	14,5 ± 0,3 a	3,22 ± 0,22 b	3,74 ± 0,17 ab

Average ± Standard deviation

Different letters indicate a significant difference ($P \leq 0.05$) for different wine treatment.

Table 3.11. Chemical analysis of Pinot Gris wines obtained after bentonite treatment at industrial scale.

Wine (dosing time)	Alcohol content (% v/v)	Volatile Acidity (g acetic acid/L)	Titrable Acidity (g/L tartaric acid)	pH	Residual Sugar (g/L)
Must	13,6 ± 0,1 a	0,39 ± 0,02 a	5,88 ± 0,38 bc	3,21 ± 0,05 b	0,99 ± 0,00 c
Start of fermentation	13,4 ± 0,1 ab	0,31 ± 0,03 b	6,73 ± 0,21 a	3,11 ± 0,08 b	1,76 ± 0,13 b
Middle of fermentation	12,9 ± 0,2 c	0,32 ± 0,02 b	6,65 ± 0,23 a	3,15 ± 0,07 b	2,21 ± 0,21 a
End of fermentation	13,2 ± 0,2 bc	0,32 ± 0,02 b	6,34 ± 0,30 ab	3,34 ± 0,02 a	0,91 ± 0,36 c
Control	13,2± 0,2 bc	0,37 ± 0,01 a	5,57 ± 0,43 c	3,35 ± 0,02 a	0,91 ± 0,36 c

Average ± Standard deviation

Different letters indicate a significant difference ($P \leq 0.05$) for different wine treatment.

In this study the resulting wines, including the control, were all stable at the end of alcoholic fermentation, not being necessary to determine a further bentonite stabilizing dose.

Table 3.12. Protein content by Bradford method, stability, bentonite stabilizing and total dose, for Pinot Gris wines.

Dosing Time	Sample	Protein Content (mg BSA/L)	Stability	Bentonite Stabilizing Dose (g/hL)	Bentonite Total Treatment (g/hL)
Must	Free run juice	79,9 ± 0,6	Stable	-	30
	Clarified must (w/bentonite)	33,9 ± 0,2			
	Dry Wine	26,1 ± 0,5			
Start of fermentation	Clarified must (w/enzymes)	36,7 ± 0,1	Stable	-	30
	Pre-treatment	26,6 ± 0,0			
	24 hours Post-treatment	25,8 ± 0,1			
	Dry Wine	19,4 ± 0,1			
Middle of fermentation	Clarified must (w/enzymes)	42,4 ± 0,0	Stable	-	30
	Pre-treatment	31,0 ± 0,2			
	24 hours Post-treatment	26,9 ± 0,2			
	Dry Wine	18,8 ± 0,0			
End of fermentation	Clarified must (w/enzymes)	36,8 ± 0,0	Stable	-	30
	Pre-treatment	23,7 ± 0,1			
	24 hours Post-treatment	22,7 ± 0,1			
	Dry Wine	21,9 ± 1,0			
Control	Clarified must (w/enzymes)	31,8 ± 0,1	Stable	-	-
	Dry Wine	19,1 ± 0,0			

As the four wines obtained with the different treatments with bentonite were stable, we can assume that the 30 g/hL bentonite dose was too high, but so did the control wine as well without any bentonite addition. We can conclude then that with the enzymatic treatment together with the discoloration the wines were able to reach stability.

Protein content and haze formation risk are characteristics of each variety but also depend on the conditions of the production years and of the techniques used to cultivate the vineyard as well as those performed before, during and after the fermentation. Looking to results of this particular year, it might be suggested to avoid any bentonite treatment so that the aromatic profile won't be affected in final fines or, if preferred use a preventive dose that moves between 5 to 10 g/hL. This particular behaviour could be associated to the mannoprotein generation (Moine-Ledoux & Dubourdieu, 1998; Waters et al., 1993; Waters et al., 1994; Dupin et al., 2000), due to the presence of barks of yeast in the nutrients added during fermentation or to the contact with lees (Pérez-Serradilla & Luque de Castro, 2008).

Protein profile evolution

Protein profile analyze for each sample obtained throughout the vinification of each treatment are shown in Table 3.13.

Table 3.13. Protein profile of musts and wines treated with bentonite at different vinification stages, by FLPC.

Dosing Time	Sample	F1 Protein Content (mg BSA/L)	F2 Protein Content (mg BSA/L)	F3 Protein Content (mg BSA/L)
Must	Free run juice	0.2 ± 0.1	32.1 ± 3.1	65.5 ± 4.5
	Clarified must (w/bentonite)	0.0 ± 0.0	0.8 ± 0.1	16.1 ± 0.3
	Dry Wine	1.5 ± 0.1	0.6 ± 0.0	18.0 ± 0.2
Start of fermentation	Clarified must (w/enzymes)	0.0 ± 0.0	0.5 ± 0.1	10.5 ± 0.1
	Pre-treatment	0.0 ± 0.0	1.6 ± 0.0	22.6 ± 1.0
	24 hours Post-treatment	0.0 ± 0.0	1.6 ± 0.2	21.2 ± 1.6
	Dry Wine	0.4 ± 0.0	0.1 ± 0.0	7.8 ± 0.1
Middle of fermentation	Clarified must (w/enzymes)	0.0 ± 0.0	0.7 ± 0.1	11.8 ± 0.7
	Pre-treatment	0.0 ± 0.0	1.4 ± 0.1	19.9 ± 1.1
	24 hours Post-treatment	0.0 ± 0.0	1.2 ± 0.1	20.8 ± 0.2
	Dry Wine	0.0 ± 0.0	0.0 ± 0.0	5.6 ± 0.4
End of fermentation	Clarified must (w/enzymes)	0.0 ± 0.0	1.1 ± 0.0	15.0 ± 0.6
	Pre-treatment	0.0 ± 0.0	0.5 ± 0.0	16.7 ± 0.2
	24 hours Post-treatment	0.0 ± 0.0	0.7 ± 0.0	18.0 ± 0.1
	Dry Wine	0.0 ± 0.0	0.0 ± 0.0	6.5 ± 0.2
Control	Clarified must (w/enzymes)	0.1 ± 0.0	1.4 ± 0.0	15.9 ± 0.2
	Dry Wine	1.0 ± 0.1	0.7 ± 0.2	17.4 ± 0.3

Macromolecular profile analysis of dry wines show that 20 – 30 kDa protein fraction (F3, more unstable), was lower in wines treated with bentonite during fermentation (range of 5 – 8 mg/L), meanwhile control wine and the one obtained from clarifying the must with bentonite had levels of this fraction around 17 – 18 mg/L. The same tendency is observed for the other two fractions. The bentonite treatment in this study was not necessary, because the winery protocol, treating musts with activated carbon, has been enough in this vintage to stabilize wines. However it can be observed the tendency that treatments with bentonite at middle and end of fermentation are more effective to eliminate unstable proteins (20 – 30 kDa). In spite of the final result and looking in detail the effect of adding bentonite during the fermentation, it can be appreciated that adding bentonite at the beginning of the fermentation the effect is not significant during the following 24 hours, but keeping bentonite in suspension (with a higher contact time) in the fermenting must, the protein levels decreased even more, till value of 7.8 ± 0.1 mg BSA/L at the end of alcoholic fermentation. Adding the bentonite at half and end of fermentation, the decrease of F3 fraction is higher at the end of the process.

The other two fractions are in small concentration during the whole fermentation, but in the free run juice fraction F2 has a high concentration (32.1 ± 3.1 mg BSA/L) that reduces drastically with the clarifying treatment. Once again this could be attributed to the effect of the activated carbon over the protein content, because in both bentonite and enzyme clarification, the content is lower than the free run juice, with values between 0.5 – 1.4 mg BSA/L. -In wines treated at beginning and middle of fermentation the content of this fraction rise again, but not up to the original levels, staying stable after 24 hours of bentonite treatment. However, keeping the bentonite in the tank during the fermentation makes this fraction to disappear almost completely in the dry

wines. In the wine treated at the end of fermentation this fraction started to decrease during fermentation, diminished a bit more with the bentonite treatment and disappeared in the dry wine. Only in control wine and in the wine obtained from the must clarified with bentonite there is a small amount of this fraction, between 0.6 – 0.7 mg BSA/L.

Fraction F1 is in very small quantities in the free run juice and the initial musts, even for some samples it is not detected. However, it appears during fermentation. In control wine it reaches 1 mg BSA/L, in the wine obtained from the must clarified with bentonite there is 1.5 mg BSA/L, being the highest concentration. The wine treated at beginning of fermentation has in the dry wine 0.4 mg BSA/L. Meanwhile in the other two wines it is not detected. From the above, it can be intuit that it is a very sensitive fraction to bentonite.

3.2.2 Sauvignon Blanc, Chile 2012.

The study was carried out during vintage 2012 in Misiones de Rengo winery (Cachapoal Valley, Chile) with Sauvignon Blanc grapes that came from Maule Valley. Vinification process was done following the usual procedure of the winery, except for the bentonite treatment. The bentonite dose used for each of the four treatments was 10 g/hL. The rest of additions, corrections and practices were decided by the technical staff following their criteria and informed to keep the record in case it influences the final result.

Tables 3.14 and 3.15 show the results of initial must and final wines chemical analyzes, respectively. Variability in each case is associated with the characteristics of the samples and the analytical methods, but they don't represent a problem when evaluating the results. Even though each tank has a same grape origin, the harvest was done through several days influencing their ripeness and sanitary level, fact that is reflected in some of these values.

Behaviour throughout the alcoholic fermentation followed the usual dynamic for these kind of wines, with no effect of the different treatments over them, what allows us to say that the use of bentonite at different stages of winemaking do not generate important differences in its development. These agree with our prior results obtained at industrial and pilot scale and also with literature available about differences on the fermentation behaviour due to different scales (Casalta et al., 2010). There is a correlation between values obtained in the original must, the ones expected in wine and those finally obtained in finished wines, with no significant difference.

Table 3.14. Chemical analysis of grape must used at industrial scale of Sauvignon Blanc grape variety.

Dosing Time	Density (kg/m3)	Degree ºBrix	Potential alcoholic degree (% v/v)	Titrable Acidity (g tartaric acid/L)	pH
Must	1089 ± 2 c	21,5 ± 0,5 b	12,9 ± 0,6 b	5,10 ± 0,38 a	3,18 ± 0,05 b
Start of fermentation	1106 ± 1 a	25,4 ± 0,6 a	15,5 ± 0,7 a	4,30 ± 0,32 b	3,31 ± 0,05 a
Middle of fermentation	1094 ± 2 b	22,6 ± 0,6 b	13,6 ± 0,6 b	4,47 ± 0,18 b	3,26 ± 0,04 a
End of fermentation	1091 ± 2 bc	21,9 ± 0,2 b	13,2 ± 0,6 b	4,99 ± 0,06 a	3,24 ± 0,06 ab
Control	1089 ± 2 c	21,5 ± 1,1 b	12,9 ± 0,7 b	4,50 ± 0,85 ab	3,35 ± 0,23 ab

Average ± Standard deviation

Different letters indicate a significant difference ($P \leq 0.05$) for different wine treatment.

Table 3.15. Chemical analysis of Sauvignon Blanc wines obtained after bentonite treatment at industrial scale.

Dosing Time	Alcohol content (% v/v)	Volatile Acidity (g acetic acid/L)	Titrable Acidity (g tartaric acid/L)	pH	Residual Sugar (g/L)
Must	12,9 ± 0,4 b	0,19 ± 0,02 d	5,67 ± 0,30 b	3,43 ± 0,08 ab	0,48 ± 0,10 b
Start of fermentation	13,7 ± 0,3 a	0,39 ± 0,01 a	6,17 ± 0,32 ab	3,40 ± 0,09 b	1,01 ± 0,09 a
Middle of fermentation	13,5 ± 0,3 ab	0,37 ± 0,03 ab	5,26 ± 0,29 c	3,59 ± 0,09 a	0,55 ± 0,06 b
End of fermentation	13,2 ± 0,3 ab	0,25 ± 0,01 c	6,31 ± 0,29 a	3,37 ± 0,12 b	1,01 ± 0,37 a
Control	13,0 ± 0,3 b	0,36 ± 0,01 b	6,45 ± 0,51 ab	3,44 ± 0,08 ab	0,86 ± 0,29 ab

Average ± Standard deviation

Different letters indicate a significant difference ($P \leq 0.05$) for different wine treatment.

Protein content evolution and stability

To every sample taken during this study the protein content was measured. Once finished the alcoholic fermentation the stability was determined for each tank, and for those unstable the stabilizing bentonite dose was also obtained.

The protein content for each sample is shown in Table 3.16 together with dry wines stability, stabilizing bentonite dose in case it was necessary and the final total bentonite dose received by each wine. Initial protein content in must is a characteristic of each variety, together with the characteristics of the vintage and the vineyard management. The content of proteins in each sample shows a picture of the moment it was obtained due to the balanced between hydrolysis and synthesis of proteins during the alcoholic fermentation (Vincenzi et al., 2011), so even though the content could be similar between the must and the wine, is the protein profile the one that changes during the vinification process.

The three wines treated during fermentation were stable at the end of it, meanwhile the must clarified with bentonite and the control wine were unstable needing an extra dose of bentonite to reach stability.

Another interesting aspect is that wine obtained from clarifying the must with bentonite required a larger dose of bentonite to reach stability than the control wine. This is coherent to other results obtained by us at pilot scale study with Albariño wines and could be because bentonite only removed proteins available in the must but not related with stability and left precursors of the unstable proteins formed later (Pocock et al., 2011).

Table 3.16. Protein content, stability, bentonite stabilizing dose and bentonite total dose, of Sauvignon Blanc wines.

Dosing Time	Sample	Protein Content (mg BSA/L)	Stability	Bentonite Stabilizing Dose (g/hL)	Bentonite Total Treatment (g/hL)
Must	Free run juice	77,2 ± 0,1	Unstable	74	84
	Clarified must (w/bentonite)	69,5 ± 0,1			
	Dry Wine	66,6 ± 0,6			
Start of fermentation	Clarified must (w/enzymes)	77,5 ± 0,1	Stable	-	10
	Pre-treatment	66,9 ± 0,1			
	24 hours Post-treatment	57,8 ± 0,3			
	Dry Wine	52,0 ± 1,1			
Middle of fermentation	Clarified must (w/enzymes)	72,4 ± 0,0	Stable	-	10
	Pre-treatment	70,0 ± 0,2			
	24 hours Post-treatment	68,6 ± 0,2			
	Dry Wine	40,8 ± 0,3			
End of fermentation	Clarified must (w/enzymes)	87,5 ± 0,1	Stable	-	10
	Pre-treatment	70,0 ± 0,1			
	24 hours Post-treatment	41,9 ± 0,2			
	Dry Wine	38,8 ± 0,0			
Control	Clarified must (w/enzymes)	73,3 ± 0,1	Unstable	47	47
	Dry Wine	68,7 ± 0,2			

Between the wines treated during fermentation there are some differences that allow distinguishing among them. In the wine treated at the beginning of fermentation after few days when it just had dropped 10 points from the initial density we can appreciate a lower protein content (66.9 ± 0.1 mgBSA/L) due to the hydrolysis of some of the proteins by the yeast action but the generation of new proteins by the same yeast had still not reached an important level to balance it (Hsu et al., 1987). Twenty four hours after adding bentonite there is an important decrease on the protein content, and later in the dry wine there is a further decrease. In this case, bentonite has more time to react with proteins but at the same time settled at the bottom of the tank, not necessarily removing proteins during the whole fermentative process, letting that proteins synthesized during the vinification are not removed from wine.

In wine treated at middle fermentation the addition of bentonite was done when protein content had not decreased significantly, which allows us to assume that balance between hydrolysis and synthesis was reached. One day after adding bentonite proteins decreased but not in a large amount due to the fact that the addition was done in a moment with great activity the removal of proteins by the bentonite was more difficult. However, with a longer time of contact up to the end of fermentation, there was a notorious decrease due to the maximization of the contact time and surface.

By other hand the wine treated at the end of fermentation show values with more significant variations allowing demonstrating better the effect of bentonite treatment over wines. In this case the total amount had already decreased comparing to the initial content, but anyway with a quieter medium the activity of the bentonite addition was more efficient removing around 40 % of proteins in the first 24 hours of treatment, and continuing till the end of the fermentation. There was no problem on the fermentation dynamic even though there could be a risk of taking yeasts to the bottom while bentonite is settling, but none of these happened finishing together with the other treated wines and the control. This is also observed in studies done with other varieties and in both industrial and pilot scale within our research. A large amount of proteins is removed being an efficient treatment, but it has to be adjusting to the objective of the wine.

Protein profile evolution

The protein profile for initial must, samples obtained during fermentation and final wines are shown in Table 3.17.

Table 3.17. Protein profile of musts and wines treated with bentonite at different vinification stages, by FLPC.

Dosing Time	Sample	F1 Protein Content (mg BSA/L)	F2 Protein Content (mg BSA/L)	F3 Protein Content (mg BSA/L)
Must	Free run juice	0.2 ± 0.0	9.5 ± 0.1	21.4 ± 0.3
	Clarified must (w/bentonite)	0.0 ± 0.0	0.2 ± 0.1	13.9 ± 0.4
	Dry Wine	0.6 ± 0.3	3.5 ± 0.1	86.4 ± 0.3
Start of fermentation	Clarified must (w/enzymes)	0.2 ± 0.0	10.0 ± 0.7	29.1 ± 0.9
	Pre-treatment	0.4 ± 0.0	6.5 ± 0.2	31.4 ± 1.1
	24 hours Post-treatment	0.0 ± 0.0	5.3 ± 0.1	18.9 ±0.2
	Dry Wine	0.8 ± 0.0	3.2 ± 0.0	25.4 ± 1.5
Middle of fermentation	Clarified must (w/enzymes)	0.0 ± 0.0	9.5 ± 0.0	22.8 ± 4.5
	Pre-treatment	0.4 ± 0.0	10.2 ± 0.4	30.6 ± 0.4
	24 hours Post-treatment	0.0 ± 0.0	1.4 ± 0.3	19.6 ± 1.5
	Dry Wine	0.5 ± 0.1	3.0 ± 0.2	22.2 ± 2.6
End of fermentation	Clarified must (w/enzymes)	0.1 ± 0.0	10.4 ± 0.9	23.6 ± 1.1
	Pre-treatment	0.0 ± 0.0	5.2 ± 0.2	24.0 ± 0.1
	24 hours Post-treatment	0.0 ± 0.0	2.8 ± 0.0	18.2 ± 1.6
	Dry Wine	0.4 ± 0.1	1.6 ± 0.0	18.1 ± 1.0
Control	Clarified must (w/enzymes)	0.2 ± 0.1	13.0 ± 0.7	27.6 ± 1.3
	Dry Wine	0.6 ± 0.0	2.4 ± 0.3	54.1 ± 7.0

The three fraction associated with the protein profile of White wines are distinguished, being the fraction F1 the one in lowest concentration and with no important changes during the evolution of the fermentation. Concentration of fraction F2 is similar in all dry wines, being lower for the one treated with bentonite at the end of fermentation, indicating that this fraction has practically no effect on the protein stability of these wines.

For wines treated during fermentation, the concentration of fraction F3 (20 – 30 kDa, chitinases and thaumatin-like proteins), usually associated with protein instability (Waters et al., 1992), diminished more as later is the treatment with bentonite. However these wines were protein stable with the 10 g/hL bentonite dose added. In the wine obtained from the must clarified with bentonite and in the control wine , both unstable, the final concentration of F3 fraction was 86.4 ± 0.3 and 54.1 ± 7.0 mg BSA/L, respectively. This higher concentration of the F3 fraction is related with the higher need of bentonite for the protein stabilization of these wines (84 and 47 g/hL, respectively).

Wines treated during fermentation show in general a lower concentration of all three fractions, which is coherent with the estimated content by the Bradford method, especially for the fraction between 20 – 30 kDa (F3).

The behaviour of the different fractions associated with protein haze in white wines give an idea of the particular characteristics of the Sauvignon Blanc grape variety, and distinguished between protein content and type of proteins. High protein contents do not necessarily mean a high risk of protein haze (Bayly & Berg, 1967). Even though these fractions are the same in every wine, their different proportion gives a special feature to each wine.

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Alcoholic fermentation

Characteristics of the initial must used for all the experiments are detailed in Table 3.18.

Table 3.18. Chemical analysis of grape must used at pilot scale of Albariño grape variety.

Density (kg/m3)	Potential alcoholic degree (% v/v)	Titrable Acidity (g tartaric acid/L)	pH
1.101 ± 0.011	14.08 ± 0.14	8.63 ± 0.09	3.30 ± 0.03

Figure 3.6 shows the evolution of the density along the fermentation as well as the temperature during the process.

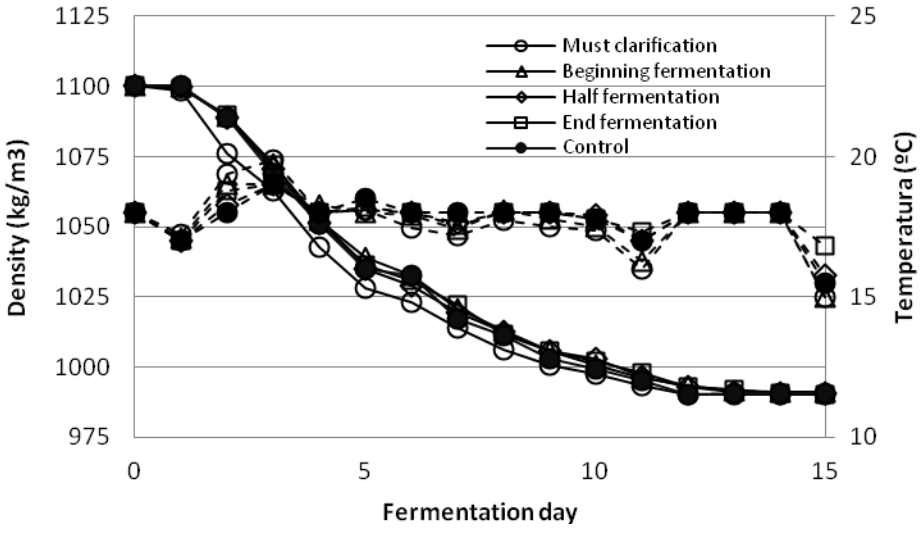

Figure 3.6.- Evolution of must density and fermentation temperature during alcoholic fermentation of Albariño wines with 30 g/hL of bentonite added at different stages of the winemaking process.

Alcoholic fermentation followed a similar tendency for each tank, except for the must clarified with bentonite that had a faster evolution in the first 2 – 3 days. This was appreciated by Casalta et al., (2010) observing a slower fermentation in must highly clarified, similar to Ferrando et al., (1998) that point the importance of solids in suspension on the acceleration at the beginning of the alcoholic fermentation at industrial scale. Fermentations last for 14 ± 2 days within a temperature range of 17 ± 2 ºC.

In Table 3.19 are shown the main analytical parameters for each wine once stabilized and bottled. Differences in analytical values are due to sampling moment and the analytical methods used for each case but do not represent a significant difference among final wines.

Table 3.19. Chemical analysis of white wines obtained after bentonite treatment at pilot scale of Albariño grape variety.

Wine (dosing time)	Alcohol content (% v/v)	Volatile Acidity (g acetic acid/L)	Titrable Acidity (g tartaric acid/L)	pH	Residual Sugar (g/L)
Must	14,01 ± 0,28 a	0,29 ± 0,01 a	6,82 ± 0,14 c	3,20 ± 0,03 a	3,15 ± 0,03 c
Start of fermentation	14,22 ± 0,28 a	0,31 ± 0,02 a	7,27 ± 0,15 a	3,11 ± 0,03 b	3,72 ± 0,04 a
Middle of fermentation	13,23 ± 0,26 b	0,31 ± 0,02 a	6,92 ± 0,14 bc	3,11 ± 0,03 b	3,74 ± 0,04 a
End of fermentation	13,93 ± 0,28 a	0,29 ± 0,01 a	7,19 ± 0,14 ab	3,10 ± 0,03 b	3,39 ± 0,03 b
Control	14,03 ± 0,28 a	0,29 ± 0,01 a	7,34 ± 0,15 a	3,09 ± 0,03 b	3,49 ± 0,03 b

Average ± Standard deviation

Different letters indicate a significant difference ($P \leq 0.05$) for different wine treatment.

Protein content evolution and stability

The protein content of the samples obtained from the initial must throughout the fermentation until the final wines is presented in Table 3.20 for each wine treatment and the control.

From the five wines elaborated three were unstable and two stable at the end of the alcoholic fermentation (dry wine). Control wine was unstable together with the wines obtained from clarifying the must with bentonite and treated at the beginning of fermentation. Meanwhile those treated at middle and end of fermentation were stable with the 40 g/hL dose applied.

When estimating the bentonite stabilizing dose for unstable wines, the one treated a beginning of fermentation just need an extra dose of 10 g/hL and the wine obtained from the must clarified with bentonite required an additional dose of 40 g/hL, with a total of 80 g/hL in the whole process. This means that this wine in total needed a higher dose of bentonite than the control wine, that reach stability with a unique dose of 70 g/hL. Tendency observed is that control wine and the wine obtained from clarifying the must with bentonite required a similar total dose of bentonite for their protein stability, meanwhile, wines treated during fermentation need a lower total bentonite dose as later is the treatment.

Table 3.20. Protein content, stability, bentonite stabilizing dose and bentonite total dose, for Albariño wines.

Dosing Time	Sample	Protein content (mg BSA/L)	Stability	Stabilizing Bentonite Dose (g/hL)	Total Bentonite Treatment (g/hL)
Must	Free run juice	40.8 ± 0.6	Unstable	40	80
	Clarified must (w/bentonite)	35.1 ± 0.4			
	Dry Wine	32.7 ± 0.1			
	Stable Wine	28.5 ± 0.6			
Start of fermentation	Clarified must (w/enzymes)	39.6 ± 0.5	Unstable	10	50
	Pre-treatment	38.7 ± 0.6			
	24 hours post-treatment	30.1 ± 1.0			
	Dry Wine	31.5 ± 0.2			
	Stable Wine	26.4 ± 0.2			
Middle of fermentation	Clarified must (w/enzymes)	39.6 ± 0.5	Stable	-	40
	Pre-treatment	38.8 ± 0.5			
	24 hours post-treatment	26.2 ± 0.4			
	Dry Wine	29.5 ± 0.1			
	Stable Wine	26.9 ± 0.3			
End of fermentation	Clarified must (w/enzymes)	39.6 ± 0.5	Stable	-	40
	Pre-treatment	36.9 ± 0.4			
	24 hours post-treatment	28.0 ± 0.2			
	Dry Wine	31.0 ± 0.2			
	Stable Wine	26.5 ± 0.3			
Control	Clarified must (w/enzymes)	39.6 ± 0.5	Unstable	70	70
	Dry Wine	38.7 ± 0.4			
	Stable Wine	26.9 ± 0.4			

Protein profile evolution

Protein profile of initial must, fermenting samples and final wines are shown in Table 3.21.

It is possible to distinguish the three mayor fractions in the different fractions, being F1 and F2 the ones with a less concentration. Fraction F1 shows a high sensibility to bentonite treatment, as it can be appreciated that independently on the dosage time, its diminution is almost complete and immediate.

Concentration of fraction F2 in free run juice and initial musts clarified with enzymes is in a range of 5.4 – 5.8 mg BSA/L. In the must clarified with bentonite it decrease a 60 % keeping stable in that values till the end of the fermentation, with new decrease when the stabilizing treatment is applied. In wines treated at beginning and middle fermentation it is possible to appreciate an increase of the concentration, probably due to yeast action, up to values between 8 – 9 mg BSA/L (Sauvage et al., 2010). In the wine treated at the end of the fermentation there is a decrease comparing to the initial content, similar to the control dry wine. The fermentation had a longer action time, following the normal evolution with an increase of the protein content due to the yeast lyses and the later diminution as an effect of the hydrolysis (Vincenzi et al., 2005). Dosage of bentonite during fermentation makes the content of F2 fraction to decrease around 50 – 60 %, and by keeping the bentonite in the medium it continue decreasing as it can be noticed in dry wines.

Table 3.21. Protein profile of musts and wines treated with bentonite at different vinification stages, by FLPC.

Dosing Time	Sample	F1 Protein Content (mg BSA/L)	F2 Protein Content (mg BSA/L)	F3 Protein Content (mg BSA/L)
Must	Free run juice	0.5 ± 0.0	5.4 ± 0.4	23.3 ± 0.3
	Clarified must (w/bentonite)	0.0 ± 0.0	2.2 ± 0.3	14.2 ± 1.4
	Dry Wine	0.0 ± 0.0	1.9 ± 0.0	11.1 ± 0.3
	Stable Wine	0.0 ± 0.0	0.4 ± 0.0	3.0 ± 0.0
Start of fermentation	Clarified must (w/enzymes)	0.6 ± 0.1	5.8 ± 0.1	26.3 ± 0.4
	Pre-treatment	0.6 ± 0.1	8.0 ± 0.5	28.4 ± 2.9
	24 hours Post-treatment	0.6 ± 0.2	4.3 ± 0.4	19.7 ± 4.0
	Dry Wine	0.0 ± 0.0	1.0 ± 0.4	4.9 ± 0.9
	Stable Wine	0.0 ± 0.0	1.1 ± 0.2	4.5 ± 0.1
Middle of fermentation	Clarified must (w/enzymes)	0.6 ± 0.1	5.8 ± 0.1	26.3 ± 0.4
	Pre-treatment	1.0 ± 0.1	9.1 ± 0.2	32.8 ± 0.4
	24 hours Post-treatment	0.2 ± 0.1	4.2 ± 0.2	23.0 ± 0.1
	Dry Wine	0.0 ± 0.0	1.8 ± 0.0	6.1 ± 0.1
	Stable Wine	0.0 ± 0.0	0.7 ± 0.1	5.7 ± 0.0
End of fermentation	Clarified must (w/enzymes)	0.6 ± 0.1	5.8 ± 0.1	26.3 ± 0.4
	Pre-treatment	1.1 ± 0.1	4.8 ± 0.7	25.3 ± 0.4
	24 hours Post-treatment	0.1 ± 0.0	2.4 ± 0.0	15.1 ± 2.1
	Dry Wine	0.1 ± 0.0	1.4 ± 0.1	5.1 ± 0.6
	Stable Wine	0.0 ± 0.0	0.7 ± 0.1	4.8 ± 0.0
Control	Clarified must (w/enzymes)	0.6 ± 0.1	5.8 ± 0.1	26.3 ± 0.4
	Dry Wine	0.5 ± 0.1	2.1 ± 0.6	17.0 ± 1.5
	Stable Wine	0.0 ± 0.0	3.0 ± 0.8	5.7 ± 0.7

The major fraction is F3, with an initial content in the free run juice of 23.3 ± 0.3 mg BSA/L and in musts clarified with enzymes of 26.3 ± 0.4 mg BSA/L. In must clarified with bentonite its content decrease down to 14.2 ± 1.4 mg BSA/L 24 hours after applying the treatment. This must after the alcoholic fermentation has a content of F3 fraction of 11.1 ± 0.3 mg BSA/L, being unstable wines. Stabilizing treatment with bentonite reduce this concentration down to 3.0 ± 0.0 mg BSA/L.

Wines treated with bentonite during fermentation, show a diminution of F3 fraction between 30 – 40 % after 24 hours of treatment, similar to F2 fraction that decrease after the addition of bentonite around 50 %. In these wines the content of this fraction keep on decreasing till the end of fermentation to values in a range of 4 – 6 mg BSA/L, with protein stability of them, except for the wine treated at beginning of fermentation, that needed a small additional bentonite dose of 10 g/hL to reach it.

In control wine the content of F3 fraction in dry wine is 17.0 ± 1.5 mg BSA/L, decreasing down to 5.7 ± 0.7 mg BSA/L, after the bentonite stabilizing treatment.

Aroma fermentative compounds

The results of the volatile composition analysis of wines were presented in Table 3.22. In addition, in order to estimate the sensory importance of each compound and the differences between treatments, the odor activity values (OAV) were also calculated by using the odor threshold values from the bibliography (Etievant, 1991; Moyano et al., 2002; Aznar et al., 2003; Culleré et al., 2004; Escudero et al., 2004; Campo et al., 2006). The OAV were also

presented in Table 3.22 [in brackets] for the compounds with OAV>0.1 units of aroma (u.a.).

As can be seen in Table 3.22, the treatments with bentonite at different stages of the fermentation, in general, affected the production of volatile fermentative compounds. It is remarkable the effect of the treatments on the contents of esters and acids that probably affect notably the wine aroma. In addition to the possible loss of volatile compounds due to adsorption on bentonite, the effects on the production of fermentative compounds could be related with the variation of nitrogen composition and other nutrients of musts and wines (Waters et al., 2005; Ugliano & Henschke, 2009).

The global effect of these differences could be evaluated by the total OAV that presented higher values for control and wines treated during fermentation (ranging from 698 to 750 u.a.) than the wine treated before fermentation which values were much more lower (552 u.a.). Therefore, with this theoretical estimation, the global aroma impact of musts clarified wines could be lower than the other ones.

Regarding the different chemical families, in the case of esters (ethyl esters and acetates), most of them presented contents higher than their odor thresholds. Esters contents and their aroma values were, in general, lower in the wines subjected to must clarification, so these wines will show fruity notes less intense than the other ones (Etievant, 1991). For volatile organic acids, also most of them were present in contents higher than their odor thresholds. The variations by effect of treatments are reflected in a lower OAV of acids for treated wines (from 79.2 to 131 u.a.) than the control wine (170 u.a.). It is remarkable this effect for acids was more important as the clarification were made before.

Therefore, the sensory impact of acids will be much more important in wines subjected to must clarification than the treated ones at the end of fermentation, and probably that produces in the first one less freshness aroma (Etievant, 1991).

On the other hand, the alcohol group did not presented important variations of OAV ranging from 6.25 and 6.83 u.a. Among higher alcohols, only isoamyl alcohols presented OAV higher than 1 u.a. Moreover, the benzenic alcohol, β-phenyl ethanol, was also presented in levels higher than its odor threshold ranging between 1.95 and 2.34 u.a, contributing with floral nuances to wine aroma. In the case of C6 alcohols, 1-hexanol and cis-3-hexen-1-ol, they were present with aroma values between 1 and 0.1 u.a., although did not influence directly with herbaceous nuances, they could contribute by additive and/or synergistic effects to the wine aroma (Etievant, 1991).

Among carbonyl compounds, quantitatively the most important was acetaldehyde that were presented in all wines at levels higher than its odor threshold with a range of OAV from 38.5 to 73.6 u.a. so it will contribute to wine aroma with ‘bruised apple’ and ‘nutty’ nuances. Finally, γ-butyrolactone was present in all wines at levels slight higher than its odor threshold, so this compound will also contribute to wine aroma with sweet notes (Etievant, 1991).

In summary, the results obtained allows to conclude that the wines with more aroma intensity and quality will be the ones treated with bentonite in the middle or and the end of fermentation. These wines seem less affected by the clarification process that produces important variation on the production of fermentative compounds such as esters and acids, affecting notably the fruity and fresh aroma nuances.

Table 3.22. Fermentative aroma compounds

Compound (odour threshold (mg/L))	Must Clarification	Beginning of fermentation	Middle of fermentation	End of fermentation	Control
Propanol (500)	40.1 [0.08]	50.5 [0.101]	52.9 [0.106]	54.0 [0.108]	50.7 [0.101]
Isobutanol (40)	19.4 [0.485]	19.1 [0.478]	20.0 [0.501]	18.5 [0.462]	19.5 [0.488]
1-Butanol (150)	1.56 [<0.1 u.a.]	1.28 [<0.1 u.a.]	1.64 [<0.1 u.a.]	1.66 [<0.1 u.a.]	1.89 [<0.1 u.a.]
(2+3)-Methyl-1-butanol (65)	213 [3.28]	220 [3.39]	218 [3.36]	217 [3.34]	226 [3.47]
Hexanol (8)	2.47 [0.308]	1.02 [0.128]	2.42 [0.302]	1.84 [0.23]	1.72 [0.216]
t-3 hexen (1)	0.013 [<0.1 u.a.]	0.010 [<0.1 u.a.]	0.013 [<0.1 u.a.]	0,018 [<0.1 u.a.]	0.020 [<0.1 u.a.]
c-3 hexen (0,4)	0.064 [0.161]	0.082 [0.205]	0.089 [0.222]	0,121 [0.303]	0.154 [0.384]
Benzyl (200)	2.25 [<0.1 u.a.]	2.204 [<0.1 u.a.]	4.52 [<0.1 u.a.]	2.38 [<0.1 u.a.]	2.66 [<0.1 u.a.]
2 Phenil ethanol (14)	32.4 [2.31]	27.3 [1.95]	32.8 [2.34]	28.0 [2.00]	29.0 [2.07]
Ethyl ester C4C2 (0,02)	0.456 [22.8]	0.600 [30.0]	0.597 [29.8]	0.694 [34.7]	0.888 [44.4]
Ethyl ester C6C2 (0,014)	0.836 [59.7]	1.210 [86.3]	1.240 [88.4]	1.220 [87.5]	1.110 [78.9]
Ethyl ester C8C2 (0,005)	1.34 [268]	1.84 [368]	1.92 [384]	1.89 [378]	1.68 [336]
Ethyl ester C10C2 (0,2)	0.413 [2.06]	0.523 [2.62]	0.564 [2.82]	0.543 [2.72]	0.560 [2.80]
Ethyl lactate (154)	14.5 [0.094]	16.8 [0.109]	17.7 [0.115]	15.8 [0.103]	16.0 [0.104]
Acetate AiC5 (0,03)	1.01 [33.5	1.66 [55.5]	1.72 [57.4]	1.80 [60.0]	1.59 [52.9]
Acetate AC6 (1,5)	0.022 [<0.1 u.a.]	0.056 [<0.1 u.a.]	0.065 [<0.1 u.a.]	0.057 [<0.1 u.a.]	0.047 [<0.1 u.a.]
Ethyl acetate (12,3)	50.6 [4.12]	67.2 [5.47]	64.9 [5.28]	67.0 [5.44]	66.1 [5.37]
Acid iC4 (2,3)	1.33 [0.579]	1,14 [0.498]	1.29 [0.561]	1.68 [0.731]	1.04 [0.453]
Acid C4 (0,173)	0.026 [0.151]	0.020 [0.117]	0.080 [0.465]	0.165 [0.952]	0.384 [2.01]
Acid iC5 (0,033)	1.64 [49.7]	1.98 [59.9]	2.04 [61.8]	2.97 [90.1]	4.34 [131.6]
Acid C6 (0,42)	5.15 [12.3]	6.75 [16.1]	7.05 [16.8]	7.90 [18.8]	7.73 [18.4]
Acid C8 (0,5)	7.17 [14.3]	9.41 [18.8]	9.73 [19.5]	9.04 [18.1]	7.80 [15.6]
Acid C10 (1)	2.19 [2.19]	2.57 [2.57]	2.65 [2.65]	2.56 [2.56]	2.29 [2.29]
Ethanal (0,5)	36.8 [73.6]	22.2 [44.4]	22.2 [44.3]	20.9 [41.8]	19.2 [38.5]
gb-Lactone (35)	39.7 [1.13]	56.4 [1.61]	55.2 [1.58]	41.4 [1.47]	49.2 [1.41]
Acetoin (150)	8.17 [<0.1 u.a.]	4.15 [<0.1 u.a.]	6.46 [<0.1 u.a.]	3.90 [<0.1 u.a.]	4.29 [<0.1 u.a.]
OAV ALCOHOLS	6,63	6,25	6,83	6,45	6,73
OAV ETHYL ESTERS	353	487	505	503	462
OAV ACETATES	37,6	61	62,7	65,5	58,3
Ratio Ethyl/Acetate	9,39	7,98	8,05	7,68	7,92
OAV ACIDS	79,2	98,0	102	131	170
OAV CARBOBYL	74,73	46,01	45,88	43,27	39,91
TOTAL OAV	552	698	723	750	737

Concentration expressed in mg/L. Odour activity values indicate in brackets.

Sensory evaluation

The tasting panel evaluated the wines obtained from the four treatments and the control in nose and palate, ranking them according to their preferences. The result was that in nose there is a difference between them, meanwhile in palate there is not.

Doing a comparative nose analyze between pair of wines there is a clear preference for the wine treated at middle fermentation with significant difference with each wine except with the wine treated at the end of fermentation that was the one chosen in second place. The other three wines had similar values between them and according to the tasters there is no difference.

In palate the comparative analyze of the wines result in clear preference of the wine treated at the end of the fermentation over the other four wines, which between them, did not have significant differences for the panel.

From the above it is possible to conclude, for this study, that wines treated with bentonite during fermentation from the half onwards, are in general better evaluated and can be highlighted the wine treated at the end of the fermentation that both in nose and palate presents a high level of acceptance among tasters. These two wines were the ones that were stable at the end of the fermentation, just receiving a treatment 40 g/hL of bentonite during the fermentation and not needing any extra bentonite dose. These mean that they were the wines with a less aggressive stabilizing treatment.

3.3 RELATIONSHIP BETWEEN THE PROTEIN PROFILE OF DRY WHITE WINES AND PROTEIN STABILITY.

In this study the relationship between protein content and stability has been analyzed for two series of white wines. In the first series single variety Italian white Pinot Gris wines and in the second series different single variety Chilean white wines were studied

3.3.1 Study about Pinot Gris wines, vintage 2012

A study to determine the relationship between protein stability, protein content and bentonite stabilizing dose for fourteen Italian Pinot Gris wines of different origin obtained once finished alcoholic fermentation was done. These wines were elaborated following the usual protocol of each winery, with different technical criteria applied in each case according to characteristics and needs of each cellar.

In Table 3.23 is shown the chemical analyze of each wine. Even though they have different origins, they move within similar parameters and the differences among them are not constant to determine that one wine or a group of wines is different from the others.

Table 3.23. Chemical analysis of different Italian Pinot Gris wines obtained after alcoholic fermentation.

Wine	Region	Alcohol content (% v/v)	Volatile Acidity (g/ acetic acidL)	Titrable Acidity (g tartaric acid/L)	pH	Residual Sugar (g/L)
PG Friuli 327	Friuli	12.8 ± 0.3	0.07 ± 0.01	4.61 ± 0.14	3.37 ± 0.01	2.83 ± 0.03
PG Friuli 35	Friuli	12.5 ± 0.3	0.14 ± 0.01	3.72 ± 0.11	3.51 ± 0.01	2.75 ± 0.03
PG Oderzo 110	Treviso	12.7 ± 0.3	0.00 ± 0.00	4.16 ± 0.12	3.35 ± 0.01	2.55 ± 0.03
PG Oderzo 116	Treviso	12.5 ± 0.3	0.02 ± 0.00	4.45 ± 0.13	3.40 ± 0.01	2.58 ± 0.03
PG Oderzo 114	Treviso	12.8 ± 0.3	0.01 ± 0.00	4.35 ± 0.13	3.38 ± 0.01	2.61 ± 0.03
PG Motta di Livenza 90	Treviso	12.7 ± 0.3	0.10 ± 0.01	4.07 ± 0.12	3.47 ± 0.01	2.63 ± 0.03
PG Motta di Livenza 91	Treviso	11.6 ± 0.2	0.11 ± 0.01	3.38 ± 0.10	3.51 ± 0.01	2.28 ± 0.02
PG Motta di Livenza 93	Treviso	12.2 ± 0.2	0.11 ± 0.01	3.81 ± 0.11	3.40 ± 0.01	2.41 ± 0.02
PG Motta di Livenza 00	Treviso	11.9 ± 0.2	0.15 ± 0.02	4.45 ± 0.14	3.35 ± 0.01	2.89 ± 0.03
PG Pramaggiore 56	Venezia	12.0 ± 0.2	0.07 ± 0.01	4.09 ± 0.12	3.38 ± 0.01	2.67 ± 0.03
PG Pramaggiore 63	Venezia	12.1 ± 0.2	0.07 ± 0.01	4.12 ±0.12	3.36 ± 0.01	2.36 ± 0.02
PG Tezze di Piave 01	Treviso	12.5 ± 0.3	0.21 ± 0.02	4.61 ± 0.14	3.58 ± 0.01	2.51 ± 0.03
PG Tezze 02	Treviso	12.5 ± 0.3	0.22 ± 0.02	4.54 ± 0.14	3.59 ± 0.01	2.59 ± 0.03
PG Cerletti	Treviso	12.3 ± 0.2	0.17 ± 0.02	5.51 ± 0.17	3.32 ± 0.01	2.56 ± 0.03

In Table 3.24 is shown a summary of the protein content of each wine, also it is point if it was stable or not and in case they were unstable the bentonite dose required to reach protein stability.

Table 3.24. Protein content by Bradford method, protein stability and stabilizing bentonite dose for 2012 Italian Pinot Gris.

Pinot Gris Wine	Protein content (mg/L BSA)	Dry wine protein stability	Stabilizing bentonite dose (g/hL)
PG Friuli 327	36.7 ± 0.1	Stable	-
PG Friuli 35	42.0 ± 0.7	Unstable	42
PG Oderzo 110	27.7 ± 0.4	Stable	-
PG Oderzo 116	37.8 ± 0.6	Unstable	40
PG Oderzo 114	31.8 ± 0.2	Unstable	4
PG Motta di Livenza 90	31.9 ± 0.4	Unstable	9
PG Motta di Livenza 91	32.2 ± 0.3	Unstable	22
PG Motta di Livenza 93	32.4 ± 0.1	Unstable	18
PG Motta di Livenza 00	35.9 ± 0.3	Unstable	16
PG Pramaggiore 56	28.6 ± 0.2	Stable	-
PG Pramaggiore 63	37.5 ± 0.2	Unstable	160
PG Tezze di Piave 01	44.2 ± 0.3	Unstable	172
PG Tezze 02	43.7 ± 0.3	Unstable	165
PG Cerletti	46.7 ± 0.7	Unstable	180

The protein profile of each of the fourteen wines was also analysed, and the main values are shown in Table 3.25.

The three usual fractions associated to white wines are appreciated, F 1 (> 100 kDa), F 2 (40 – 60 kDa) y F 3 (20 – 30 kDa). Concentrations are different among wine samples, being F3 the highest and F1 the lowest. F2 also had low concentrations with values between 0.2 – 1.3 mg BSA/L.

Table 3.25. Protein profile of musts and wines treated with bentonite at different vinification stages, by FLPC.

Wine sample	F1 Protein Content (mg BSA/L)	F2 Protein Content (mg BSA/L)	F3 Protein Content (mg BSA/L)
PG Friuli 327 - FRIULI	0.9 ± 0.1	0.7 ± 0.1	25.8 ± 0.8
PG Friuli 35 - FRIULI	1.3 ± 0.1	1.0 ± 0.0	30.1 ± 0.1
PG Oderzo 110 - TREVISO	0.3 ± 0.0	0.4 ± 0.1	19.6 ± 2.3
PG Oderzo 116 - TREVISO	0.2 ± 0.0	0.3 ± 0.0	20.4 ± 1.3
PG Oderzo 114 - TREVISO	0.4 ± 0.0	0.3 ± 0.0	20.7 ± 0.3
PG Motta di Livenza 90-TREVISO	0.0 ± 0.0	0.2 ± 0.1	21.5 ± 0.6
PG Motta di Livenza 91-TREVISO	0.2 ± 0.0	0.3 ± 0.0	22.7 ± 0.8
PG Motta di Livenza 93-TREVISO	0.1 ± 0.0	0.2 ± 0.0	18.3 ± 1.4
PG Motta di Livenza 00-TREVISO	0.8 ± 0.2	0.9 ± 0.2	30.3 ± 1.3
PG Pramaggiore 56 - VENEZIA	0.0 ± 0.0	0.2 ± 0.1	13.7 ± 0.8
PG Pramaggiore 63 - VENEZIA	0.3 ± 0.1	0.6 ± 0.1	24.5 ± 4.3
PG Tezze di Piave 01 - TREVISO	0.4 ± 0.2	2.1 ± 0.0	43.5 ± 1.0
PG Tezze 02 - TREVISO	0.6 ± 0.1	1.3 ± 0.5	46.2 ± 2.1
PG Cerletti - TREVISO	0.5 ± 0.1	1.3 ± 0.0	51.9 ± 1.1

A Pearson analysis was done to establish correlations between the total protein content, the fractions content and the bentonite dose required to stabilize wines. The bentonite dose needed to reach protein stability of Pinot Gris wines is highly correlated with the total protein content ($R=0.814$, $p=0.01$), with the F2 protein fraction content ($R=0.755$, $p=0.01$) and with the F3 protein fraction content ($R=0.820$, $p=0.01$).

In the published literature the relationship between protein content and different protein fractions with the white wine instability, and consequently with the bentonite dose to wine fining is diverse (Waters et al., 2005). The hazing potential of a wine does not seem to be correlated with its total protein concentration (Bayly & Berg, 1967), suggesting a differential contribution of individual proteins to the phenomenon of haze formation (Hsu et al, 1987; Waters et al., 1992). Nevertheless the linear correlation of protein content and

bentonite dose is in agreement with Dawes et al. (1994). They found that there was no bentonite selectivity based on isoelectric point, and that bentonite fining resulted in the removal of all the different protein fractions. The diminution of protein content was correlated linearly with the bentonite addition. These different conclusions in the published literature might be attributed to the different methods used to fractionate proteins and assess their levels (Waters et al., 2005).

3.3.2 Study about Chilean white wines, vintage 2012

Six wines of six different white grape varieties from the Chilean Central Valley (Viña Cousiño Macul, Valle del Maipo, Chile) were analyzed concerning about their protein content, stability level, bentonite requirements for its stability and protein profile.

Each sample was obtained at the end of alcoholic fermentation, without any stabilizing treatment applied and following the usual vinification protocols of the winery.

Chemical characteristics of each must and wine are shown in Tables 3.26 and 3.27, respectively.

Table 3.26. Chemical analysis of grape must used at industrial scale of six different Chilean white grapes varieties.

Must sample	Density (kg/m3)	Degree ºBrix	Potential alcoholic degree (% v/v)	Titrable Acidity (g tartaric acid/L)	pH	Free amino Nitrogen (mg/L)
Chardonnay	1105 ± 3	24,5 ± 0,6	15,2 ± 0,3	4,31 ± 0,33	3,69 ± 0,11	180,9 ± 28,5
Sauvignon Gris	1104 ± 3	23,8 ± 0,6	14,9± 0,3	4,87 ± 0,38	3,64 ± 0,11	204,5 ± 32,2
Semillon	1102 ± 3	23,8 ± 0,6	14,6 ± 0,3	3,67 ± 0,28	3,75 ± 0,12	128,8 ± 20,3
Sauvignon blanc	1091 ± 3	21,2 ± 0,5	12,8 ± 0,2	7,05 ± 0,54	3,50 ± 0,11	134,4 ± 21,1
Viognier	1120 ± 5	27,6 ± 1,1	17,6 ± 0,7	4,35 ± 0,62	3,79 ± 0,14	151,0 ± 29,4
Riesling	1099 ± 3	23,0 ± 0,6	15,1 ± 0,3	5,01 ± 0,39	3,31 ± 0,10	88,1 ± 13,9

Table 3.27. Chemical analysis of white wines obtained at industrial scale of six different Chilean white grapes varieties.

Wine sample	Alcoholic degree (% v/v)	Volatile acidity (g acetic acid/L)	Titrable Acidity (g tartaric acid/L)	pH	Residual sugar (g/L)
Chardonnay	14.4 ± 0.2	0.40 ± 0.02	6.37 ± 0.27	3.37 ± 0.03	1.99 ± 0.13
Sauvignon Gris	14.6 ± 0.2	0.43 ± 0.02	6.75 ± 0.28	3.29 ± 0.03	2.00 ± 0.13
Semillon	14.4 ± 0.2	0.48 ± 0.03	6.30 ± 0.26	3.51 ± 0.04	1.67 ± 0.11
Sauvignon blanc	13.4 ± 0.2	0.37 ± 0.02	7.50 ± 0.31	3.33 ± 0.03	0.99 ± 0.07
Viognier	15.1 ± 0.3	0.36 ± 0.2	7.12 ± 0.30	3.32 ± 0.03	2.15 ± 0,14
Riesling	14.6 ± 0.2	0.39 ± 0.02	7.30 ± 0.30	3.03 ± 0.03	1.95 ± 0.13

For each one of these wines the total protein concentration, bentonite stabilizing dose and protein profile was determined. Results are shown in Tables 3.28 and 3.29.

Table 3.28. Protein content by Bradford method, stability, bentonite stabilizing and total dose, of six different Chilean white wines

Wine sample	Protein Content (mg BSA/L)	Stability	Bentonite Stabilizing Dose (g/hL)
Chardonnay	35,1 ± 0,1	Unstable	45
Sauvignon Gris	38,6 ± 0,3	Unstable	170
Semillon	40,3 ± 0,4	Unstable	90
Sauvignon blanc	47,4 ± 0,7	Unstable	135
Viognier	36,6 ± 0,2	Unstable	10
Riesling	32,6 ± 0,3	Unstable	55

All wines were unstable, though at different levels. These could be noticed in the bentonite required to stabilize each one.

Table 3.29. Protein profile of musts and wines treated with bentonite at different vinification stages, by FLPC.

Wine sample	F1 Protein Content (mg BSA/L)	F2 Protein Content (mg BSA/L)	F3 Protein Content (mg BSA/L)
Chardonnay	0.1 ± 0.1	1.7 ± 0.1	26.2 ± 0.4
Sauvignon Gris	0.5 ± 0.3	1.9 ± 1.0	64.9 ± 1.3
Semillon	1.5 ± 0.1	7.1 ± 0.1	49.3 ± 2.9
Sauvignon Blanc	3.1 ± 0.0	6.5 ± 0.0	60,2 ± 2,6
Viognier	0.0 ± 0.0	0.0 ± 0.0	8.1 ± 0.4
Riesling	0.4 ± 0.0	1.3 ± 0.3	19.0 ± 0.3

Pearson analyze was performed to establish correlations between total protein content, protein profile fractions and required bentonite dose to reach stability. The bentonite dose use for stabilization is highly correlated with the F3 protein fraction content ($R=0.963$, $p=0.01$), and fair correlated with the total protein content ($R=0.597$, not significant).

Analyzing together both studies with both series we can observe that fraction F3 (protein range of 20 – 30 kDa) is the most involved in instability of wines, thing that agrees with most of the published scientific literature (Waters et al., 2005)

Chapter 4

CONCLUSIONS

The main finding of this thesis is that if bentonite is added during alcoholic fermentation, and especially during the half/end, the total dose required for the protein stabilization of white wines is reduced and the sensorial characteristics of each variety are respected.

We also conclude that:

- For practical purposes the oenological parameters of the fermentations were not affected by the treatment or the scale of winemaking.
- This study shows that the fermenter size (industrial or pilot scale) has no significant effect on the kinetics of alcoholic fermentation and, therefore, from a practical point of view, pilot scale fermentations satisfactorily reproduce industrial scale fermentations.
- However, scale effects were observed in the effectiveness of bentonite addition and in the parameters of foam quality.

- Considering the theoretical estimation of the sensory impact of the wine aroma, no important differences have been found between winemaking scales.
- If they are treated with bentonite during the fermentation process, wines with low protein instability present a final lower protein concentration. For this kind of wine a greater and more extensive ageing on lees could be an alternative to additional treatment with bentonite, as demonstrated by the complete protein stability of the bentonite-free wines.
- The addition of bentonite before alcoholic fermentation during must clarification does not effectively achieves protein stabilization or reduces the total bentonite dose, unless the doses are high or it acts together with other products that can remove proteins or other macromolecules that affect wine stability. The resulting wines are less aromatic, either because bentonite is used twice or because aromatic precursors are removed from the initial must.
- The use of bentonite in conjunction with discoloration products such as activated carbon produces stable wines with a very low protein concentration. This leads to stability and removes colour but it also affects both the aromatic profile and the varietal characteristics of the wines.
- The use of bentonite in appropriate doses from the very beginning of fermentation gives stable wines. The content of protein is higher and, therefore, respecting the features associated with them as the aromatic profile.

- The three usual protein fractions associated with white wines were found in both musts and wines: F1 (>100 kDa), F2 (60 – 40 kDa, invertase and β-glucanases) and F3 (20 – 30 kDa, chitinases and TLP). Fraction F3 was the largest in all cases, and was related directly with wine instability and with the bentonite dose required to reach the protein stability.
- Wines that were stabilized with the bentonite treatment during fermentation showed a higher aromatic profile and were preferred by the tasting panels.

Nevertheless further work must be carried out in the future with other varieties if this protein stabilization technique is to be adjusted, improved and adapted to the requirements of the winemaking industry.

Chapter 5

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