Addition of Carrageenan at Different Stages of Winemaking for White Wine Protein Stabilization

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ABSTRACT: Carrageenan added at different stages of winemaking was assessed for its protein removal and impact on wine heat stability and on the chemical and sensorial profile of the wines. Carrageenan was added to a Semillon during fermentation and after fermentation and to finished wines, and the effect of each addition was compared to that of bentonite fining at the same time point. Data on protein concentration, heat stability, and bentonite requirement indicate that when added at the correct dosage carrageenan was very effective in stabilizing wines at dosages at least three times lower than those of bentonite. In addition, carrageenan treatment did not cause an increase in lees volume relative to bentonite and resulted in very similar chemical parameters to the unfined and bentonite-treated wine. Sensorially, although carrageenan-treated wine was significantly different from the unfined wine, the magnitude of difference did not vary significantly when compared to bentonite treatment. The feasibility of carrageenan use in a winery production setting will need to be determined by individual wineries, as technical issues including frothing, slower filterability, and risk of overfining will need to be considered relative to the benefits, particularly when carrageenan is used before or during fermentation.

KEYWORDS: wine, protein, haze, stability, bentonite, carrageenan

◼ INTRODUCTION

Securing wine stability is essential in winemaking. Among the possible instabilities that can occur, protein haze formation is the most important instability of nonmicrobial origin, particularly for white, rosé, and sparkling wine production.[1−3] Proteins are found in wines at 10−500 mg/L,[4] among which the grape pathogenesis-related proteins, thaumatin-like proteins and chitinases, are the major soluble proteins in grape juice[5] and are those directly involved in haze formation in wines.[6−8] Under certain conditions, grape proteins in wines can unfold and aggregate into light-dispersing particles to make wines appear turbid.[9] Hazy wines are not saleable because consumers perceive them as faulty, and therefore proteins need to be removed before bottling.

To prevent haze formation, the wine industry uses bentonite, a clay negatively charged at wine pH, which binds to the positively charged wine proteins and settles to the bottom of the tanks. Bentonite is an efficient fining agent, but its application has several drawbacks, as its use tends to extend time in tank, causes volume and quality loss, and presents waste disposal challenges.[2] A recent study estimated the hidden cost of bentonite fining to be around $1 billion worldwide.[10] Consequently, winemakers either aim to use the minimum amount of bentonite for wine quality, cost, and environmental reasons or would welcome the introduction of alternatives with fewer drawbacks than the current practice.

For these reasons, alternative approaches for protein removal from wine have been extensively investigated, with the list of proposed alternatives including ultrafiltration,[11] heat treatments,[12,13] use of proteolytic enzymes,[14−22] combination of flash pasteurization and proteases,[23,24] and use of polysaccharide-rich proteins with a protective effect against haze

formation.[25−28] Removal of proteins via adsorption onto materials such as immobilized phenolic compounds,[29,30] chitin,[4] and adsorbent and metal oxide materials[31−36] has also been proposed. A promising alternative to bentonite is carrageenan, a cell wall hydrocolloid found in some species of red algae (class: Rhodophyceae); it is a high molecular weight linear polysaccharide comprising repeating galactose units and 3,6-anhydrogalactose, both sulfated and nonsulfated, joined by alternating α-(1,3) and β-(1,4) glycosidic links.[37] Carrageenan contains a large number of sulfate groups, and this makes it very negatively charged at acidic pH, a fact that has been exploited for its proposed application in wine, where it showed the ability to bind the positively charged wine proteins.[38]

In previous work,[39] the addition of carrageenan to juice before fermentation resulted in wines that were partially stable but with some modification of wine composition and sensory properties depending on the timing of addition. However, no information regarding the volume of lees produced was reported. Other authors demonstrated that the timing of addition of bentonite results in different stabilization performances,[40,41] but the effects of timing have not been assessed for carrageenan.

The knowledge gaps highlighted above were addressed in the current work by investigating how the addition of carrageenan at three different stages of winemaking affects the chemical and sensory characteristics of a Semillon wine, with a focus on heat stability and lees production.

Table 1. Schematic Representation of the Treatments

| code	carrageenan during fermentation	bentonite during fermentation	carrageenan after fermentation	bentonite after fermentation	carrageenan on finished wine	bentonite on finished wine
unfined	n.a.[a]	n.a.	n.a.	n.a.	n.a.	n.a.
Car wine	n.a.	n.a.	n.a.	n.a.	250 mg/L	n.a.
Car wine 1/2	n.a.	n.a.	n.a.	n.a.	125 mg/L	n.a.
Bent wine	n.a.	n.a.	n.a.	n.a.	n.a.	800 mg/L
Car ferm	250 mg/L	n.a.	n.a.	n.a.	n.a.	n.a.
Bent ferm	n.a.	800 mg/L	n.a.	n.a.	n.a.	n.a.
Car post ferm	n.a.	n.a.	250 mg/L	n.a.	n.a.	n.a.
Bent post ferm	n.a.	n.a.	n.a.	800 mg/L	n.a.	n.a.

[a] n.a., fining agent was not added.
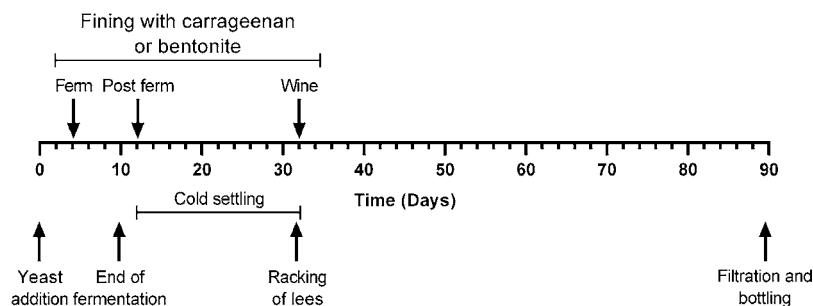
Figure 1. Timeline of operations and fining agents addition in large-scale experiment.

MATERIALS AND METHODS

Materials. Three types of carrageenan were used and named A (kappa form, the same used in a previous work[39]) and B and C (lambda form). Solutions of 2% carrageenan (Genuvisco, CPKelco ApS, Lille Skensved, Denmark) in distilled water were freshly prepared before use. The bentonite used was a Granular Sodic Plusgran gel (Enologica Vason S.p.A, San Pietro in Cariano, Italy) prepared at 50 g/L in distilled water. The yeast strain used was EC1118 (Lallemand, Montreal, Canada).

Protein Concentration Determination. Protein concentration was determined by EZQ protein quantitation kit (Invitrogen, Mt Waverley, VIC, Australia) following the manufacturer's instructions. The calibration curve was built using serial dilution from 0 to 250 mg/L of ovalbumin provided in the EZQ kit. Fluorescence measurements were taken using excitation/emission settings of 485/590 nm with a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Heat Test. Wines were heated at 80 °C for 2 h and cooled on ice for 2 h. After equilibration at ambient temperature the haze was measured by calculating the difference between heated and unheated samples in nephelometry turbidity units (NTU) by means of a nephelometer.[42] Samples with differences in NTU < 2 were considered heat stable.

Bentonite and Carrageenan Fining Trials. Fining trials for determination of the amount of bentonite and carrageenan required to achieve heat stability of the juice or wine were performed by adding increasing dosages of fining agent to 50 mL of juice and mixing well. After 2 h, bentonite or carrageenan B was removed by centrifugation (4000g, 3 min, 10 °C) and filtration of the supernatant (0.22 μm), samples were submitted to heat test, and their residual protein concentration was measured by EZQ.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed with NuPage 12% Bis–tris, 1.0 mm thick, 15-well gels (Invitrogen), and an XCell SureLock Mini Cell (Invitrogen) following the manufacturer's instructions. Approximately 50 mg of $Na_2S_2O_5$ was added to the top reservoir prior to running to prevent cysteine oxidation. Samples were prepared by precipitating proteins with four volumes of cold ethanol from 200 μL of wine. The pellet was collected by centrifugation (14000g, 15 min, 4 °C) and dissolved in 20 μL of loading buffer (Invitrogen NuPage recipe) with 3% 2-mercaptoethanol. Standard molecular weight used was the BenchMark Protein Ladder (Invitrogen). Proteins were stained with Pierce Imperial Protein Stain (Quantum Scientific, Sydney, NSW, Australia) according to the manufacturer's microwave instructions.

Volume of Lees. In the small-scale experiments the volume of lees was measured by transferring 100 mL of each wine at the end of fermentation to cold settle at 4 °C into 100 mL graduated measuring Duran glass cylinders (Witeg Labortechnik GmbH, Wertheim, Germany). The volume of gross (or heavy) was recorded after 28 h, while that of the fine (or light) lees was recorded regularly until settling was completed after 125 h.

In the large-scale experiment the volume of compact lees remaining in the vessel at racking of the wine (3 weeks after cold settling) was recorded.

Analytical Methods. Alcohol, specific gravity, pH, titratable acidity, glucose/fructose, and volatile acidity analysis were performed using a Foss WineScan FT 120 as described by the manufacturer (Foss, Hillerød, Denmark). Free and total SO_2 were measured by the aspiration method.[43] Sugar levels were measured by refractometry (Brix) and by densitometry (Baumé). Glucose and fructose concentrations were determined spectrophotometrically using a Randox kit (Randox Laboratories Ltd., Crumlin, Antrim, UK) with adaptations as described by Vermeir et al.[44] for performance of 200 μL assays in 96-well microtiter plates.

Organic Acids and Glycerol Quantification by High-Performance Liquid Chromatography (HPLC). The concentration of organic acids (citric, tartaric, malic, succinic, and lactic) and glycerol was determined by high-performance liquid chromatography as described by Marangon et al.[35]

Experimental Wine Samples. The small-scale and large-scale experiments were conducted using a Semillon juice (2500 L, vintage 2012) from the Adelaide Hills region (South Australia).

Small-Scale Experiment. Three carrageenan types (named A, kappa form; B and C, lambda form) and a sodium bentonite were tested. The four fining agents were added at dosages able to fully stabilize the juice as assessed by fining trials and confirmed by the absence of protein left in supernatants (determined by EZQ assay). In particular, carrageenan was added at 250 mg/L and bentonite at 800 mg/L. Additions were made at two time points: prefermentation (at day 0, without removal of the fining agents before fermentation) and postfermentation (at day

11). Fermentation took place in 250 mL flasks (filled with 150 mL of juice) placed on an orbital shaker (at 100 rpm) at 18 °C. An unfined juice was used as control. After yeast inoculation (EC1118 at 200 mg/L), the fermentation rate was monitored daily by measuring the residual glucose/fructose content of the fermentations, which were considered finished when glucose/fructose was below 1 g/L. Finished wines were transferred to 100 mL glass cylinders and stored at 4 °C for cold settling. Each of the nine fermentations was performed in triplicate, and analyses for each replicate were performed in triplicate unless otherwise stated.

Large-Scale Experiment. The Semillon juice was initially homogenized to the same turbidity (207 NTU) and divided into eighteen 78 L vessels. Rehydrated yeast was added at 200 mg/L, and fermentations were conducted between 15 and 18 °C. When residual sugar reached <2 g/L, SO_2 levels were adjusted and the wine was chilled to 0 °C for 3 weeks of cold settling. After racking, wines were filtered (0.45 μm) and bottled under Saran tin laminate screw-caps in 750 mL bottles 89 days after yeast inoculation. Carrageenan type B and bentonite were added separately at three stages: during fermentation after sugar had been consumed from 19.5° to 7° Brix (Car ferm, Bent ferm), after fermentation but before racking (Car post ferm, Bent post ferm), and to finished wines (Car wine, Bent wine) (Table 1 and Figure 1).

Carrageenan additions made during fermentation had to be done very slowly to minimize excessive frothing issues. For additions made after fermentation the fining agents were added when the residual sugar content of wines was below 2 g/L. Initially, six fermentations were conducted in the absence of fining agents. After the end of fermentation, three of the unfined fermentations were subdivided in three ways into 18 L vessels. Of the resulting nine vessels, three were treated with 250 mg/L carrageenan after racking (Car wine), three with 800 mg/L bentonite after racking (Bent wine), and three with a half-dosage of carrageenan after racking (Car wine 1/2, 125 mg/L). Since fining agents were dissolved in water, and since bentonite finings were the treatments where the highest amount of water was introduced to fermentations, variable amounts of distilled water were added to the unfined controls and to carrageenan-treated wines to achieve the same dilution factor of fermentations treated with bentonite. Overall a total of eight treatments in triplicate were made, yielding 24 finished wines.

Sensory Assessment. Wines were initially assessed at a bench tasting that was attended by staff from the AWRI and senior winemakers from the winery collaborator. This tasting allowed discussion of the treatments and confirmed that wines were free from off-flavors and suitable for sensory analysis. All replicates were also assessed in a separate session and were considered virtually identical.

In formal sensory analysis sessions, 16 assessors evaluated the wines, with the test conducted in duplicate on the same day. Assessors were professional winemakers with extensive wine tasting experience, who were familiar with the difference from control method and whose performance was found to be acceptable from previous tests. The assessors were aware that there may be a blind control included in the test.

Sensory analysis was conducted in a well-ventilated temperature-controlled sensory laboratory with daylight lighting at ambient temperature (approximately 21−24 °C). Tasters were not in isolated booths, but were closely supervised to minimize any verbal or nonverbal interaction. Presentation order was randomized across assessors and presentation replicates. Samples (approximately 30 mL) were presented in 3-digit-coded, covered ISO tasting glasses. Three bottles were used for each test, with bottles randomly selected from the three replicates of each treatment and were not blended.

The difference from control method was applied,[45] with three treatments selected from the study compared to the untreated wine (no bentonite or carrageenan, unfined control). The treatments selected were bentonite on finished wine (Bent wine), carrageenan added during fermentation (Car ferm), and carrageenan added on finished wine (Car wine). A blind coded control was included in the test, so that four coded samples were each compared to the labeled

control. The magnitude of difference from control was determined on the basis of overall difference and was rated on a 10-point category scale from 0 to 9, where 0 was "No Difference" and 9 was "Extremely Large" difference.

Statistical Analyses. Sensory data were analyzed using JMP Statistical Discovery software (version 5.0.1a, SAS Institute, Cary, NC, USA). A three-way analysis of variance (ANOVA) was initially conducted to determine whether there were significant effects of the treatment, assessor or presentation replicate, and the two-way interactions, followed by a two-way ANOVA with interaction for treatment and assessor, with Dunnett's means comparison test applied to compare means with the control and Tukey−Kramer honestly significant difference (HSD) test ($p = 0.05$) to compare all means.

All other data were analyzed by one-way completely randomized ANOVA, and data significance assessed by the HSD test. Each measure was the result of at least three replicates unless otherwise stated.

■ RESULTS AND DISCUSSION

The general composition of the Semillon juice used in the small- and large-scale experiment is shown in Table 2. The juice was heat unstable (24.7 NTU), with required dosages of fining agents to achieve juice heat stability identified as 250 mg/L for carrageenan and 800 mg/L for bentonite.

Table 2. Enological Parameters of the Semillon Juice at Day 0

parameter	value
yeast assimilable nitrogen[a]	233 mg/L
ammonia[a]	97 mg/L
alpha amino nitrogen[a]	153 mg/L
Brix[a]	19.5°
Baumé[a]	10.8°
pH[a]	3.27
total acidity (as tartaric acid)[a]	6.9 g/L
SO_2 (total)[a]	31 mg/L
glucose + fructose[a]	181.3 g/L
initial turbidity[a]	207 NTU
total protein concentration (by EZQ)	58 ± 1.3 mg/L
haze after heat test	24.7 ± 1.4 NTU
required fining dosage, carrageenan	250 mg/L
required fining dosage, bentonite	800 mg/L

[a]Data from single replicates.

Small-Scale Experiment. Three types of carrageenan (named A, B, C) and one type of bentonite were added before or after fermentation of a Semillon juice. The fermentation rate of the four prefermentation additions plus that of the unfined control was monitored daily (Figure 2).

Addition of bentonite to juice has been reported to slow the fermentation, but only when the lees are removed before yeast inoculation.[46] Fermentation data shown in Figure 2 indicated minimal differences between treatments. The unfined control (green line) completed the fermentation in 7 days, while the presence of carrageenan or bentonite reduced the fermentation time to about 6 days. These findings are consistent with those of others[40,47] describing an increase of the rate of fermentation in the presence of bentonite. The increased rate in fermentations containing carrageenan could be due to a similar effect of that played by bentonite in acting as a support for yeast and/or as a nucleation point for CO_2.[47]

A key parameter in the evaluation of the efficiency of a fining agent is the assessment of the amount of lees that it produces
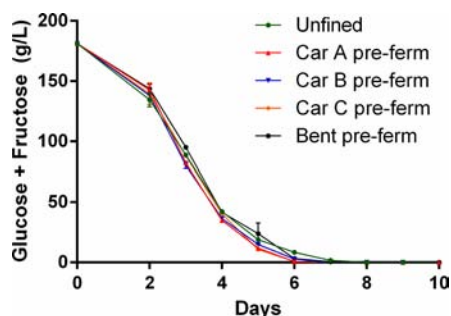
Figure 2. Fermentation rate of unfined juice (control), of juice treated with carrageenan (A, B, and C), and of bentonite in prefermentation. Samples with additions made in postfermentation (day 11) are not reported, as they behaved exactly as the unfined control.

and of the time required to complete the settling, as both characteristics have a direct impact on the costs associated with its application due to wine loss and tank occupancy. After the fermentation was completed and the postfermentation additions were made, the 27 wines were transferred at 4 °C for lees settling. The cold settling took place in 100 mL graduated cylinders, and measurements of the volume of both gross and fine lees were taken regularly until settling was completed (Figure 3).

Figure 3A shows a significant differentiation between the four fining agents in terms of gross lees volume. The unfined control produced the lowest amount of lees (2%), while carrageenan B and C added in both pre- and postfermentation increased the volume of gross lees to 2.7–3.7%, but is insignificant relative to unfined. Conversely, carrageenan A and bentonite behaved very similarly: when either was added in prefermentation, the lees production for both was significantly higher than the control (around 6%), while when added postfermentation the volume of lees rose to around 9% for both. After 28 h most of the settling of the gross lees was completed (data not shown), while the fine lees required up to 6 days to fully settle (see Figure 3B). In general, every addition of fining agents in prefermentation resulted in a faster settling of the fine lees when compared to the unfined control (Figure 3B). The unfined control required 100 h for complete settling, while when bentonite was added prefermentation, the settling time was halved (51.5 h). Carrageenan A and B slightly reduced the settling time (93 h), while carrageenan C did not modify it

(100 h). Postfermentation addition of carrageenan B and C slowed the settling by about one day, while carrageenan A settled in the same time as when added prefermentation (93 h).

Generally bentonite had the advantage of speeding up the settling (50% in prefermentation, 25% in postfermentation); however, the three carrageenans tested did not modify the settling time in comparison to the unfined control and generally gave better results when added in prefermentation.

The residual protein concentration (Figure 4) and heat stability (Figure 5) of the finished wines were measured after cold settling.
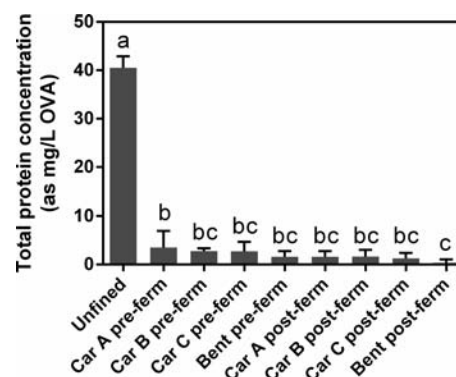

Figure 4. Protein concentration of wines after cold settling. Results are expressed as an average of each treatment replicate plus the average of the three experimental replicates of the analyses (at least 9 values for each bar). Bars with different letters are significantly different according to the Tukey−Kramer HSD test ($p \leq 0.05$).

The dosages of fining agents used (250 mg/L for carrageenan, 800 mg/L for bentonite) were sufficient to almost completely remove the proteins from the wines, with additions made after fermentation resulting in a slightly higher protein removal.

In terms of heat stability, every prefermentation addition of fining agents resulted in stable wines (Figure 5). When added postfermentation, only carrageenan A and bentonite gave a wine that was almost stable, while carrageenan B and C failed the heat test despite their protein concentration being negligible (Figure 4). Wines with minimal protein levels are expected to be stable, but, as this was not the case, it is possible
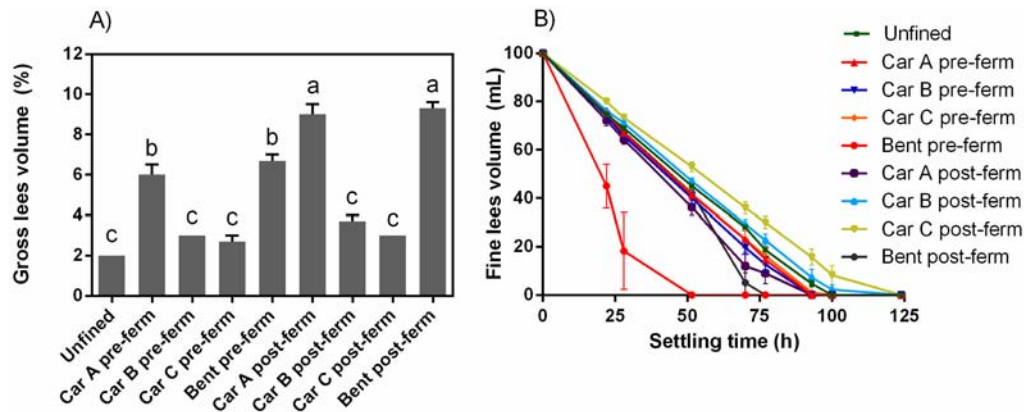

Figure 3. Volume measurements of lees during static settling of 100 mL of wine in a glass cylinder: (A) volume of gross (heavy) lees (as a percentage of the total wine volume) after 125 h of cold settling; (B) volume of fine (light) lees (in mL) for the 9 treatments. Mean values are shown ($n = 3$). Bars with different letters are significantly different according to the Tukey−Kramer HSD test ($p \leq 0.05$).
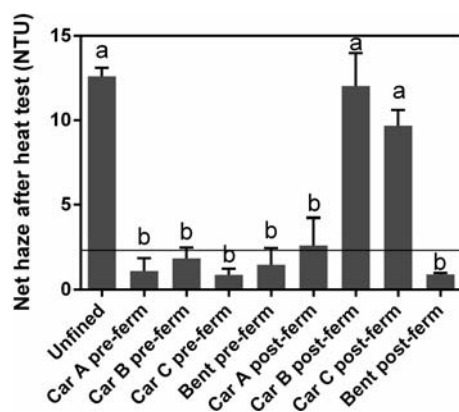
Figure 5. Heat stability of wines after cold settling. Results are expressed as an average of each treatment replicate plus the average of the three experimental replicates of the analyses (at least 9 values for each bar). Bars with different letters are significantly different according to the Tukey–Kramer HSD test ($p \leq 0.05$).

that this haze is attributable to the presence of residual carrageenan in the wines. In a winery situation, without knowledge of the amount of remaining protein, such a result confounds the interpretation of the test results, as it becomes unclear whether the failure of the heat test is due to the presence of protein or carrageenan.

A simple experiment was made to confirm this hypothesis. Three new wines (two heat unstable and one heat stable) and one model wine (12% ethanol, 4 g/L tartaric acid, pH 3.0) were treated with increasing dosages of carrageenan B. After filtration (at 0.22 μm) the samples were heat tested (Figure 6).
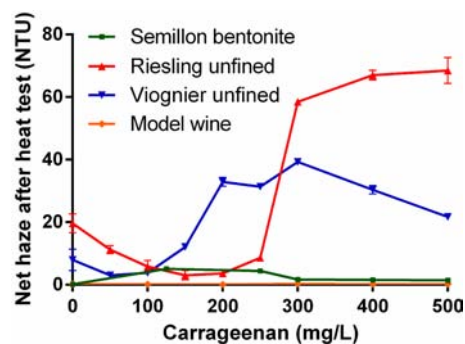

Figure 6. Haze formation after heat test of two unfined wines (Riesling and Viognier), one bentonite stabilized wine (Semillon), and one model wine at increasing addition dosages of carrageenan B. Results are expressed as an average of three experimental replicates.

The Semillon wine was stable in the absence of carrageenan, as it had been previously treated with bentonite. Adding carrageenan triggered the formation of haze (about 5 NTU), confirming the hypothesis that carrageenan can pass through filters and contribute to haze formation during the heat test. The same experiment was performed with addition of increasing dosages (0 to 500 mg/L) of carrageenan to two heat-unstable wines (Riesling and Viognier). Haze data showed a U-shape behavior, with the turbidity initially decreasing until reaching a bottom indicating the optimal protein:carrageenan ratio. Past the optimal point, the haze level increases until reaching a plateau. This behavior is a clear indication that haze formation upon heat test for samples of carrageenan B and C added postfermentation (see Figure 5) is due to residual

carrageenan in the product. Interestingly, if carrageenan was added in model wine, no haze was formed, indicating that, in the absence of proteins, carrageenan reacts with other wine components to form haze and that heat alone does not denature it.

In general, additions of carrageenan prefermentation seemed preferable because this resulted in an increased fermentation rate, produced less lees volume without modifications of the settling time, and removed proteins as effectively as for additions made postfermentation.

Large-Scale Experiment. The same Semillon juice used in the small-scale experiment was used during the experimental winemaking trial. Carrageenan B was selected because of its effective protein removal combined with its low production of lees. The experiment was set up following the design shown in Table 1 and Figure 1.

Chemical Parameters and Lees Volume. The general composition of the wines produced during this study is shown in Table 3.

Parameters such as alcohol, volatile acidity, free and total SO_2, time for completing fermentation, citric acid, succinic acid, and lactic acid did not show any significant differences among the treatments. In only six instances the parameter measured differed significantly from the unfined control: Car wine 1/2 had a significantly lower amount of malic acid than the unfined wine, even though its total acidity was not different from that of the control. When added during fermentation, carrageenan-treated wines (Car ferm) had significantly less residual sugars and glycerol than the control, while when added postfermentation carrageenan significantly affected the pH and the total acidity of the resulting wine. Bentonite fining caused a significant decrease in the amount of glycerol, but only when added postfermentation. In general, when compared to the unfined wine, the seven treatments did not cause any major effect on the common winemaking parameters measured.

One important parameter to consider when assessing a new fining agent is the production of lees associated with its use and how this compares to the lees volume from the common practice of bentonite fining. After the end of fermentation wines were cold settled at 0 °C for 3 weeks before racking, and the volume of lees produced is shown in Table 4.

In general, the addition of carrageenan increased the lees volume in comparison to the control, but this increase was not statistically significant. The addition of carrageenan and bentonite during fermentation resulted in the same increase (+1.5%) in lees volume when compared to the unfined control. Carrageenan added after the end of fermentation (5.6%) produced lower lees volume than bentonite did at the same addition point (7.3%).

Protein Concentration and Heat Stability. Once the wines were filtered and bottled, their protein concentrations were measured (Figure 7).

The unfined control wine contained ~30% less protein than the starting juice (compare Figure 4 with Table 2), confirming that winemaking processes lead to the degradation or precipitation of a portion of the starting protein of the wine.[2,39,48] Every wine treated with carrageenan or bentonite yielded significant protein reductions ranging between 75% and 90% of protein removed, confirming that the amount of fining agent determined to be used in the experiment (see Table 2) to achieve wine stability was sufficient.

In small-scale experiments (see Figure 5) it was proven that carrageenan added prefermentation resulted in heat-stable

Table 3. Effect of the Treatments with Carrageenan and Bentonite on the Chemical Parameters of Bottled Wines[a]

parameter	unfined	Car wine	Bent wine	Car wine 1/2	Car ferm	Bent ferm	Car post ferm	Bent post ferm
alcohol (% v/v)	11.70 a	11.70 a	11.70 a	11.67 a	11.70 a	11.70 a	11.60 a	11.67 a
glucose + fructose (g/L)	0.20 a	0.20 a	0.20 a	0.13 ab	**0.10** b	0.20 a	0.17 ab	0.13 ab
volatile acidity (g/L)	0.26 a	0.26 a	0.26 a	0.30 a	0.34 a	0.32 a	0.26 a	0.28 a
total acidity (as tartaric acid) (g/L)	5.90 b	5.97 b	5.90 b	5.90 b	6.00 ab	5.90 b	**6.13** a	5.93 b
pH	3.16 b	3.17 ab	3.17 ab	3.17 ab	3.16 b	3.17 ab	**3.21** a	3.18 ab
free SO_2 (mg/L)	38.33 a	35.67 a	37.33 a	36.33 a	37.67 a	37.00 a	36.67 a	37.00 a
total SO_2 (mg/L)	100.67 a	98.00 a	102.33 a	99.67 a	101.67 a	98.33 a	97.00 a	96.67 a
fermentation time (days)	7.00 a	7.00 a	7.00 a	7.00 a	7.00 a	7.00 a	7.00 a	7.00 a
citric acid (g/L)	0.26 a	0.25 a	0.26 a	0.24 a	0.25 a	0.27 a	0.29 a	0.26 a
tartaric acid (g/L)	2.76 ab	2.78 ab	2.79 ab	2.61 b	2.82 a	2.68 ab	2.81 a	2.66 ab
malic acid (g/L)	2.87 a	2.87 a	2.92 a	**2.68** b	2.83 ab	2.89 a	2.79 ab	2.85 ab
succinic acid (g/L)	2.01 a	1.98 a	2.03 a	1.90 a	1.97 a	1.99 a	1.93 a	1.97 a
lactic acid (g/L)	0.56 a	0.52 a	0.56 a	0.50 a	0.54 a	0.53 a	0.51 a	0.51 a
glycerol (g/L)	6.79 a	6.49 ab	6.64 a	6.34 abc	**5.84** bc	6.68 a	6.59 a	**5.70** c

[a]Mean values are shown ($n \geq 3$). Within each row, means followed by a different lowercase letter are significantly different ($p \leq 0.05$) according to the Tukey−Kramer HSD test. Numbers in bold indicate significant differences from the unfined control.

Table 4. Effect of Fining Agent Addition on Lees Volume[a]

treatment	lees volume (L)	% of lees on total volume
unfined	3.7 b	4.7%
Car wine	n.m.	n.m.
Bent wine	n.m.	n.m.
Car wine 1/2	n.m.	n.m.
Car ferm	4.9 ab	6.2%
Bent ferm	4.9 ab	6.2%
Car post ferm	4.3 ab	5.6%
Bent post ferm	5.7 a	7.3%

[a]Summary of lees produced upon racking in absolute value (L) and in % of the total volume of wine (78 L). Mean values ($n \geq 3$) followed by a different letter are significantly different according to the Tukey−Kramer HSD test ($p \leq 0.05$). n.m., not measured, as the three treatments made in wine were done post-racking and post cold settling. Treatments in post-racking with carrageenan and bentonite were done in smaller vessels (18 L), so data on lees are not necessarily comparable with those obtained in 78 L vessels.
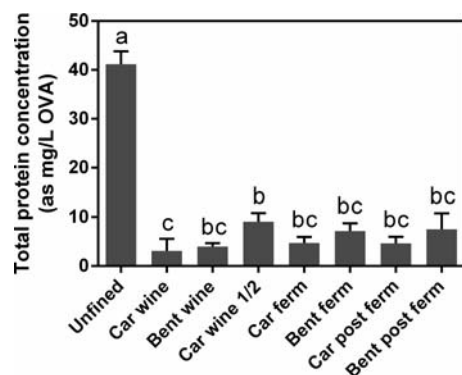

Figure 7. Protein concentration of the wines after bottling. Results are expressed as an average of each treatment replicate plus the average of the three experimental replicates of the analyses (at least nine values for each bar). Bars with different letters are significantly different according to the Tukey−Kramer HSD test ($p \leq 0.05$).
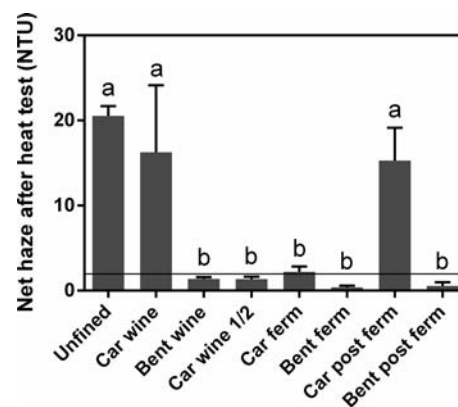

Figure 8. Heat stability results of the bottled wines expressed in NTU. Results are expressed as an average of each treatment replicate plus the average of the three experimental replicates of the analyses (9 values for each bar). Bars with different letters are significantly different according to the Tukey−Kramer HSD test ($p \leq 0.05$).

fermentation (Car ferm). As expected, bentonite always resulted in stable wines independently of the time of addition. Interestingly, carrageenan added after the end of fermentation (Car wine and Car post ferm), despite resulting in wines with minimal protein concentration (∼5 mg/L, see Figure 7), yielded wines deemed not stable when subjected to the heat test (Figure 8). Since the amount of protein in these wines is too low to cause instability, the observed haze was attributed to carrageenan overfining, as demonstrated in Figure 6.

When carrageenan was added post-racking at half-dose (125 mg/L, Car wine 1/2), the wine was heat stable. This dosage was selected by carrageenan fining trial on wine, and it was the amount required to remove all the proteins from the wine without causing overfining (see Table 5), as discussed for Figure 6. Indeed carrageenan added in excess (i.e., not reacting with proteins), as with Car post ferm, could remain in the wine and pass through the filters to potentially form cloudiness in bottled wines.

During the preparation of the samples for the heat test wine had to be sterile filtered, and the filtration was observed to be slower in wines treated with carrageenan, particularly for postfermentation treatments. The same issue was encountered

wines, an outcome similar to that obtained in a previous project,[39] where no indication on the effects of carrageenan addition during fermentation was possible because this treatment was not included. Data from Figure 8 showed that carrageenan can stabilize wines also when added during

Table 5. Bentonite Fining Dosages of Bottled Wines

treatment	fining agent added prior bottling (mg/L)	bentonite requirement (mg/L)	amount required in finished wines if no fining was done (mg/L)
unfined	0	433	433[a]
Car wine	250	n.d.[b]	125
Bent wine	800	n.d.	<600
Car wine 1/2	125	0	125
Car ferm	250	0	250
Bent ferm	800	n.d.	800
Car post ferm	250	n.d.	125
Bent post ferm	800	n.d.	<600

[a]Expressed as an average of three treatment replicate plus the average of the three experimental replicates of the analyses. [b]n.d., not determined, as wines were already stable after treatments made during the experiment.

during filtration of the wines prior to bottling, indicating that carrageenan can clog the filtration membranes.

Figure 9 gives indications of both the magnitude of protein removal as well as the classes of protein involved. The unfined
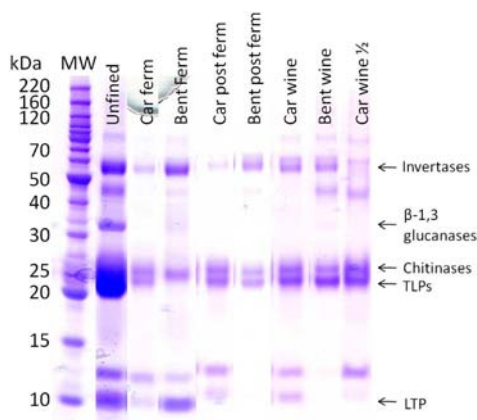

Figure 9. Protein profiles by SDS-PAGE of bottled wines with tentatively assigned identity based on protein profiles established in previous work.[23] MW, molecular weight standard.

sample represented a typical wine profile; it had the highest band intensity, in particular in the MW around 22 kDa (tentatively thaumatin-like proteins, TLPs) and 26 kDa (tentatively chitinases), but also contained bands at 34 kDa (tentatively β-1,3-glucanases), at 60 kDa (tentatively invertases), and at 11 kDa (tentatively lipid transfer proteins, LTP). The largest decrease in intensity was observed when bentonite and carrageenan were added during or postfermentation. In general bentonite seemed to remove more protein in the 20–40 kDa range, while carrageenan was less specific and yielded a more homogeneous protein removal across all MW ranges. The protein profiles of Car wine and Car post ferm are very similar to that of Car ferm, supporting the theory previously discussed that the haze formed upon heat test of these treatments is not protein-related.

A key parameter in assessing the performance of a new stabilization treatment consists of measuring the amount of bentonite still needed to stabilize a wine after the treatment. Therefore, bentonite fining dosages were measured on the wines after bottling (Table 5).

Results showed that the unfined control wine needed 433 mg/L of bentonite to be heat stable. All the other treatments resulted in heat-stable wines, as fining agents were added during winemaking at doses that resulted in heat stability. For Car post ferm and Car wine the heat test failed (Figure 8), but, as previously explained, this was the result of carrageenan-induced haze.

Sensory Assessment. The treatments chosen for sensory evaluation were Bent wine, as it is a reflection of current industry practice, Car Ferm and Car wine, and the unfined control, as it was the only sample that was independent of a timing effect in our experiment and therefore the most appropriate control for the type of test selected. Difference from control test was performed to assess the magnitude of difference that the selected fining treatments had in comparison to the unfined control (Figure 10).
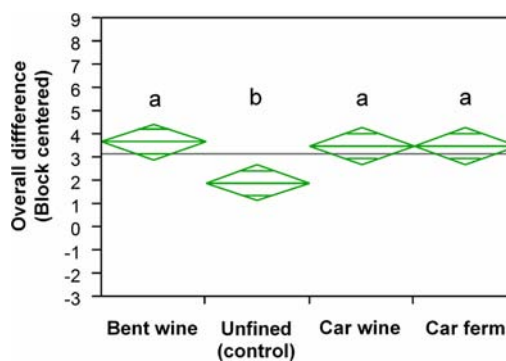

Figure 10. Control and treatment means, shown as diamonds—group mean (middle lines within diamonds), overlap points (top and bottom lines within diamonds), and 95% confidence interval (top and bottom point of the diamonds)—for each treatment, including unfined control. Different letters above the diamonds indicate significant differences between means by the Tukey–Kramer HSD test ($p <$ 0.05).

From a three-way ANOVA to test the effects of treatment, taster, and tasting replicate, there was a significant treatment effect ($p < 0.01$) and no taster or presentation replicate effect, together with no significant two-way interactions. A two-way ANOVA with the main effects treatment and assessor confirmed the significant treatment effect ($p < 0.01$). All treatments were significantly different from the control (unfined), but the magnitude of difference from the control did not vary significantly between treatments, as shown in Figure 10. In practical terms, the carrageenan tested produced a similar magnitude of difference from the unfined wine to that of the current industry stabilization practice (Bent wine), suggesting that, in terms of sensory effects, this material may be able to compete with bentonite as a possible alternative for protein fining of wines. It is important to consider that this analysis characterizes the magnitude of difference between treatments and the control, but does not provide information about the nature of the differences.

Carrageenan has previously shown potential to reduce the need for bentonite in wines by partially removing their proteins.[38,39] The carrageenan used in this study fully removed the need for bentonite in wine and was particularly effective when added during fermentation or to wine, providing that no over fining occurred. When added postfermentation the dosage of addition played a critical role, as an excess of carrageenan

added at this stage can slow the filtration process and can result in wines deemed unstable in a heat test despite being almost protein free.

Carrageenan did not slow the fermentation rate, as observed previously,[39] and actually increased it in the small-scale experiment. In addition, the use of carrageenan caused no significant modifications of the chemical composition of the wines and a similar magnitude of difference from the unfined wine to that of bentonite-treated wine.

Data on a previously unexplored feature of carrageenan treatment, volume of lees produced, were very encouraging. In controlled laboratory conditions carrageenan B was shown to produce a significantly lower amount of lees than bentonite, while in small-scale winemaking carrageenan B and bentonite additions yielded lees volumes that were not significantly different from each other. This is a critical feature influencing the possible adoption of this treatment by the wine industry, as losses of wine trapped on the lees is a direct cost for the wineries.

The timing of application proved to be very important for the final outcome. Carrageenan additions made before or during fermentation gave stable wines, with the advantage of reducing or not increasing the lees production in comparison to bentonite fining. On the other hand, although less carrageenan is required for postfermentation additions, it is associated with the risk of obtaining wines that fail the heat test due to carrageenan remaining products contributing to haze in the heat test. This poses a practical consideration to wineries in terms of establishing that their product is always dosed with the precise amount of carrageenan fining agent in order to manage the risk of a wine that may not be heat stable. However, this risk may be deemed manageable by performing carrageenan fining trials prior to additions, particularly postfermentation, in a manner similar to that currently used to establish bentonite fining doses. Alternatively, residual protein reductions may be confirmed by other methods (as HPLC and SDS-PAGE) to confirm protein removal by finings with carrageenans.

Some technical issues that need management in practical wine production settings are the possibility of excessive frothing for additions made during fermentation and the potential for some difficulties in filtration. However, as these issues were found in a small-scale winery environment, it may be that in more common wine production settings they could be minimized, e.g., by using antifoaming agents, centrifugation, and cross-flow filtration. Further studies are required to address these technical production issues as well as studies on the effect of different carrageenan types on different types of wines. The feasibility of carrageenan use in a winery production setting will need to be determined by individual wineries, as such technical issues will need to be considered relative to the benefits of using it as an alternative to bentonite. The regulatory status relating to the legality of carrageenan use in winemaking should also be established prior to application.

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Notes
The authors declare no competing financial interest.

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