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The Effect of Dicarboxymethyl Cellulose on the Prevention of Protein Haze Formation on White Wine

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Abstract: Wine clarity is a critical aspect in the commercialization of white wines. The formation of wine haze can be attributed to the aggregation and precipitation of heat-unstable wine proteins. Bentonite fining is the commonly used method in winemaking for protein removal, but it is responsible for loss of wine volume and quality. Dicarboxymethyl cellulose (DCMC) was developed as a potential alternative to bentonite. Water-insoluble DCMC was prepared via catalyzed heterogeneous etherification using sodium chloromalonate and potassium iodide. White wine fining trials were benchmarked with different dosages of DCMC against a bentonite. A high-performance liquid chromatography method was optimized for protein quantification. The samples underwent heat stability tests to evaluate wine turbidity before and after fining. Results show that DCMC successfully reduced the wine protein content and turbidity. DCMC produced heat-stable wines with dosages higher than 0.25 g/L. The innovative application of DCMC in the wine sector shows potential due to its ability to stabilize white wines while overcoming problems associated with bentonite, such as lees production and loss of wine, contributing to a more sustainable process.

Keywords: bentonite; dicarboxymethyl cellulose; wine protein haze; cellulose derivative; sustainability

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1. Introduction

Wine turbidity is a serious aesthetic problem that affects the commercialization of white wines. Wine stabilization and clarification has been thoroughly studied over the years [1]. One of the most prevalent causes of white wine haze occurs from the aggregation and denaturation of grape pathogenesis-related proteins, namely thaumatin-like (TLP) and chitinase proteins. The conditions associated with protein haze are the exposure of wines to high temperatures and long-term storage [2]. To avoid this phenomenon, proteins are frequently removed through bentonite fining.

Bentonite is a negatively-charged clay at wine pH that binds to proteins positively charged at wine pH [3]. Though effective, bentonite presents several drawbacks that include lees formation, loss of wine and stripping of natural aromas of the wine [4,5]. Bentonite is a non-specific fining agent, removing compounds besides proteins, which may directly affect sensory qualities of the wine [5]. Nonetheless, it is still the predominant process used in the winemaking industry [4]. These drawbacks drive the search for new wine protein removal methods and materials. Several techniques and materials have been studied as alternatives to bentonite fining [3,6–9].

Dicarboxymethyl cellulose (DCMC) is a cellulose derivative prepared via heterogeneous catalyzed etherification. The polymer is negatively charged at wine pH due to the presence of malonic groups, which makes it suitable for removing positively charged compounds such as dyes [10]. DCMC has similar structure and properties to carboxymethyl cellulose (CMC), a compound commonly used for wine tartrate stabilization [11]. CMC, having an acidic group with a pKa near 4.5, becomes mostly uncharged at a pH below

3.5, which is common in white wines [12], failing to promote ionic exchange and remove positively charged proteins. Nevertheless, the malonic carboxylate groups are more acidic than the one present in CMC and, consequently, more prone to be deprotonated at wine pH due to its first pKa (approx. 3) [13].

The aim of this work was to evaluate the use of dicarboxymethyl cellulose in wine stabilization as an alternative to bentonite fining. The ability of DCMC to remove wine proteins and, consequently, produce heat-stable wines was studied. A protein quantification method by high-performance liquid chromatography (HPLC) was developed.

2. Materials and Methods

2.1. Materials and Equipment

Malonic acid (99% purity), anhydrous sodium carbonate and potassium iodide at commercial grade were purchased from Panreac (Barcelona, Spain). Trichloroisocyanuric acid (95% purity) was purchased from TCI Chemicals (Zwijndrecht, Belgium). Sodium hydroxide (97% purity), bromine (reagent grade, minimum 98% purity) and thaumatin (from *Thaumatococcus daniellii*) were purchased from Sigma Aldrich (Dasmstadt, Germany). Methanol (ACS reagent), isopropanol (ACS reagent) and acetic acid (glacial) were purchased from Supelco (Bellefonte, PA, USA). Anhydrous sodium carbonate and potassium iodide were preexistent in the laboratory without a commercial reference. Food grade cellulose and bentonite (Pluxbenton N, L53739–12/2022) were obtained from ESSECO (San Martino di Trecate, Italy). Regenerated cellulose dialysis tubing (Spectra/Por® 4, 12–14 kDa MWCO, Spectrum Laboratories Inc. Rancho Dominguez, CA, USA) was purchased from Fisher Scientific (Waltham, MA, USA). Trifluoroacetic acid, acetonitrile and water of HPLC grade were obtained from Carlo Erba reagents (Cornaredo, Italy). A white wine 2019 vintage from a blend of varieties was gifted by the Primavera winery, Bairrada region, Águeda, Portugal.

Infrared spectra were recorded on a Perkin-Elmer FT-IR Spectrometer Spectrum Two (Waltham, MA, USA), equipped with an attenuated total reflection (ATR) cell. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker ARX400 (Karlsruhe, Germany) at 400 and 100 MHz, respectively. Inductively coupled plasma-atomic emission spectroscopy (ICP-AES) was performed using a Horiba Jobin–Yvon Ultima (Kyoto, Japan) model equipped with a 40.68 MHz RF generator, a Czerny–Turner monochromator with 1.00 m (sequential), and an autosampler AS500. X-ray diffraction (XRD) was determined using a Rigaku D/Max-C Wide Angle automated X-ray diffractometer (Tokyo, Japan) with a vertical goniometer (Cu, 30 kV, 15 mA). HPLC analyses were carried out in a Waters Alliance system (Waters, Milford, MA, USA), consisting of a 2695 Separation Module, 2996 Photodiode Array detector, and Empower 3 software.

2.2. Dicarboxymethyl Cellulose Synthesis

2.2.1. Synthesis of Sodium Chloromalonate (NaCMA)

The preparation of chloromalonic acid was based on the monohalogenation of β-dicarbonyl compounds described in the literature [14,15]. Malonic acid was dissolved in deionized water (250–400 molar eq). The solution was stirred and trichloroisocyanuric acid (0.66 molar eq) was added. The mixture was stirred for 5 h at 15 °C. After this time, the reaction mixture was neutralized with the appropriate quantity of sodium carbonate. Then, the mixture was filtered, and the filtrate was dried under vacuum at room temperature (conversion: 97%). ^{1}H NMR (400 MHz, D_2O) δ 6.20 (–CClH–) ^{13}C NMR (100 MHz, D_2O) δ 168.91 ($–COO^-$), 65.79 (–CClH–). IR (FTIR-ATR) 3410, 1650, 1620, 1390, 1330, 1190, 1170, 920, 788, 726 cm^{-1}.

2.2.2. Synthesis of Dicarboxymethyl Cellulose

Cellulose was vigorously stirred in isopropanol and three molar equivalents of an aqueous solution of sodium hydroxide (40%) were slowly added. The mixture was stirred for 1 h at room temperature. Then, the appropriate amount (see Table 1) of solid sodium

chloromalonate was added. Sodium hydroxide (1 eq) and potassium iodide (0.1 eq) were dissolved in water and added dropwise to the mixture, resulting in an isopropanol/water mixture of 88:12 and in a relation of 40 mL of isopropanol/g of cellulose. After complete dispersion, the mixture was placed on a water bath at 60 °C for 5 h with vigorous stirring. After this time, the reaction mixture was filtered and the solid suspended in 70% (*v*/*v*) methanol. The pH was adjusted to 5 with glacial acetic acid. The product was washed with 70% (*v*/*v*) aqueous methanol followed by pure methanol. After drying, the product was suspended in water and sodium carbonate was added until neutral pH. The resulting deprotonated product was dried under vacuum at room temperature. For characterization purposes, a small sample was purified by dialysis against deionized water for 48 h, using a dialysis tubing with a molecular weight cut-off of 12–14 KDa, and then lyophilized.

Table 1. Conditions for dicarboxymethyl cellulose (DCMC) polymers production.

Sample	Molar Ratio AGU:NaCMA:NaOH [1]	$m_{Cellulose}$ (g)	m_{NaCMA} (g)	m_{NaOH} (g)
DCMC 1	1:1:4	3.00	3.53	2.94
DCMC 2	1:0.3:4	3.00	1.06	2.94

[1] anhydroglucose units/sodium chloromalonate/sodium hydroxide.

2.2.3. Degree of Substitution Determination

The degree of substitution (DS) is the average number of hydroxyl groups replaced by sodium carboxymethyl groups (-CH (COO^-Na^+)$_2$) at C2, C3, and C6 in the cellulose structure [16]. The DS was calculated from the sodium content in samples based on a procedure previously described in literature [17].

2.2.4. Structural Characterization of Dicarboxymethyl Cellulose

FTIR spectra of dicarboxymethyl cellulose were recorded in a spectral range of 4000 to 400 cm^{-1}. All spectra were normalized to minimize errors related to differences in mass, pressure, and dryness of the sample. X-ray diffractograms of DCMC samples were analyzed in a 2θ angle range of 5° to 60° at a scan rate of 1°/min and with a step size of 0.02°.

2.3. Wine Fining Trials

2.3.1. Wine Characterization

The producer sent us an unstable non-fined wine that was used in this trial. Analysis data: pH = 3.03, ethanol (% *v*/*v*) 12.8, total phenols (g GAE/L) 7.888, total SO_2 (mg/L) 143, free SO_2 (mg/L) 63, total acidity (g tartaric acid/L) 6.55, and free acidity (g tartaric acid/L) 0.35.

2.3.2. Adsorption of Wine Proteins

The wine was centrifuged at 5230× *g* for 15 min in a Universal Centrifuge Z306 (Hermle LaborTechnik, Wehingen, Germany). This procedure assured that all suspended solids were removed from the wines by decantation. Wine adsorption experiments were performed in 15 mL falcons with 10 mL of wine in the presence of increasing polymer and bentonite concentration (0, 0.125, 0.25, 0.5, 1.0, and 2.0 g/L). The falcons were placed horizontally on an orbital shaker and kept at room temperature (25 °C) for 66 h. All experiments were performed in triplicate.

2.3.3. Heat Stability Test

Following contact with the polymers or bentonite, the wines were filtered (0.45 µm Nylon syringe filters, Filter-Lab (Barcelona Spain) and transferred to new 15 mL falcons. The samples were heated at 80 °C for 2 h and subsequently cooled in ice for 2 h. After equilibration at room temperature, the increase in turbidity was detected spectrophotometrically at 540 nm. All measurements were performed in triplicate for posterior statistical

analysis. Before any contact with the fining agents, the wine presented an Δabs (540 nm) of 0.23. Since it is considered unstable, the wine was suitable for this study.

2.3.4. Protein Quantification

The protein content of the wine samples was determined by reverse-phase HPLC. All samples were filtered (0.45 µm Nylon syringe filters, Filter-Lab, Barcelona Spain) prior to injection. Samples (50 µL) were loaded at 1 mL/min onto a Vydac® C18, 5 µm, 4.6 × 150 mm. Separation was achieved with a solvent system of 0.1%TFA/H_2O (solvent A) and 0.1% TFA/ACN (solvent B). The method was adapted from Marangon et al. [18] and the mobile phase gradient was as follows: 0–7 min, 20.2–43.3% B; 7–15 min, 43.3–49.0% B; 15–16 min, 49.0–54.8% B; 16–30 min, 54.8–66.3% B; 30–32 min; 66.3–66.3% B; 32–35 min, 66.3–80.0% B; 35–37 min, 80.0 –80.0% B; 37–40 min, 80.0–90.0% B; 40–42 min, 90.0–90.0% B; 42–44 min, 90.0–20.2% B; and 44–50 min, 20.2–20.2% B. Quantification was achieved using a standard curve of thaumatin (Thaumatin from *Thaumatococcus daniellii*, Sigma, Castle Hill, NSW, Australia). The retention time of this commercial thaumatin was also confirmed using protein isolated from white wine. Results are provided as mg/L of thaumatin equivalents. Peaks eluting between 8 and 12 min were considered thaumatin-like proteins (TLPs), whereas peaks between 18.5 and 24.5 min were considered chitinases [18].

2.4. Statistical Analysis

Statistical analyses were performed using Microcal Origin software (OriginLab, Northampton, MA, USA) One-way analysis of variance (ANOVA) was conducted to compare the difference among the different dosages (control, 0,125 g/L, 0.25 g/L, 0.5 g/L, 1 g/L, and 2 g/L) of DCMC and bentonite addition over white wine. The means were separated using the Tukey method and different letters represent significant differences at $p \leq 0.05$. The standard deviation was calculated from the analysis of three replicates within one treatment.

3. Results and Discussion

3.1. Synthesis and Characterization of Dicarboxymethyl Cellulose Sodium Salt

The preparation of DCMC followed a method previously described, with some modifications [10,17,19]. Dicarboxymethyl cellulose (DCMC) as its sodium salt (NaDCMC) was prepared by reacting sodium chloromalonate (NaCMA) with cellulose. A schematic diagram of the reaction is shown in Figure 1.

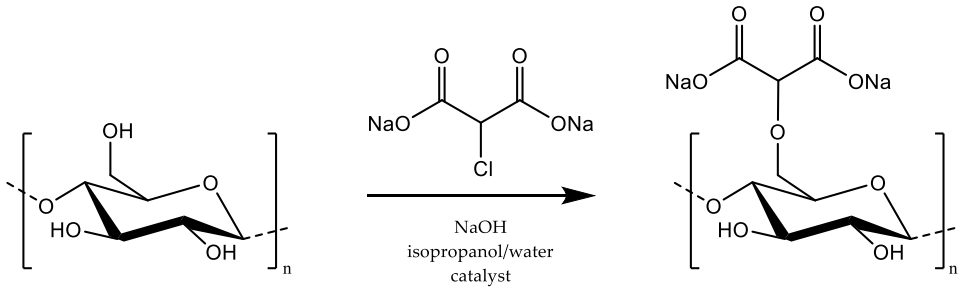


Figure 1. Schematic representation of the synthesis of dicarboxymethyl cellulose sodium salt.

The synthesis of NaDCMC is a two-stage process that includes mercerization and etherification of cellulose. The mercerization step promotes the swelling of cellulose, increasing the availability of hydroxyl groups [6]. In the etherification step, the mercerized cellulose was treated with sodium chloromalonate (NaCMA).

NaCMA was produced through the α-chlorination of malonic acid with trichloroisocyanuric acid (TCICA). TCICA has three transferable chlorines, and the ratio of reactants is usually based on the number of chlorine atoms needed (0.33 or 0.66 molar equivalents for mono- and di-halogenation, respectively). However, since TCICA reacts slowly with water,

electrophilic chlorine atoms are not readily available. For this reason, the monohalogenation of malonic acid was performed using 0.66 molar equivalents of trichloroisocyanuric acid [14]. Detailed information on the spectral characterization of sodium chloromalonate can be found in the supplementary materials. For ^{1}H NMR, ^{13}C NMR and FTIR spectra of NaCMA, see Figures S1–S3, respectively.

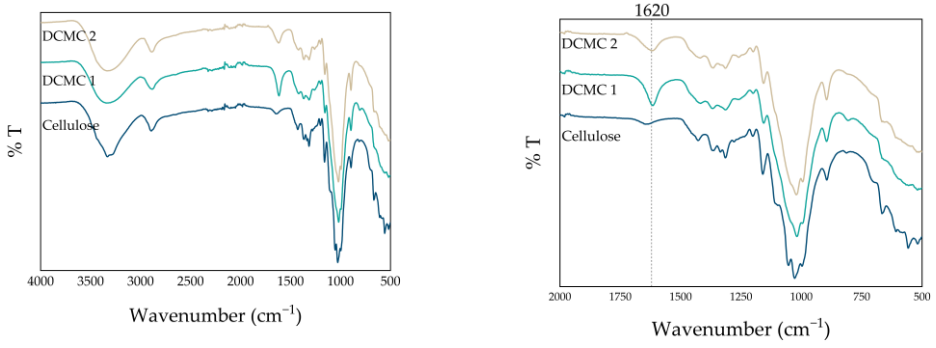
DCMC was synthesized with 1 and 0.3 molar equivalents of sodium chloromalonate. Results obtained on the sodium content and varying degrees of substitution of the synthesized polymers are given in Table 2. DCMC 1 and DCMC 2 had a DS of 0.03 and 0.01, respectively. From these results, we could assume a direct correlation between NaCMA equivalents and DS. The three-fold increase in NaCMA equivalents tripled the DS value. Both polymers have a low degree of substitution, well below the limit of water solubility (DS of approximately 0.5). Therefore, DCMC 1 and 2 are water-insoluble. The resulting polymers developed with this synthetic process must be heavy metals-free, which is of great importance for their application in the food industry.

Table 2. Degree of substitution of synthesized dicarboxymethyl cellulose sodium salt.

Sample	NaCMA eq [1]	% Na	DS
DCMC 1	1	0.85	0.03
DCMC 2	0.3	0.32	0.01

[1] sodium chloromalonate molar equivalents to anhydroglucose units.

The infrared spectra of NaDCMC and pure cellulose are shown in Figure 2. The following results confirm the presence of carboxylate groups, which is evidenced by an increase in intensity and broadening of the peak at 1620 cm^{-1}. NaDCMC polymers with a higher DS have a higher number of carboxylate groups [10]. Figure 2 shows that DCMC 1 has a more intense peak than DCMC 2, which corroborates the results obtained for the degree of substitution of the polymers. Table 3 presents the peak assignment of the functional groups, according to the literature.



(**a**) (**b**)

Figure 2. (**a**) Full FTIR spectra; (**b**) zoom-in region for the carbonyl stretching vibrations.

Table 3. FTIR peak assignments of DCMC.

Wavenumber (cm^{-1})	Assignment	Reference
3300	-OH stretching	[20,21]
2890	C-H stretching	[20,21]
1620	-COO^{-} asymmetric stretching	[21]
1429	-COO^{-} symmetric stretching	[21]
1100	-C-O-C	[20,21]
1020	-C-O-C	[20,21]

The XRD patterns of cellulose and synthesized dicarboxymethyl cellulose sodium salt samples are presented in Figure 3. The observed peaks at 16.1° and 22.4° are characteristic of cellulose I type diffractograms [22–25]. Diffraction patterns of DCMC show that the aforementioned peaks disappeared completely, indicating that the hydrogen bonds between celluloses were weakened and the crystal structures were destroyed. DCMC samples showed new signals at 12.5° and 20.5°, which are attributed to a less crystalline structure resulting from a successful etherification of cellulose. The peak at 22.4° shifted to 20.5° and presented lower intensity for DCMC 1 than DCMC 2. This decrease is consistent with the results obtained for the increasing degree of substitution. Increasing replacement of hydroxyl groups by carboxylate groups reduces crystallinity and increases the amorphous matrix of the samples. Therefore, since DCMC 1 has a higher DS than DCMC 2, its structure is more amorphous, an indicator for the derivatization of the cellulose backbone.

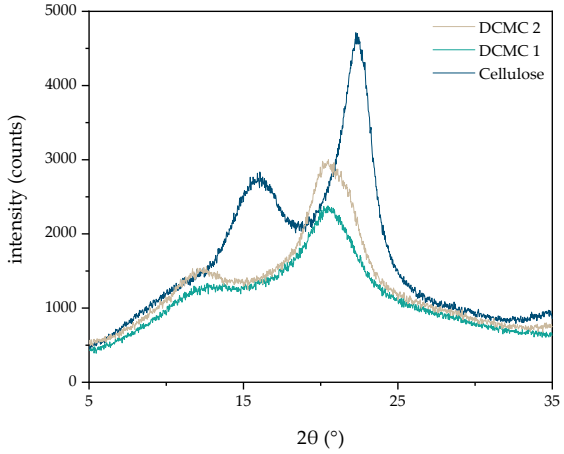


Figure 3. XRD pattern of cellulose and dicarboxymethyl cellulose sodium salt.

3.2. Wine Fining Trials

The effect of heating on the wine samples was investigated by performing a heat stability test (HST). Differences in wine turbidity (before and after the heat treatment) have been shown to correlate directly with wine protein instability [26]. In practical terms, a wine is considered unstable when the difference in absorbance between heated and unheated controls is higher than 0.02 absorbance units [27]. Following the HST, the control wine was considered unstable since the Δabs at 540 nm was of 0.23 ± 0.0011. The wine produced a significant amount of haze and was, therefore, considered heat-unstable.

The adsorption experiments were carried out by adding different amounts of dicarboxymethyl cellulose sodium salt or bentonite to the sample wine. The experiments were conducted in 15 mL falcons placed horizontally in an orbital shaker, since DCMC sediments easily and this display allows a better contact between wine and DCMC. Figure 4 shows the result of the HST applied to the sample wine after contact with the stabilizing agents. The heat stability test provides information on the haze potential of the wine based on the turbidity data. A correlation between adsorbent concentration and wine haze is evident. With increasing dosages, the turbidity of the resulting wine is reduced. Based on the heat test, wines treated with all concentrations of DCMC 02 remained unstable, whereas wines treated with more than 0.25 g/L of DCMC 01 were stabilized. Bentonite fining required more than 0.5 g/L to stabilize the wine. The difference in adsorption capacity for the two DCMC samples can be explained by the number of carboxylate groups present on the polymer. Increasing the molar equivalents of the etherification agent increases the degree of substitution. An increase in the degree of substitution (DS of 0.03 and 0.01 for DCMC 1 and 2, respectively) allowed for a higher adsorption of positively charged wine proteins due to the increased availability of binding sites. Therefore, DCMC promoted wine stabilization as verified by heat stability test. Contrarily to other methods described in the literature, DCMC can be used in very low doses and without heating the samples [3,8].

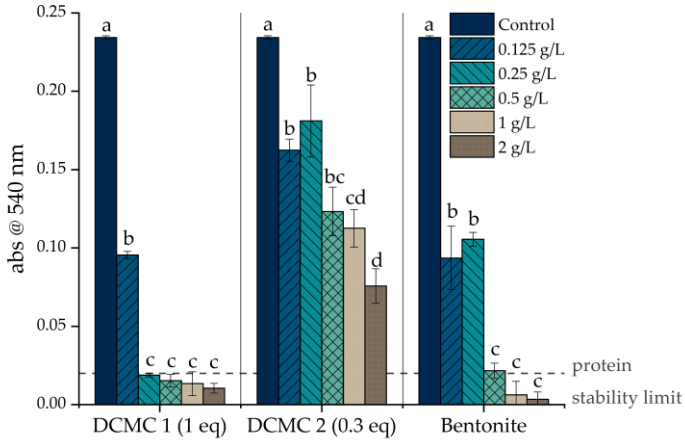


Figure 4. Heat stability test results of Primavera wine treated with DCMC 1, DCMC 2, and bentonite. Different letters represent significant differences among different homogeneous subsets for $p = 0.05$.

To understand the impact of the protein concentration in the wine stability, total protein was quantified in both control and fined wines. To surpass some problems inherent to colorimetric methods applied to wines, namely failure to correctly estimate protein content with the Bradford assay and overestimation of protein content with Smith and Lowry assays [5], an HPLC protein quantification method was applied. A standard calibration curve was made using standard concentrations of thaumatin (from 15 to 250 mg/L) dissolved in water. Relative protein quantification of the wine samples was carried out by comparing the area of the peaks eluted from min 8 to min 11.5 and from min 18.5 to min 24.5 (for TLPs and chitinases, respectively) against the area of the thaumatin standard [18].

To determine which peaks in the wine chromatogram could be attributed to proteins, control wine samples were passed through a gel permeation chromatography column (PD-10) to remove all compounds smaller than 5 kDa. The majority of peaks disappeared after the gel permeation. Only the remaining peaks at 7.6 and 10 min were considered when calculating peak integration of the wines. Analysis of the control wine showed that it had a total concentration of 97.82 mg/L of TLPs and no proteins eluted after 12 min. For this reason, it was considered that the concentration of chitinases in this wine sample was negligible.

Following fining, the wines were analyzed by the same HPLC method. The concentrations of TLPs decreased with increasing adsorbent dosages, for both wines treated with DCMC and bentonite. The results show that, after treatment with the polymer (DCMC 01 and DCMC 02) and bentonite, the peak at min 9 was significantly reduced in all chromatograms. Fining induced a protein content reduction in the ranges of 72–75%, 57–66%, and 64–71% for wines treated with DCMC 1, DCMC 2, and bentonite, respectively. Figure 5 shows the HPLC chromatograms of the heat-stable wine fined with the minimum doses of DCMC 1 and bentonite (0.25 and 0.5 g/L, respectively).

Results from protein stability and quantification analyses are summarized in Table 4. When both analyses are considered, the results show that DCMC 1 performed better than all the alternatives tested. Fining with 0. 25 g/L of DCMC 1 and 0.25 g/L of bentonite removed 75% and 77%, respectively, of the total protein. However, according to the HST, DCMC produced stable wine whereas bentonite did not. The results also show that wines fined with DCMC are stable when protein content is below 27 mg/L, whereas bentonite fining can stabilize wines with up to 35 mg/L. The different protein content limits required for wine stabilization may be explained by the non-specificity of bentonite. If bentonite removed other compounds besides protein, wine haze potential was reduced even with a higher protein content. These results support the claims that haze formation is a multifactorial process and not only linked to protein concentration. Protein content plays a significant role in wine turbidity, but other factors (wine pH, phenolic compounds,

alcohol levels) also have impact wine haze [1,28,29]. The impact of DCMC on these factors must be accounted for in future works.

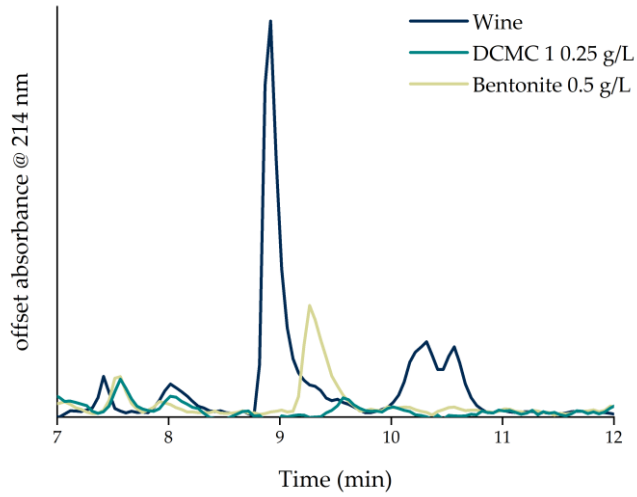


Figure 5. HPLC chromatograms of Primavera wine and the same wine treated with 0.25 g/L of DCMC 1 and 0.5 g/L of bentonite.

Table 4. Summary of the protein stability and protein quantification results.

Sample	Dosage (g/L)	Heat Test (Δabs)	Protein Content (mg/L)	Protein Removal (%)	Results (S/U) [1]
Control	NA	0.23	97.82	NA	U
DCMC 1	0.125	0.10	27.68	72%	U
	0.25	0.02	24.79	75%	S
	0.50	0.02	26.67	73%	S
	1.00	0.01	24.50	75%	S
	2.00	0.01	26.55	73%	S
DCMC 2	0.125	0.16	34.93	64%	U
	0.25	0.18	38.21	61%	U
	0.50	0.12	41.79	57%	U
	1.00	0.11	42.03	57%	U
	2.00	0.08	33.47	66%	U
Bentonite	0.125	0.09	28.42	71%	U
	0.25	0.11	22.70	77%	U
	0.50	0.02	35.07	64%	S
	1.00	0.01	31.92	67%	S
	2.00	0.00	32.08	67%	S

[1] Stable (S) or Unstable (U) wine.

4. Conclusions

Sodium chloromalonate was successfully synthesized and used as an etherification agent in the preparation of dicarboxymethyl cellulose. DCMC sodium salt showed a high protein removal efficiency (up to 75% for DCMC 1 above 0.25 g/L of wine), reducing the haze formation of these wines. A minimum dosage of 0.25 g/L of DCMC was required to produce heat-stable wines. The results demonstrate that dicarboxymethyl cellulose can be used for white wine stabilization. DCMC produced heat-stable wine with low doses and without heating the samples. The HPLC protein quantification method was established for wine analysis, providing a fast and reliable method for protein quantification, and a correlation between protein instability and protein quantification was verified.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/beverages7030057/s1, Figure S1: ^{1}H NMR (D_2O, 400 MHz) spectrum of NaCMA; Figure S2: ^{13}C NMR (D_2O, 100 MHz) spectrum of NaCMA; Figure S3: FTIR (ATR) spectrum of NaCMA.

Author Contributions: D.G., R.C. and L.M.F. conceived and designed the experiments. D.G. performed the experiments and analyzed the data. D.G. wrote the paper with contributions from the remaining authors. R.C. and L.M.F. supervised the execution of analyses and revised the data and the manuscript. All authors have read and agreed to the published version of the manuscript.

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