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Evaluation of Carboxymethyl Cellulose as an Additive for Selective Protein Removal from Wine

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Abstract: Achieving protein stability is one of the main objectives before bottling wine. Traditionally, this is accomplished via bentonite fining, but the application has drawbacks and is not the most sustainable practice. A promising alternative was previously identified in modified cellulose, which is approved for tartrate stabilization but, as a side activity, could also help remove protein from wine. This study was designed to evaluate powdered carboxymethyl cellulose (CMC) and a liquid formulation in model wine using bovine serum albumin (BSA) and egg white as model proteins. The solubility of BSA proved to be insufficient, so experiments in wine were conducted with egg white protein only. Low-addition levels of liquid CMC showed the highest protein removal rates in real wine, so final trials were conducted with 13 commercial wines to evaluate the performance in different wine styles. The protein removal rate ranged from 12% to 84%, with an overall average of 57%. While these results do not reach the efficiency of bentonite, CMC is showing promise as an additional stabilization tool for a wide variety of wines. It can stabilize over the entire pH range of wine between 2.9 and 4.1, which is a unique feature of this method.

Keywords: bovine serum albumin; egg white; zeta potential; precipitation; stability

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1. Introduction

Protein stability is one of the main challenges when wines are finished and prepared for bottling, as denaturing proteins, for example, in the hot trunk of a customer's car, can cause haze formation in the bottle. The reasons why grapes and wines accumulate proteins are complex and can range from environmental factors [1,2] to the fermentation and aging strategies of the wines [3,4]. While proteins that are released from yeast cells are not responsible for haze formation due to their better solubility in water [5], grape proteins, especially pathogenesis-related proteins, can be sensitive to heat, denature and precipitate in the finished product [6–10]. While this haze poses no risk for the consumer, it does not meet the quality standards and expectations for commercial wine. The removal of protein material prior to packaging is, therefore, necessary for most wines.

The standard method for protein removal from wine is the addition of bentonite, followed by filtration. There are various bentonites with slightly different functionalities as well as chemical and physical properties [11]. However, the main mode of action is based on a negative surface charge that attracts positively charged proteins, a mechanism that works better at lower pH when more proteins carry a positive net charge [12]. This can lead to challenges with wines that naturally have a higher pH due to factors like cultivar, growing conditions, or vintage variations [4]. In addition to that, bentonite is mined and is not renewable, its dust can cause respiratory complications, the disposal of used bentonite is

challenging and the effect on wine aroma and mouthfeel can be negative [13–16]. All these factors have led to attempts to find more sustainable alternatives for its use in wine [17].

One of the more promising additives that is already approved for use in food and wine and has shown its potential to bind proteins is carboxymethyl cellulose (CMC) [17–20]. Modified cellulose has the advantage that it is a renewable resource that can be chemically altered to possess desirable functions [21,22]. In the case of CMC, the molecule can bind proteins based on charges similar to bentonite as well as hydrophobic interactions [18]. This could allow for protein removal across a wider pH range and a different protein fraction that cannot be removed by bentonite. A previous study in model wine demonstrated an inverse concentration effect where the lowest CMC addition led to the best protein removal results [17]. The reasons for that behavior are unknown but can be speculated to be related to a better unfolding of the cellulose strand at low concentrations, which could then lead to better interaction efficiency with proteins. Currently, CMC can legally be used to stabilize potassium bitartrate in wine by preventing crystal growth and, therefore, precipitation [23]. While this mechanism is fundamentally different from the protein binding capacity described above, using one additive for multiple stability purposes would benefit winemakers greatly.

Studying effects with complex influencing factors can be challenging if the observation cannot be directly linked to a source. Using model systems can help with that since the complexity of the system can be continuously adjusted until it is reasonably close to reality. Basic wine-like models have been used in the past to study molecular interactions involving CMC [18,19,24]; however, these models have limitations as they usually draw conclusions for limited conditions. Transferring these assumptions to a wider range of wine styles is a necessary next step, especially in the case of a fining agent or additive that could be used on all wines. The minimum matrix for meaningful studies just contains alcohol and acid with a buffered pH [25]; however, for physical stability studies, there are known matrix interactions that play an important role [26]. When considering protein stability questions, phenolic material should be added to the model, since it is naturally involved in aggregation reactions in real wine [27]. For more complex fining agent simulations, it might even be necessary to add more complex macromolecule mixtures like polysaccharides to the matrix, such as mannoproteins, if these were shown to have an effect [28].

The term zeta potential (ζ-potential) describes the overall stability of a colloidal system by analyzing the electrokinetic potential of a dispersion [29]. Larger values, positive or negative, indicate that particles are unlikely to aggregate. The nominal value of the ζ-potential at a given pH indicates the overall particle charge. The isoelectric point of a molecule is the same pH where the ζ-potential would equal zero. This is an important indicator for the reactivity of a macromolecule since most interactions are based on the attraction of opposite charges. The concept of ζ-potential has been used before to predict interactions of complex systems like wine [24,30] and can offer valuable insight into how additives and fining agents work under different conditions.

The objective of this study was to explore the use of carboxymethyl cellulose as a protein removal tool in wine and to identify mechanisms that might help to optimize the application process as an alternative to bentonite.

2. Materials and Methods

In order to evaluate the effects of CMC on different wine types and protein sources, this study was divided into pre-trials with a wine-like model system and two real wines, followed by a more refined application study in commercial wines with the best performing formulation from the pre-trials. All wine fingerprinting analyses were performed using FT-MIR spectroscopy (FT2 WineScan™, FOSS, Hillerød, Denmark). The zeta potential was

analyzed between pH 8 and pH 2 using a Mutek SZP-06 Meter (BTG, Chattanooga, TN, USA). The CMC concentration for the analysis was 20 g/L and the pH was adjusted using 1 M hydrochloric acid first and 1 M sodium hydroxide (both Sigma-Aldrich, St. Louis, MO, USA) to raise the pH stepwise.

2.1. Formulation Pre-Trials in Model System and Wine

Experiments were conducted in a wine-like model system, as previously used in a similar study [17] with a slight modification. In addition to the protein dosage of 1 g/L (either bovine serum albumin (VWR, Radnor, PA, USA) or egg white protein (dried powder, Barry Farm, Wapakoneta, OH, USA)), the model contained 350 mg/L of grape skin tannins (Uva'Tan™, Scott Laboratories, Petaluma, CA, USA). The solid CMC used was a food-grade sodium salt powder from Modernist Pantry LLC (Portsmouth, NH, USA) and the liquid formulation was VinoStab® (Erbslöh Geisenheim GmbH, Geisenheim, Germany). The different CMC addition levels to each protein model are given in Table 1. All experiments were conducted in duplicate.

Table 1. Experimental design for the wine model system as well as real wines with two different carboxymethyl cellulose (CMC) formulations (ppm: parts per million, mg/L).

Matrix	Protein	CMC Formulation	CMC Addition
Model wine	Bovine serum albumin	liquid	0, 25, 50, 100, 200, 500 ppm
		solid	0, 25, 50, 100, 200, 500 ppm
	Egg white protein	liquid	0, 25, 50, 100, 200, 500 ppm
		solid	0, 25, 50, 100, 200, 500 ppm
White wine	Egg white protein	liquid	0, 25, 50, 100, 200, 500 ppm
		solid	0, 25, 50, 100, 200, 500 ppm
Rose wine	Egg white protein	liquid	0, 25, 50, 100, 200, 500 ppm
		solid	0, 25, 50, 100, 200, 500 ppm

Following the model wine trials, a white and a rose wine (Table 1) were selected from a previous project as a matrix example before exploring the application in commercial wines. The white wine was a Muscat Canelli with 16.9% alcohol, titratable acidity of 4.8 g/L and a pH of 3.93. The rose wine was a Barbera with 12.6% alcohol, titratable acidity of 8.9 g/L and a pH of 3.01. The wines were selected for their large differences in composition and their chemical profile.

Fining efficiency was evaluated using a photometric Bradford protein assay [31] on a microplate scale (discrete analyzer Gallery, Thermo Scientific, Waltham, MA, USA).

2.2. Application Study in White and Rose Wines

A total of 13 commercial wines were selected, 11 white and 2 rose wines, representing 2021 to 2024 vintages and a wide range of compositional properties. The most relevant attributes for this study are listed in Table 2.

The wines were spiked with 1 g/L egg white in addition to the naturally occurring protein to create a saturated solution and mixed thoroughly. After 24 h, the wines were clarified via centrifugation (11,000 rpm for 10 min, 5804 R–Benchtop Centrifuge, Eppendorf, Hamburg, Germany) and divided into 15 mL centrifuge tubes (control and CMC treatment in duplicates). The liquid CMC (5% concentration) was added to the treatment tubes to a final concentration of 100 mg/L and incubated for 7 days. All tubes (including controls) were inverted daily for mixing. For the final evaluation, all wines were filtered through 0.45 µm syringe filters (nylon 25 mm diameter, Biomed Scientific Ltd., Colombo, Sri Lanka), Bentotest® solution (Erbslöh Geisenheim AG, Geisenheim, Germany)

was added according to the manufacturer's instructions as a chemical alternative to heat treatment and the resulting turbidity caused by precipitating proteins was analyzed via spectrophotometer (Genesys 150, Thermo Scientific, Waltham, MA, USA) at 860 nm, as previously described [17]. Protein removal efficiency was calculated by subtracting the average turbidity in the treatment vials with CMC addition from the controls, everything containing the same amount of Bentotest® solution.

Table 2. Commercial wines selected for the study due to a wide range of the most important analytical attributes in the context of carboxymethyl cellulose functionality.

ID	Wine Description	Alcohol [%]	Titratable Acidity [g/L]	pH	Tartaric Acid [g/L]	Malic Acid [g/L]	Lactic Acid [g/L]	Glycerol [g/L]
W#1	2021 White Blend	12.9	6.6	3.43	2.5	3.3	<0.2	4.5
W#2	2022 Chardonel	12.1	5.8	3.71	1.9	3.2	0.2	6.2
W#3	2020 Chardonel	12.8	5.9	3.54	1.5	0.4	2.4	5.6
W#4	2024 White Blend	14.8	6.3	3.53	2.8	1.8	<0.2	8.3
W#5	2022 Traminette	10.2	6.4	3.98	1.6	0.3	5.2	5.7
W#6	2023 Vidal Blanc	13.5	5.9	3.39	1.9	2.2	<0.2	7.7
W#7	2021 White Blend	12.1	6.5	3.41	1.6	3.0	0.2	7.7
W#8	2023 White Blend	11.0	8.4	3.24	3.4	2.8	0.8	7.4
W#9	2023 Vignoles	12.0	9.8	3.62	1.7	6.4	<0.2	6.3
W#10	2022 Vignoles	11.7	8.8	3.47	1.7	5.5	<0.2	6.2
W#11	2023 Vidal Blanc	11.1	6.7	3.25	2.9	2.6	<0.2	4.2
R#1	2023 Rose Blend	10.9	6.3	3.26	3.3	0.5	1.1	7.3
R#2	2024 Concord Rose	11.2	6.6	3.29	3.5	1.7	<0.2	4.8

2.3. Data Analysis

Data analysis and visualization as well as statistical analyses via one-way Analysis of Variance and paired *t*-test were performed using SigmaPlot 15.0 (Systat Software Inc., San Jose, CA, USA).

3. Results

Previous studies suggested a correlation between pH, the surface charge of CMC and any resulting interactions in the complex system like wine [18,30]. If CMC should be used as an additive to remove protein from wine, the surface charge and reactivity need to be stable in the pH range of wine. A tool to monitor that behavior is the ζ-potential of the pure modified cellulose over a wider pH range. Figure 1 shows the results of that analysis.

One critical point is the pH at which the ζ-potential equals zero, since it represents the isoelectric point of the system. Similar to tannins, CMC is a diverse mix of different molecule sizes and molecular modifications, so knowing the average charge distribution provides a reference point to estimate the net charge at any given pH. In our analysis, CMC has an isoelectric point that is below the pH range of wine; in fact, the ζ-potential is dropping leading up to the isoelectric point, then showing an almost flat curve until it drops again sharply beyond pH 4.1. This indicates that CMC maintains a stable negative ζ-potential under wine pH conditions. High negative values would indicate that particles are unlikely to aggregate [29]; however, the value does not drop below −100, meaning that CMC is reactive due to its net charge without the tendency to self-aggregate and form a gel. This has been demonstrated at neutral pH before [30], showing that CMC can stabilize macromolecules under the right conditions. For this study, however, the ζ-potential indicates ideal conditions for the reaction with positively charged proteins and the removal of this group of compounds. The secondary reaction mechanism that is based on hydrophobic interactions [18] cannot be evaluated by analyzing the ζ-potential. This

could be an additional advantage of CMC over bentonite as a protein fining agent. While the attraction of opposite charges works as a function of pH in both additives, modified cellulose can bind hydrophobic proteins independent of their charge [32]. This effect can be evaluated in wine-like model systems with more complex protein compositions.

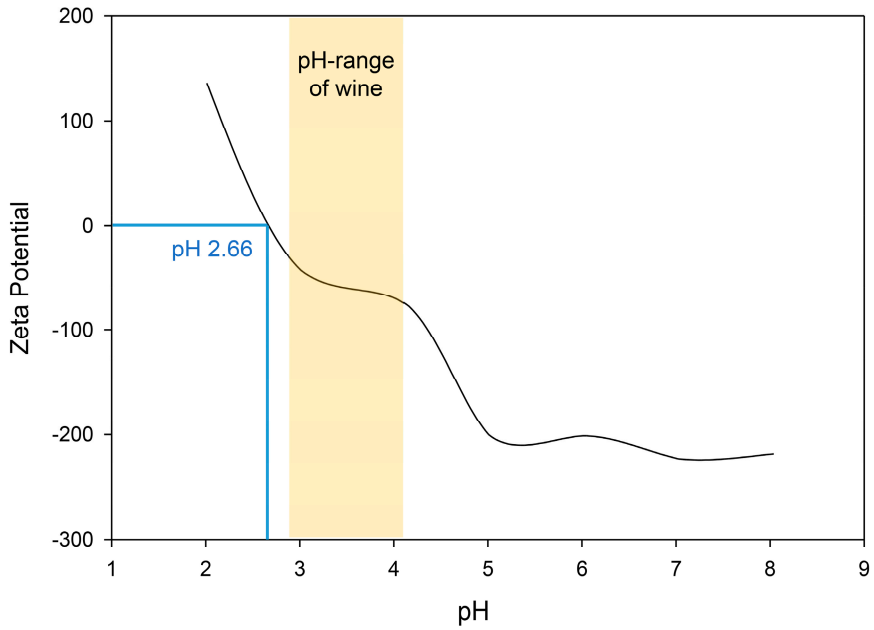

Figure 1. Zeta potential (ζ-potential) of a 2% (*w*/*v*) solution of carboxymethyl cellulose in water between pH 2 and pH 8.

3.1. Application Pre-Trials

Bovine serum albumin (BSA) and egg white protein have both been used in wine models before [17,18,24,33]. While BSA has similar properties to the proteins found in wine, it only represents a limited bandwidth of chemical and physical properties. It also shows substantial confirmational changes at wine pH [34], which limits its usefulness as a model protein. It was still included in the pre-trials due to its widespread use in the literature. The results of the model trials using both BSA and egg white, as well as two different CMC formulations, are shown in Figure 2.

Considering the total protein concentration at the beginning of the experiment, it becomes obvious that BSA does not dissolve as well as egg white protein at wine pH. Of the 1 g/L BSA that was added to the model, the photometric assay only shows 0.1 g/L. While there is a decrease in protein concentration after the addition of CMC in all cases, the level in the experiments with BSA (Figure 2a,b) is generally too small to see a real effect. It seems like the protein concentration even increases between day 5 and day 9, but the difference is minimal and does not lead to statistically significant differences in the end.

Egg white protein, on the other hand, shows higher dissolved protein starting values in the model wine (Figure 2c,d), which makes this a better model for this study. An interesting difference occurs between the addition of a dry powdered CMC (Figure 2c) and the liquid formulation (Figure 2d). The standard deviation and inconsistent behavior seem to be larger with the powdered form, which is consistent with previous observations [17–19]. CMC displays moderately hydrophobic behavior until it is completely dissolved and has formed a gel. This process might take a few days depending on the concentration and solvent composition. While the variability after 5 days can be explained by a mixing effect, there are no statistically significant differences between the CMC addition levels after 9 days. With the liquid CMC, however, the control is significantly higher after that time, even though there are no differences between the treatment levels. This indicates

that the reaction between proteins and CMC works in the model but it is not showing the concentration effect that was previously described [17].

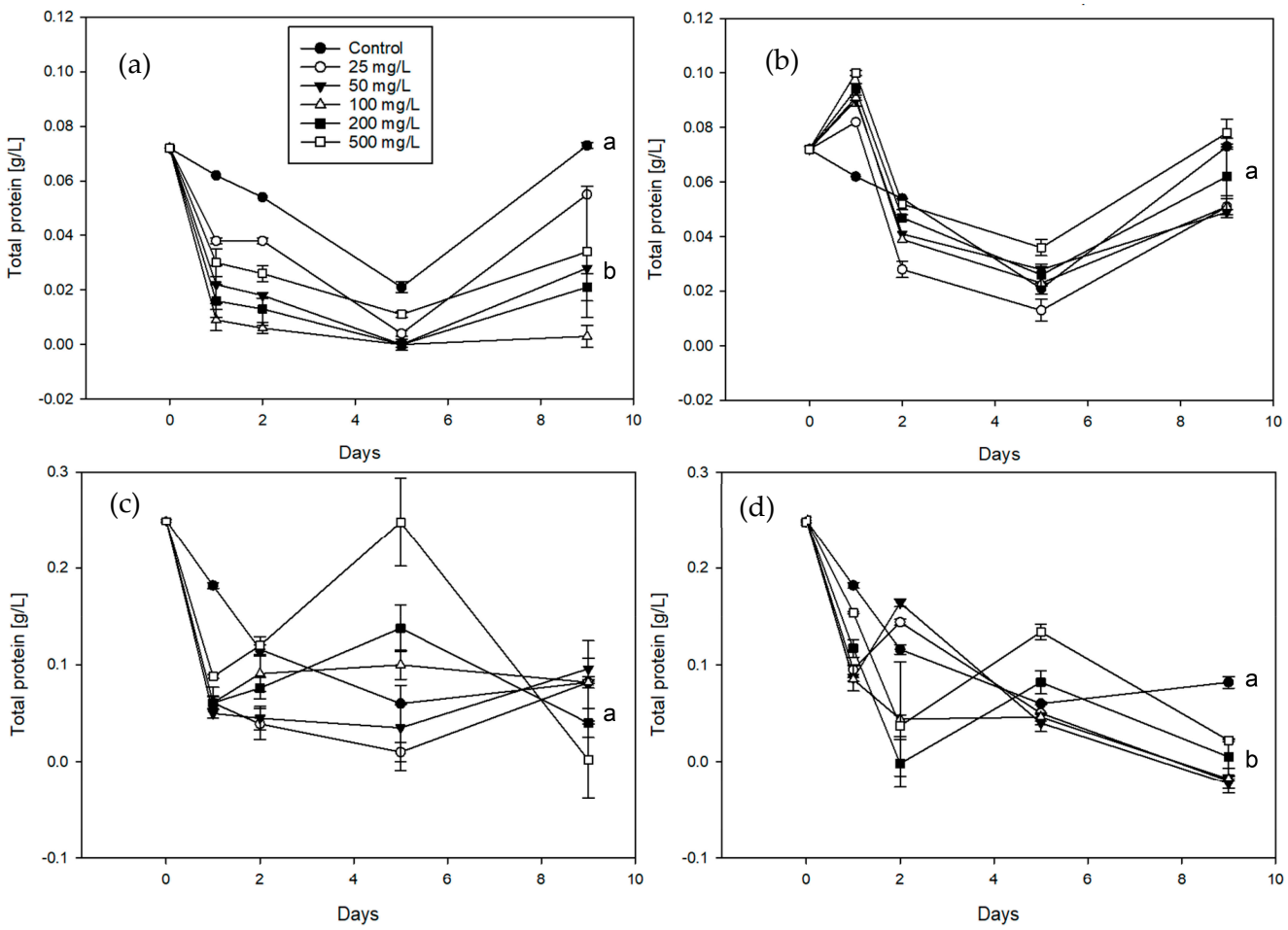

Figure 2. Residual protein analyses after the addition of carboxymethyl cellulose (CMC) to model wine over the duration of nine days ((**a**) egg white protein with powdered CMC; (**b**) egg white protein with liquid CMC; (**c**) bovine serum albumin with powdered CMC; (**d**) bovine serum albumin with liquid CMC). Statistically significant differences ($p < 0.05$) within each dataset are indicated using letters ab.

Since the model trials with BSA were inconsistent, the follow-up experiments in real wines were only performed with egg white protein. The results in a white and a rose wine are shown in Figure 3.

Interestingly, the solubility in real wine is very comparable to the model system, so the experiments start at a very similar level. The further drop in protein concentration, however, is following a somewhat different pattern than in the model wine. The use of powdered CMC (Figure 3a,c) leads to a quick reduction in protein concentration after only one day; however, after that, the level remains relatively unchanged. In white wine (Figure 3a), the concentration seems to fluctuate more, which is consistent with the data obtained in the model system. In the rose wine matrix, however, the protein concentrations remain very stable after day 1 of the experiment and show a clear concentration effect. With the control losing almost no protein over the course of nine days, an increase in CMC addition leads to a decrease in protein. There seems to be correlation where more CMC removes more protein, very similar to other fining agents like bentonite. Previous studies have found an inverse effect [17], suggesting that mixing and the three-dimensional structure of CMC

could lead to this phenomenon; however, this study shows that, at least for this specific rose wine, the reactivity of CMC follows the concentration effect.

The addition of liquid CMC (Figure 3b,d) shows a very different reaction behavior with protein. While the control and the high CMC addition rates lead to the highest protein retention, the lower CMC concentrations are responsible for significantly decreased final protein levels. The 25 ppm experiment especially leads to the lowest protein readings after 9 days, actually reaching that level after only 2 days in rose and 7 days in white wine. This confirms the previous findings mentioned above but raises the question as to whether there is a matrix effect that leads to that difference between white and rose wine and also why the liquid formulation behaves so differently from the powder. While the higher CMC addition rates remove more protein when added as a solid, they seem to stabilize the protein when added in a liquid form.

Since the objective of this study was to evaluate the use of CMC for protein removal, the experiments with commercial wines were performed with liquid CMC at low addition rates.

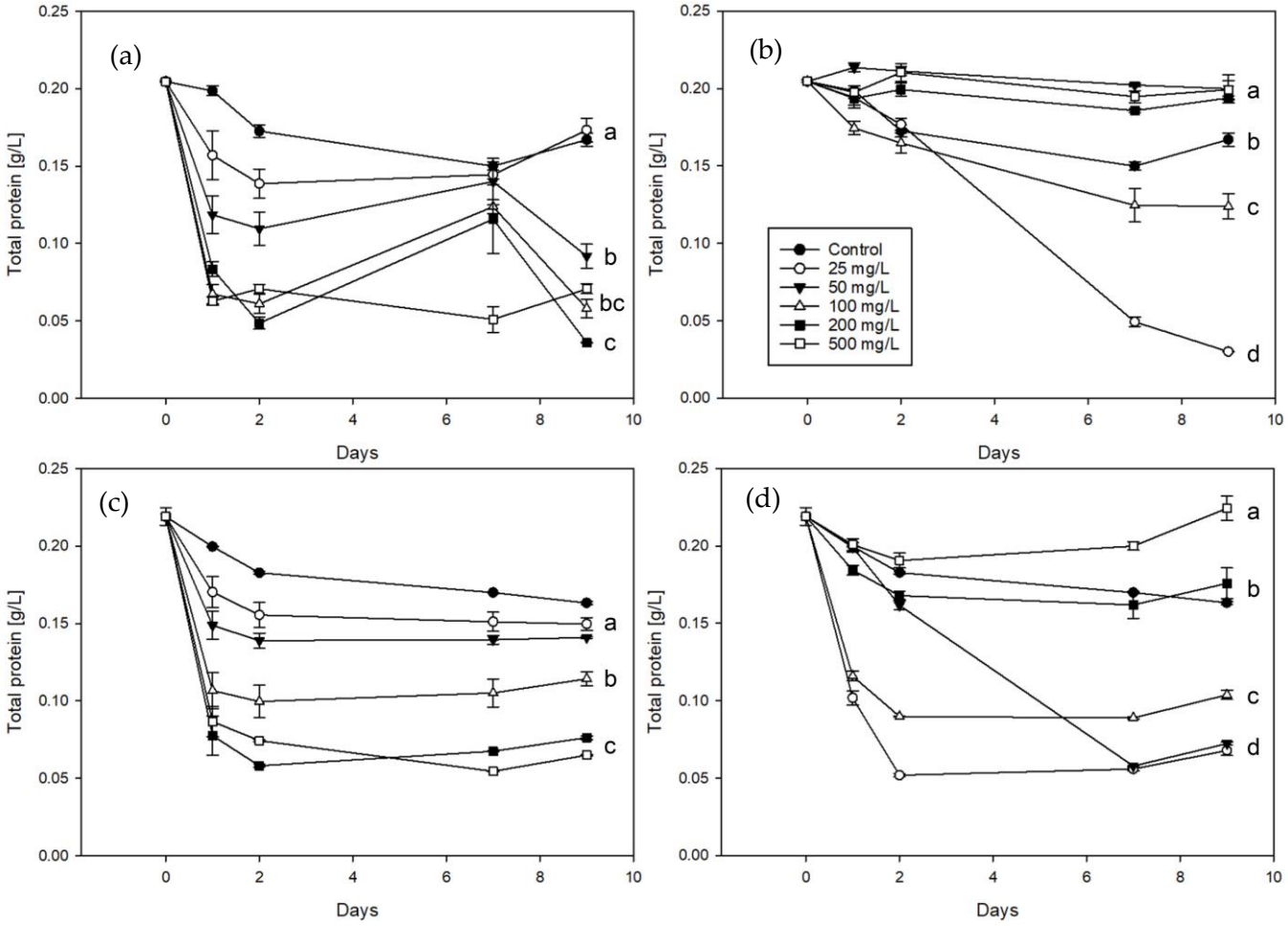

Figure 3. Residual protein analyses after the addition of carboxymethyl cellulose (CMC) to real wine over the duration of nine days ((**a**): egg white protein in white wine with powdered CMC; (**b**): egg white protein in white wine with liquid CMC; (**c**): egg white protein in rose wine with powdered CMC; (**d**): egg white protein in rose wine with liquid CMC). Statistically significant differences ($p < 0.05$) within each dataset are indicated using letters abcd.

3.2. Application in Commercial Wines

The results of all 13 commercial wines are shown in Figure 4 in a pairwise comparison between the control and the 100 ppm CMC treatment after seven days.

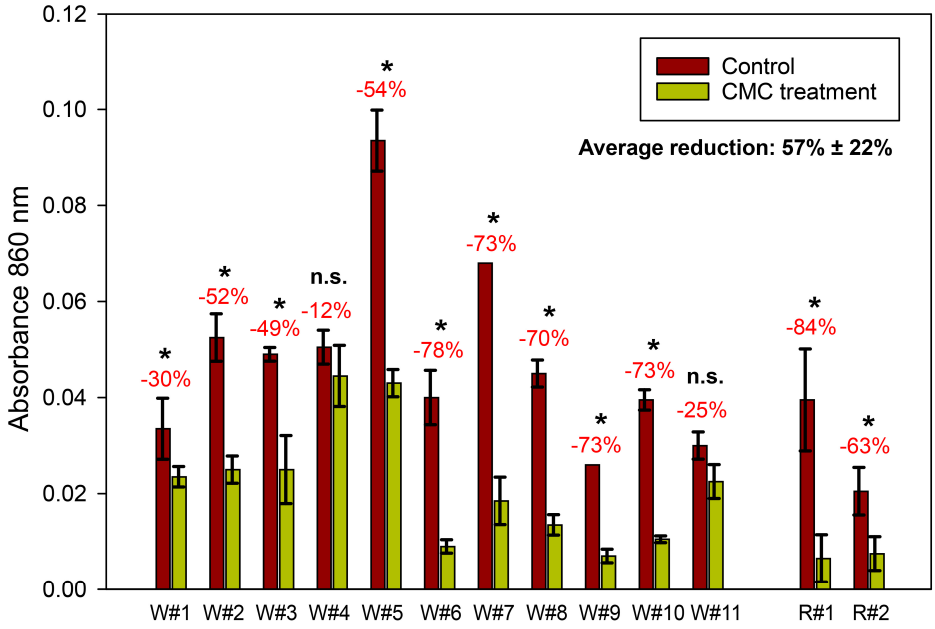

Figure 4. Reduction in haze formation in 13 commercial wines after the addition of carboxymethyl cellulose (CMC) and Bentotest® solution. Statistically significant differences ($p < 0.05$) between the control and the CMC treatment are indicated with an asterisk (n.s.: not significant). The average reduction in haze formation in percent is given for each wine.

The reduction in haze formation ranges from 12% to 84%, with an overall average of 57%. In comparison to the pre-trials, that range could be expected, even though the pre-tests were looking at total protein content while the commercial wine trials were assessing protein instability through precipitation. The range, however, is comparable and proves the concept of the use of CMC for protein precipitation.

Only two of the white wines (W#4 and W#11) showed a haze reduction that was not statistically significantly different from the control. It is unclear why the reaction did not work as well in these two samples. Correlation tests with any of the analytical results did not provide an answer. While the alcohol concentration in W#4 is very high and could be speculated to have an impact, W#6 is not much lower in alcohol and shows a fining efficiency of 78%. Acid strength and pH are also well within the range of the other wines and do not provide an explanation. However, with 70% of the wines showing an efficiency well over 50%, the use of CMC for selective protein removal can be adapted to finishing and stabilizing wines before bottling.

4. Discussion

While carboxymethyl cellulose is widely used in the food and bioengineering industries [35–37], its use in wine is limited to tartrate stabilization [38,39]. Similar to other additive options [40–42], the mechanism is described as preventing crystal growth by coating the potassium bitartrate nucleation points and keeping them in solution. The legal limit in wine is 100 mg/L [43], which is a fairly low concentration considering the amount of potential tartrate crystal material in wine. CMC has also been used to precipitate and purify proteins in applications unrelated to wine [44,45]. The reaction mechanism between CMC and proteins in wine has been shown before [18,19]; however, an application recommendation has not been made before. Any further information on the dosage levels and reaction patterns that would be necessary to achieve protein stability is missing. Previous application trials, in comparison to bentonite [17], revealed that the fining efficiency can be better than for other alternative additives, even though it might not reach the protein

removal rate of bentonite in all wines. Assuming that CMC can be legally used in wine at a concentration of 100 ppm, as shown for cold stability, this concentration was chosen as a middle level in these experiments. The data suggest that a lower concentration could be more efficient, while addition levels over 100 mg/L might be counterproductive. This at least seems to be true for a liquid formulation of CMC that had time to fully expand and form a gel. This behavior has been described in the literature for a wide variety of CMC concentrations [46].

Solubilizing a gel-forming, moderately hydrophobic molecule like CMC depends on various factors that all influence the use as a fining agent. Tannins, as a more common wine additive, are equally diverse in size and can be mildly hydrophobic as well [46], so pre-solubilizing them in wine or water is a viable strategy before adding them to the larger volume of wine. This could be an option for CMC as well, since it reduces viscosity and would make it easier to handle. The pH of the solvent, especially, as well as the matrix in which CMC is used determine its reactivity and chemical behavior [47]. It was shown with the analysis of ζ-potential that modified cellulose experiences molecular changes and expansion in an acidic environment. At a neutral pH, it behaves entirely different than in a wine environment [24,30], which creates a challenge in the context of its formulation. Liquid CMC was shown to be more efficient and effective here than the addition in a powdered form. The liquid formulation at a concentration of 5% uses water as a solvent, which creates a viscous gel at a neutral pH. In this state, the CMC mixes easily with other fluids like wine and is not going through conformational unfolding and uncoiling changes that were suspected to be responsible for the lower efficiency of powdered CMC [17]. When liquid CMC is mixed into wine at a much lower pH, however, the molecules will still undergo acidic expansion, which could explain the longer reaction time of several days that was observed in these experiments. The solubility of modified cellulose in water and water–ethanol mixtures is complex [48] and might need modification in low-alcohol wines or wines with high ethanol concentrations like Port-style dessert wines.

With an average protein removal efficiency of 57%, CMC proved to be a sufficiently active additive to be used in wine. It still remains unclear why two of the wines did not show the same protein binding capacity as the others. Matrix factors like polysaccharides were shown to interfere with macromolecular interactions depending on their net charge at wine pH [19,30,49], which could also be the reason why the precipitation did not work as well in those wines. Polysaccharides, due to their diverse molecular structure, can interact with molecules and chemical compounds of all sizes and functionalities in wine [50,51]. Examples for polysaccharides that have shown their potential as protective colloids working on a wide range of molecules are mannoproteins as natural wine constituents [24,52,53] and gum arabica as an additive [54]. A different wine that still showed substantial protein haze after CMC addition (W#5) started at a much higher level in the control, most likely due to insufficient protein removal before bottling. Muscat varieties like Traminette are known for their tendencies of protein instability [55], and egg white spiking results in the highest protein level of all wines in this experiment. The removal rate was still at 54%, indicating that, even when the wines might not be heat stable after CMC addition, it can still help with lowering the use of other fining agents like bentonite. The wider pH range in which CMC is reactive underlines the advantage that this strategy would have in finishing wines. Analyzing the ζ-potential of different CMC preparations and additional modifications, as suggested in the literature before [22,56], with various degrees of substitution [32] can ensure that the reactivity is stable in the pH range of wine, as seen in these experiments. Additional trials with liquid CMC formulations at a lower pH could help increase the efficiency by shortening the reaction time in wine.

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