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Addressing Enzymatic Clarification Challenges of Muscat Grape Juice

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Abstract: Winemakers use technical enzymes to assist with clarification, extraction, and other processes in winemaking. In some cases, enzyme mixes are found to be ineffective for a variety of reasons. This study characterizes difficult-to-clarify juices from the Muscat family, examines the effects of pasteurization, and classifies these juices based on cultivar, harvest date, geographical location, and harvesting technique. In addition to studying the chemical compositions of different Muscat juices, enzyme testing was performed by creating enzyme cocktails and evaluating their functionality. The data suggest a distinct matrix effect on juice clarification that can be influenced during juice processing. Berry proteins, polysaccharides, and native enzymes play an important role during the clarification process, influencing the efficiency of technical enzymes. On the other side, high macromolecule extraction from the grape material, through excessive shearing forces in machine-harvested and processed fruit, for example, can have a negative effect, especially in ripe and overripe grape material. Based on these findings, the winemaking strategy and use of technical enzymes need to be adapted to the incoming grapes. Besides adjusting the mechanical forces to the level of ripeness, avoiding native fermentation prior to clarification should be prioritized. The enzyme mixes developed and tested in these experiments show a high degree of efficiency in the majority of juices that were evaluated.

Keywords: polysaccharides; turbidity; protein; polyphenols; pectinase

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1. Introduction

White wines are produced from grapes without long maceration times, usually directly after destemming, crushing, and pressing. Rapid processing limits the extraction of polyphenols and polysaccharides that would otherwise impact the flavor and mouthfeel of the finished wine [1]. White wines are expected to be fresh and aromatic without noticeable bitterness or astringency. In particular, flowery cultivars of the Muscat family—for example, Muscat Canelli, Muscat of Alexandria, or Orange Muscat—need to be fruit-forward to match consumers' stylistic expectations [2]. Enological practices to achieve this goal include gentle processing of the grapes to minimize shearing forces [3], stabilizing juice with sulfur dioxide to prevent aroma and phenolic oxidation [4], the use of technical enzymes to increase clarity prior to fermentation [5], and rapid onset of fermentation with a specialized yeast strain that develops and preserves the typical aroma character [6]. Studies have shown that the clarification step, in particular, is essential for aroma development and flavor preservation [7,8].

Various methods exist in the wine industry to clarify grape juice prior to fermentation, ranging from technological solutions like centrifugation, filtration, and flotation to simple physical settling of the solids [9]. Variations of the settling technique also exist, for example, applying low temperatures to prevent native fermentation, the use of technical enzymes to speed up the process and reduce juice losses [5], or adding a fining agent to increase

efficiency [9]. Over-clarification, however, is associated with a loss of varietal aroma [10] and should be balanced according to the stylistic goal for each specific wine [3]. For that reason, settling juices with the addition of enzymes is probably the most widespread clarification method in the wine industry. The most common class of enzymes used for juice clarification prior to fermentation is pectinases [5]. Due to the complex structure of a pectin molecule, a large variety of different enzymes and activities fall under that category. Examples are pectin esterases, which remove methyl side chains, and polygalacturonases, which are responsible for breaking up the main pectin chain [5,11,12]. Polysaccharides can form substantial networks in solution and keep solids and other macromolecules in suspension [13]. With the degradation of these gel networks, the settling process is quicker and more thorough than clarification without enzymes, leading to a cleaner juice with a more compact deposit of solids [12]. The most common commercial clarification enzymes that are available for wine contain a mixture of these activities and are expected to break a variety of different pectin structures into small soluble fragments that no longer form gels [14]. Some preparations also have other main or side activities, such as cellulase, hemicellulase, and glucanase activities, which can help to break down other structural polysaccharides and metabolites from microbial infections like *Botrytis* or various lactic acid bacteria [5].

Enzyme function and clarification problems can occur if, for example, the juice is microbially impacted by native yeast and bacteria, leading to a spontaneous fermentation that prevents the solids from settling. Other organisms like *Botrytis cinerea* can produce glucanes that increase the juice viscosity and impact the clarification and filterability [15]. Physical properties like temperature and pH can also affect the functionality of technical enzymes [16]. While these factors are fairly well understood, certain cultivars like Muscat varieties can cause unexpected challenges during processing. For reasons that have not been previously described, the addition of technical enzymes might not have consistent effects throughout the harvest season. According to observations from the wine industry, enzymes generally work better early in the season, and their efficiency can decrease with increased ripening (unpublished industry communication). While this observation seems to be consistent over multiple vintages, no pattern has been shown or investigated up to this point to the best of our knowledge.

The objective of this study was to analyze the settling behavior of the most common Muscat varieties with and without enzyme addition, with and without pasteurization, and with different enzyme formulations, and correlate observations during clarification with processing techniques, cultivars, and analytical properties of the juice.

2. Materials and Methods

2.1. Sample Collection and Preparation

Grape acquisition occurred within the central valley and central coastal regions of California during the 2020 vintage. Machine-harvested samples were collected in 23 L food-grade plastic buckets at specific points of the winery operation, either from the gondola or the hopper sump, depending on the site-specific workflow and accessibility. For handpicked samples, 19 L of juice was either taken from the press pan of the winery in 19 L-sized stainless steel kegs or self-pressed with approximately 50 kg of grapes (pressed with a 40-L hydro basket press up to 2 bar, Speidel Tank- und Behälterbau GmbH, Ofterdingen, Germany). All samples from the cooperating wineries were acquired before the addition of a technical enzyme or sulfur dioxide. Juices were transferred through a stainless-steel mesh screen (Universal sediment filter, A.O. Smith Water Technologies, Istanbul, Turkey). The juices were then pressure filtered through another 45 µm sediment home water filter up to 1.4 bar under CO_2 into 950-mL canning jars (Ball and Kerr, Westminster, CO, USA). Half of the sample jars were pasteurized at 78 °C for 30 s and immediately cooled to below 20 °C in an ice bath. The other half of the samples were stored on ice and analyzed in an unpasteurized condition.

2.2. Clarification Tests

Three pasteurized and three unpasteurized sample jars were homogenized and poured into 500-mL graduated cylinders for enzymatic testing. The cylinders were grouped in duplicates representing pasteurized and unpasteurized juice and set up as a control, Enzyme A (Lafazym® Press (5 g/100 kg) and Lafazym® CL (2 g/100 L)), and Enzyme B (Lafase® XL Press (4 mL/100 kg) and Lafazym® 600XL ICE (2 mL/100 L)). All enzymes used in these preparations are commercially available from Laffort USA, Petaluma, CA, USA. The decision to use two enzymes in combination was made based on preliminary trials that showed a synergistic effect where one enzyme has a broad mix of different activities and the other is fairly specific. Lafazym® CL, for example, hydrolyzes all parts of the pectin molecule under a wide range of conditions, and Lafazym® Press improves the juice's clarity and settling behavior. To evaluate the effect of enzyme formulation, enzyme A was created from powdered products and enzyme B from liquid formulations. Turbidity analysis (in Nephelometric Turbidity Units; NTUs) was carried out using a TN400 Portable Turbidity Meter (Apera Instruments LLC, Columbus OH, USA). Pectin tests (2.5 mL of clear juice in a test tube with 5 mL of acidified ethanol; 1% concentrated HCl in ethanol) were performed every 60 min over a four-hour period [17]. All chemicals for the clarification tests were purchased from a winemaking supply laboratory (Lodi Wine Labs, Lodi, CA, USA).

An enzyme efficacy index was created to describe the clarification behavior of grape juice over a four-hour period. This index describes the enzyme's ability to clarify the juice and the relative speed of settling the solids. The index is defined as K for *i* samples in the dataset using the following equation:

$$K_i = \frac{NTU_{max} - NTU_{min}}{NTU_{max}} \quad (1)$$

The turbidity measurements represent a fractional change in NTU with a value of 1 representing 100% clarification. However, this number should be taken in the winemaking context as some juices are more turbid than others and total clarification becomes relative as most winemakers do not desire an NTU of zero.

2.3. Juice Characterization

For chemical analyses, all Muscat grape juices were settled at 4 °C for at least two hours. The pre-clarified juice was then divided into smaller portions for further analysis. Total phenolics were analyzed using the Folin-Ciocalteu method according to Singleton and Rossi (1965) [18] with modifications from Möbius and Görtges (1974) [19]. The protein concentration was determined with a colorimetric assay as suggested by Bradford (1976) [20] with minor modifications. First, 100 µL of the juice sample was filtered through a 0.45 µm syringe filter and added to a 10-mL centrifuge tube. Then, Bradford Reagent (Alfa Aesar, purchased through VWR International, Radnor, PA, USA) was pre-filtered through a cellulose filter and 5 mL was added to each sample tube. The samples were incubated for 45 min at room temperature and analyzed at 595 nm. A calibration curve was constructed with bovine serum albumin (VWR International, Radnor, PA, USA).

The total polysaccharides were analyzed using an ethanol precipitation method. The juices were centrifuged at 6000 RPM for 10 min and decanted. 50 mL of juice was pipetted into a 100 mL shaking flask and 50 mL of 96% ethanol (Koptec, purchased through VWR International, Radnor, PA, USA) was added. The flask was closed and agitated for ten seconds, followed by at least eight hours in the fridge to allow alcohol-insoluble polysaccharides to separate. The cold samples were filtered through a pre-weighted cellulose filter (Whatman® #1, 110 mm, Millipore Sigma, Darmstadt, Germany) and the wet weight was recorded as well as the dry weight after treatment in a drying oven at 70 °C until the paper was completely dry. The polysaccharides were calculated according to the dry weight per liter of juice.

The total soluble solids (Brix), titratable acidity, pH, total yeast assimilable nitrogen (YAN), volatile acidity, and gluconic acid were analyzed via an FT2 Winescan (FOSS, Denmark). The juice samples were centrifuged at 6000 RPM for 10 min and filtered through a 2-µm cellulose filter prior to analysis. UV-Vis spectroscopy was used to screen for the effects of native enzymes and pasteurization. The juice samples were centrifuged at 6000 RPM for 10 min and filtered through a 0.45 µm syringe filter prior to analysis. The absorbance was checked at 280 nm and 420 nm, representing the total macromolecules and yellow/brown color, respectively. The spectrophotometer was a Lambda 25 instrument (PerkinElmer Inc., Waltham, MA, USA).

2.4. Statistical Analysis

Tests for normality and significant differences were performed. Correlations were examined via heat maps and cluster analysis. All the data analyses and descriptive statistics were performed using Python™ (Python Software Foundation, Beaverton, OR, USA).

3. Results

The most common Muscat cultivars that are used for wine production in Central California are Muscat of Alexandria and Muscat Canelli. Orange and Golden Muscat are less frequently found but were also included in this study to identify a possible cultivar effect. Figure 1 shows the locations of all samples that were collected in 2020.

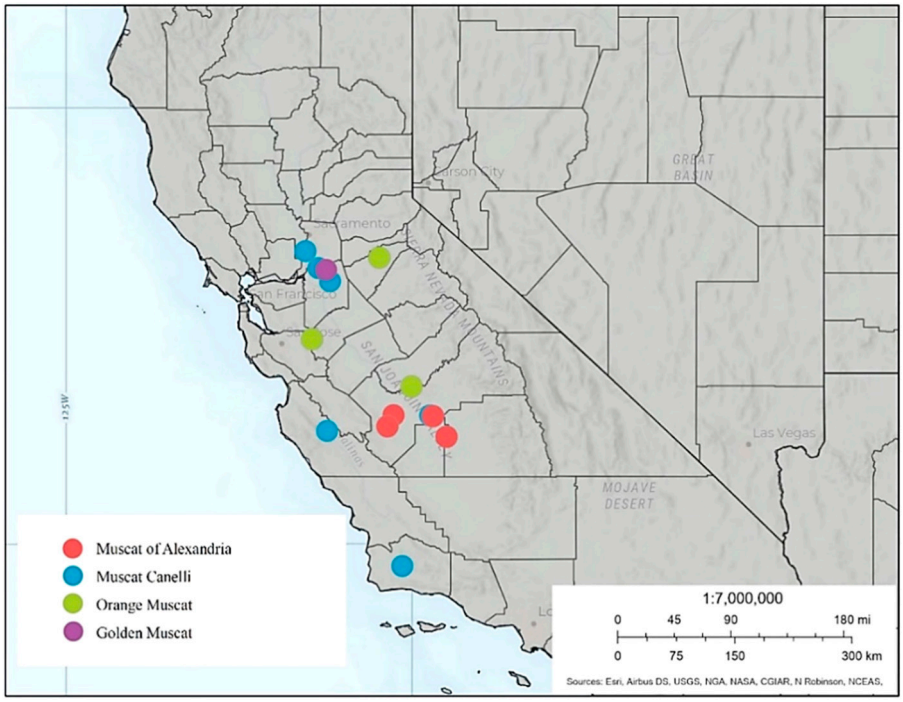


Figure 1. Vineyard locations for all Muscat varieties included in this study.

The working hypothesis that the travel time of the grape material from the vineyard location to the winery influences the microbial condition of the grapes and could lead to an early onset of fermentation, could not be confirmed. Even though daytime temperatures in Central California can exceed 40 °C on a regular basis during the summer, there were very few incidents of native fermentation that could interfere with the juice clarification process, with no correlation to the travel distance of the grape material.

Any yeast or bacterial activity in that stage could lead to increasing carbon dioxide release, preventing the solids from settling out of suspension. This was seen as a possible explanation for why the clarification efficiency decreases as the season progresses, since more mature grapes with higher sugar concentrations are more likely to show signs of microbial activity, especially when transported over long distances in a warm environment.

This aspect, however, did not have a significant influence on the clarification behavior (Spearman correlation coefficient −0.088).All juices were divided into two separate batches, one of which was pasteurized while the other one was stored on ice until further analysis. The goal of the pasteurization step was to exclude any native enzyme activity that could potentially influence the technical clarification enzymes. Table 1 shows all the data that were collected to characterize the juices, also comparing the pasteurized and unpasteurized samples of the same juice.

The main indicator that the pasteurization indeed inhibited the activity of berry enzymes is the difference in the brown color analyzed at 420 nm. While unpasteurized samples turned brown due to polyphenol oxidase activity [21], the pasteurized version of the same juice had significantly lower absorbance readings, indicating less browning ($p < 0.001$). While the degree of polyphenol oxidation was significantly affected by pasteurization, the total concentration of phenolic compounds was not ($p = 0.212$). This indicates that the overall composition of the juices was not altered to a degree where the effect of pasteurization on the enzyme efficiency could not be evaluated. The other attribute that is significantly influenced by pasteurization is the total protein concentration ($p = 0.008$). The pasteurized juices contained significantly less protein compared to the unpasteurized samples. While this observation is a logical consequence of the heat treatment during pasteurization, the loss of proteins potentially changes the equilibrium of macromolecules in the samples prior to enzyme treatment. The pasteurization step was originally included in this study to evaluate the influence of native enzymes on the activity of technical enzymes. However, Figure 2 reveals an unexpected effect of pasteurization on the clarification behavior by making a comparison of both technical enzymes to the control and differentiating between pasteurized and unpasteurized juices. While the control samples are spread relatively equally between low and high clarification efficiencies, the technical enzymes mainly show high-efficiency clarification behavior with a few samples with low efficacy ratings. Interestingly, the samples that responded poorly to the enzyme treatment were exclusively from the pasteurized group.

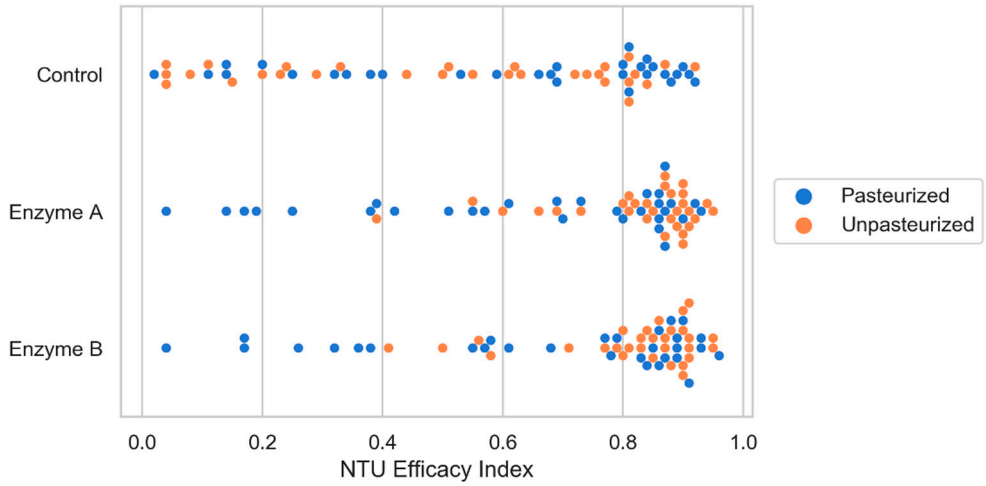


Figure 2. Effect of juice pasteurization on clarification behavior and enzyme efficiency.

Since this observation cannot be caused by any microbial activity, it can be hypothesized to be related to the missing synergistic effect of native and technical enzymes in the presence of other macromolecules like proteins. Another possible hypothesis regarding the low clarification rates of pasteurized samples is that they may be related to non-enzymatic transformations in the structure of pectin at high temperatures. It may be possible that the pasteurization temperature was too high, causing the formation of a gel that could have inhibited the settling process. Since none of the general chemical markers like pH, soluble solids, or acids varied significantly between the pasteurized and unpasteurized samples, a matrix effect seems to be the most logical explanation.

Table 1. All characterization data collected for pasteurized and unpasteurized Muscat juice samples in this study.

Cultivar	Condition	Pick	Enzyme Efficacy Index			Total Soluble Solids	Titratable Acidity	pH	Yeast Assimilable Nitrogen	Volatile Acidity	Gluconic Acid	A420	Total Polyphenols FolinC	Total Proteins Bradford Assay	Ethanol-Insoluble Polysaccharides
			Control	EnzymeA	EnzymeB	(°brix)	(g/L)		(mg/L)	(g/L)	(g/L)	(AU)	(mg/L)	(mg/L)	(g/L)
Orange	pasteurized	Machine	0.14	0.25	0.17	26.7	2.8	3.81	292	0.24	0.3	0.230	383	534	0.00
Orange	unpasteurized	Machine	0.15	0.39	0.41	27.2	2.5	3.83	298	0.22	0.4	0.563	349	552	0.00
Canelli	pasteurized	Machine	0.14	0.14	0.17	21.6	4.4	3.46	136	0.30	0.3	0.126	633	529	5.56
Canelli	unpasteurized	Machine	0.62	0.55	0.56	21.2	4.1	3.45	135	0.28	0.4	0.475	469	564	4.13
Canelli	pasteurized	Machine	0.69	0.61	0.57	21.6	4.6	3.43	128	0.41	0.7	0.145	523	521	4.33
Canelli	unpasteurized	Machine	0.63	0.69	0.50	21.6	4.7	3.41	132	0.40	0.6	0.537	373	539	4.61
Canelli	pasteurized	Machine	0.66	0.92	0.93	24.6	2.3	3.58	133	0.10	0.0	0.058	323	471	7.17
Canelli	unpasteurized	Machine	0.20	0.92	0.91	24.2	2.0	3.58	132	0.10	0.1	0.388	311	678	3.65
Canelli	pasteurized	Machine	0.89	0.87	0.84	23.4	3.3	3.60	200	0.11	0.3	0.125	297	683	0.00
Canelli	pasteurized	Machine	0.85	0.87	0.86	24.1	3.5	3.61	215	0.13	0.2	0.083	377	489	5.59
Canelli	unpasteurized	Machine	0.72	0.91	0.91	23.8	3.0	3.61	227	0.13	0.4	0.233	305	542	0.00
Canelli	unpasteurized	Machine	0.61	0.91	0.90	23.0	3.0	3.60	216	0.11	0.3	0.185	306	514	0.00
Canelli	pasteurized	Machine	0.25	0.86	0.86	22.3	3.8	3.42	162	0.09	0.1	0.158	165	496	6.46
Canelli	unpasteurized	Machine	0.11	0.90	0.90	22.1	3.7	3.41	161	0.08	0.1	0.546	203	554	7.92
Orange	pasteurized	Hand	0.38	0.39	0.38	22.9	2.5	3.72	270	0.08	0.1	0.145	318	562	3.11
Orange	unpasteurized	Hand	0.76	0.87	0.85	22.2	2.1	3.72	271	0.09	0.2	0.553	243	595	0.00
Orange	pasteurized	Hand	0.34	0.42	0.58	23.9	2.7	3.79	313	0.10	0.2	0.190	367	579	2.36
Orange	unpasteurized	Hand	0.81	0.82	0.85	23.5	2.4	3.79	320	0.11	0.3	0.616	257	589	0.00
Alexandria	pasteurized	Machine	0.69	0.73	0.77	17.0	1.1	3.77	114	0.07	0.0	0.217	235	380	1.76
Alexandria	unpasteurized	Machine	0.23	0.60	0.58	16.8	1.1	3.78	117	0.10	0.0	0.406	230	479	1.52
Golden	pasteurized	Hand	0.87	0.70	0.89	18.7	1.9	3.69	231	0.02	0.0	0.060	269	428	2.02
Golden	unpasteurized	Hand	0.92	0.95	0.95	18.4	1.8	3.68	230	0.02	0.0	0.101	250	436	1.89
Orange	pasteurized	Hand	0.84	0.87	0.88	22.6	2.5	3.76	284	0.01	0.0	0.108	321	375	3.64
Orange	unpasteurized	Hand	0.74	0.90	0.91	22.3	2.3	3.76	279	0.02	0.1	0.362	233	428	0.00
Canelli	pasteurized	Machine	0.32	0.90	0.93	24.6	3.6	3.42	168	0.13	−0.1	0.180	433	368	2.57
Orange	pasteurized	Machine	0.81	0.86	0.85	24.2	2.8	3.69	259	0.10	0.0	0.251	325	388	2.75
Canelli	unpasteurized	Machine	0.08	0.88	0.86	24.2	3.2	3.42	171	0.14	0.0	0.630	347	431	4.49
Orange	unpasteurized	Machine	0.33	0.88	0.88	23.8	2.3	3.71	276	0.09	0.1	0.388	300	469	6.08
Alexandria	pasteurized	Machine	0.91	0.86	0.89	21.8	1.2	3.99	145	0.06	0.0	0.388	237	456	3.77
Alexandria	unpasteurized	Machine	0.24	0.81	0.77	20.4	1.2	3.96	144	0.05	0.0	0.401	263	519	3.08
Canelli	pasteurized	Machine	0.83	0.88	0.91	25.2	3.1	3.48	149	0.11	0.1	0.175	410	378	4.72
Canelli	unpasteurized	Machine	0.51	0.90	0.91	24.7	2.8	3.49	153	0.10	0.3	0.374	387	441	2.99
Alexandria	pasteurized	Machine	0.88	0.86	0.87	19.4	1.9	3.74	131	0.15	0.4	0.281	240	388	2.34
Alexandria	pasteurized	Machine	0.80	0.84	0.68	19.8	1.8	3.73	132	0.14	0.3	0.216	237	365	2.75
Alexandria	unpasteurized	Machine	0.77	0.89	0.88	19.3	2.0	3.73	140	0.15	0.4	0.340	245	448	0.00
Alexandria	unpasteurized	Machine	0.50	0.90	0.90	19.7	1.9	3.71	132	0.14	0.3	0.316	229	416	0.00
Alexandria	pasteurized	Machine	0.53	0.51	0.55	20.2	1.9	3.76	135	0.22	0.6	0.240	277	363	0.00
Alexandria	pasteurized	Machine	0.59	0.57	0.61	21.1	1.8	3.75	136	0.19	0.5	0.226	278	365	0.00
Alexandria	unpasteurized	Machine	0.77	0.80	0.80	20.1	1.9	3.75	135	0.20	0.6	0.381	272	443	0.00
Alexandria	unpasteurized	Machine	0.84	0.89	0.83	20.9	1.9	3.74	137	0.17	0.5	0.408	252	438	0.00
Alexandria	pasteurized	Machine	0.90	0.55	0.90	17.3	2.4	3.76	202	0.03	−0.1	0.311	211	448	1.93
Alexandria	unpasteurized	Machine	0.04	0.87	0.87	16.9	2.3	3.75	210	0.03	0.0	0.411	222	511	0.00
Alexandria	pasteurized	Machine	0.92	0.93	0.96	19.0	1.8	3.89	201	0.01	−0.2	0.466	273	469	1.69

Table 1. *Cont.*

Cultivar	Condition	Pick	Enzyme Efficacy Index			Total Soluble Solids	Titratable Acidity	pH	Yeast Assimilable Nitrogen	Volatile Acidity	Gluconic Acid	A420	Total Polyphenols FolinC	Total Proteins Bradford Assay	Ethanol-Insoluble Polysaccharides
			Control	EnzymeA	EnzymeB	(°brix)	(g/L)		(mg/L)	(g/L)	(g/L)	(AU)	(mg/L)	(mg/L)	(g/L)
Alexandria	unpasteurized	Machine	0.04	0.94	0.95	18.7	1.4	3.90	210	0.01	−0.1	0.539	279	537	0.00
Orange	pasteurized	Hand	0.02	0.04	0.04	26.4	1.3	4.00	210	0.03	0.0	0.448	341	411	3.52
Orange	unpasteurized	Hand	0.04	0.81	0.80	26.2	1.3	3.94	204	0.04	0.0	0.530	278	461	0.00
Alexandria	pasteurized	Machine	0.81	0.83	0.89	20.4	2.3	3.80	162	0.09	−0.1	0.409	254	365	2.59
Alexandria	pasteurized	Machine	0.20	0.17	0.26	20.4	2.3	3.80	169	0.08	−0.2	0.373	263	363	2.56
Alexandria	unpasteurized	Machine	0.44	0.85	0.83	20.1	2.0	3.80	176	0.07	−0.1	0.428	292	469	0.00
Alexandria	unpasteurized	Machine	0.29	0.66	0.71	20.1	2.0	3.80	171	0.07	−0.1	0.437	249	443	0.00
Canelli	pasteurized	Machine	0.80	0.69	0.79	25.9	2.2	3.57	92	0.11	0.1	0.177	384	534	3.37
Canelli	unpasteurized	Machine	0.82	0.90	0.90	25.6	1.8	3.57	104	0.10	0.2	0.198	347	552	4.21
Alexandria	pasteurized	Machine	0.40	0.38	0.32	23.9	1.9	3.84	151	0.11	0.4	0.290	369	512	0.00
Alexandria	pasteurized	Machine	0.11	0.19	0.36	23.6	1.8	3.83	154	0.12	0.3	0.252	385	498	0.00
Alexandria	unpasteurized	Machine	0.81	0.84	0.81	23.6	1.4	3.86	163	0.10	0.5	0.369	348	567	0.00
Alexandria	unpasteurized	Machine	0.81	0.84	0.84	23.3	1.5	3.85	168	0.09	0.4	0.369	358	526	0.00
Alexandria	pasteurized	Machine	0.84	0.80	0.83	23.1	1.9	3.87	152	0.10	0.7	0.113	578	325	0.00
Alexandria	pasteurized	Machine	0.68	0.79	0.78	22.0	1.9	4.00	172	0.10	0.2	0.327	519	303	0.00
Alexandria	unpasteurized	Machine	0.87	0.87	0.87	22.7	1.7	3.87	157	0.08	0.9	0.121	500	411	0.00
Alexandria	unpasteurized	Machine	0.55	0.73	0.79	21.8	1.7	4.00	183	0.11	0.4	0.409	633	427	0.00

If that assumption is correct and a reduced level of matrix macromolecules and/or the absence of native berry enzymes can lead to a decrease in technical enzyme efficiency, this would influence the way grape juice is clarified. It can be hypothesized that the native berry enzymes support the function of the technical enzymes, and that the overall reduction of proteins through pasteurization hinders the clarification process. There is no significant difference between the two enzyme preparations that were used in this study. The working hypothesis that a liquid formulation would lead to a different clarification behavior compared to a powdered enzyme could not be confirmed. Both enzyme formulations showed a very similar performance in this study.

The total concentration of soluble and insoluble polysaccharides, proteins, and phenolic material is mostly influenced by the harvesting and processing methods of the grapes [22,23]. There is a possibility that high levels of macromolecules in the juice also influence the efficiency of enzymes and the settling behavior prior to fermentation [24,25]. Table 1 shows that 45% of the samples did not precipitate any polysaccharides after ethanol treatment. In particular, samples with later harvest and processing dates show very little to no ethanol-insoluble polysaccharides, indicating that the berries were fully ripe and starting to degrade in terms of their structural cell wall material. Figure 3 shows all Muscat samples sorted by harvesting method and relates those data points to the efficiencies of the two different enzymes used in this study.

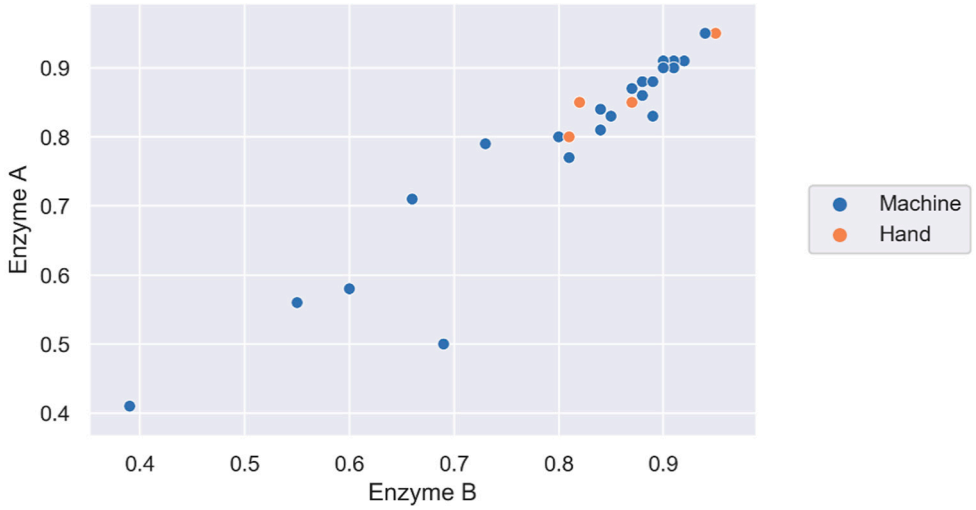
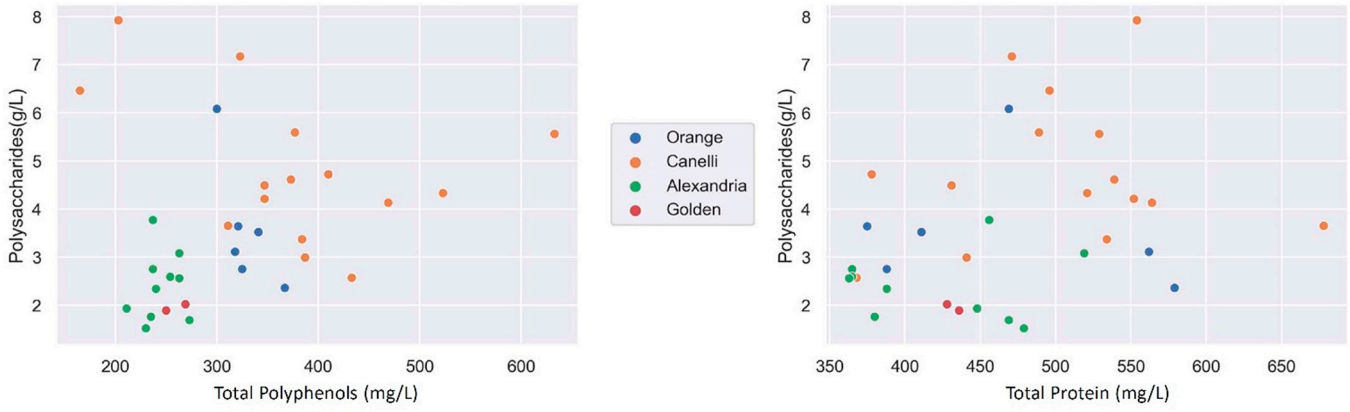


Figure 3. Relationship between harvesting method (hand vs. machine harvested) and enzyme efficiency. The axes show the enzyme efficiency index for each enzyme to allow for a sample-specific comparison.

Even though the number of hand-harvested samples was relatively small compared to machine-harvested grapes, no manually harvested unpasteurized sample showed an enzyme efficiency below 80%. On the other side, about 20% of the unpasteurized machine-harvested grape material displayed an enzyme efficiency below 80%. The reasons for that can be speculated to be related to both the mechanical shearing forces during processing and the resulting increase in suspended solids, and an early onset of natural fermentation. Polysaccharides and berry skin fragments can cause the formation of gels in aqueous solution [25], which could affect enzyme functionality and prevent solids from settling out of suspension, especially if premature yeast activity is creating carbon dioxide. The machine-harvested samples with the lowest enzyme efficiency displayed various levels of microbial activity when the grape material reached the winery test stand.

The total level of ethanol-insoluble polysaccharides depends, among other factors, on the grape cultivar and the ripeness level of the berry [26]. When clusters exceed the stage of full ripeness, native enzymes start to degrade cell wall polysaccharides from within the berry, making it softer until it starts to disintegrate. Polyphenols are stored

in the berry early on to discourage animals from eating the grape before the embryos in the seeds are fully mature, while other compounds serve as UV light protection [27] or reaction towards fungal infections. Two classes of compounds, polysaccharides and polyphenols, are the most important indicators of physical stability in combination with proteins. Figure 4 shows all three classes of macromolecules in relation to the grape cultivar. While polysaccharides and polyphenols cluster within one cultivar (Figure 4a), the relationship between polysaccharides and polyphenols is less cultivar-specific (Figure 4b).



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

Figure 4. Ethanol-insoluble polysaccharides with total polyphenols (**a**) and total proteins (**b**) grouped by grape cultivar.

The reasons for that could be related to the growing conditions having a much bigger influence on the phenolic profile than on the proteins in the berry. As stated earlier, the ethanol-insoluble polysaccharide fraction changes with the ripeness of the grape, so earlier cultivars are expected to show higher levels of these structural cell wall components in the juice. Examining berry development and the level of ripeness could help to draw distinctions among varietal classes and ripeness character. If over-ripe juices are more difficult to clarify, there could be a problem with the main activity of the pectinase. Conversely, if must from under-ripe grapes is more difficult to clarify, then the problem may be related to the activities regarding the degradation of the side chains, thereby limiting the main activity through physical inhibition, and thus preventing the exposure of the active site or target component. This inhibition can occur by physically blocking the enzyme with a side chain, thus preventing the formation of the enzyme-substrate complex.

The influences and interactions of all factors can be evaluated in a Principal Component Analysis (PCA) as shown in Figure 5. Since pasteurized and unpasteurized juices were significantly different in some respects, as discussed above, separate PCAs are shown. Generally, both enzyme formulations tested in this study showed very similar behaviors and a positive correlation, independent of the pasteurization status of the juice. The factors correlated with the enzyme efficacy are provided in subfigures a and b. In the unpasteurized juices, the phenolic content and gluconic acid provide possible areas of interest, with Spearman correlation coefficients of -0.20 and -0.22, respectively, representing a weak, low, negative linear correlation ($n = 30$). While the pasteurization process sees greater confounding variables in the negatively correlated quadrant, the polysaccharide content correlation increases from 0.07 to 0.29, illustrating barely any correlation to a near-moderate positive linear correlation with enzyme efficacy when pasteurized. If proteins are partially removed from the juice, the positive correlation between polysaccharides and proteins disappears, as can be seen by the 90 °C angle between the two vectors. In pasteurized juice, however, a positive correlation between polysaccharides and enzyme efficiency can be observed. This again indicates that proteins are playing a critical role in the clarification process.

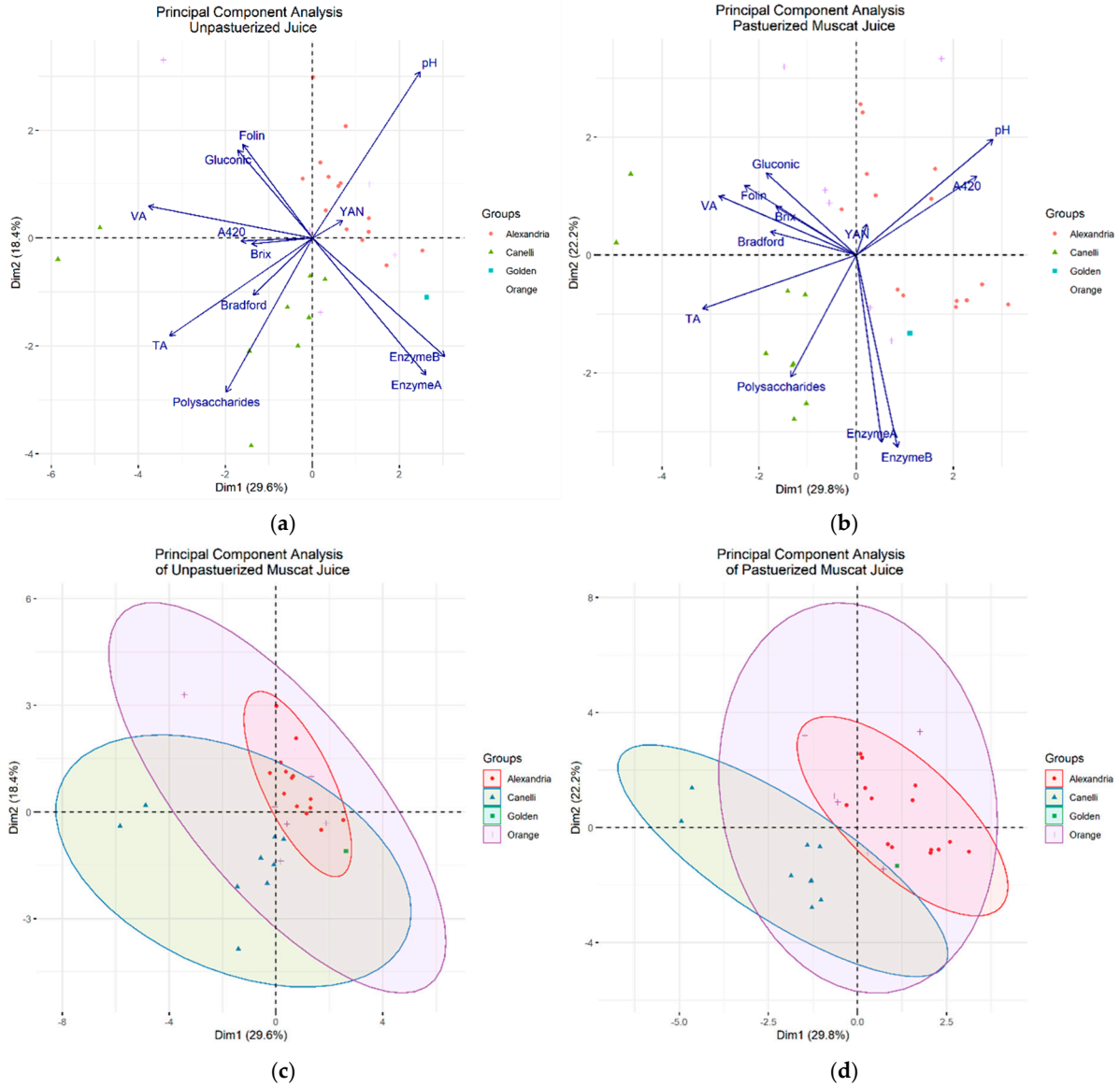


Figure 5. Principal component analyses of all Muscat juices separated into unpasteurized (**a**,**c**) and pasteurized (**b**,**d**) samples.

It is important to note that, in complex systems, a perfect linear correlation (a score of 1 or -1) is very unlikely. Furthermore, values between ±0.3 and ±0.7 indicate a moderate linear correlation, albeit positive or negative. In subfigure c, there is no differentiation between the groups of Muscat. However, there is a significant sparsity in the Orange Muscat samples, which may be due to the sample size. Subfigure d represents pasteurized Muscat juice and a clearer separation of the Alexandria and Canelli samples. This observation is potentially based on the browning behavior that was analyzed via the spectrophotometric absorbance at 420 nm, which separates the samples between the bottom-left and top-right quadrants.

4. Conclusions

In this study, the use of blended commercial technical enzymes was evaluated for musts and juices described as difficult to clarify. There seems to be a distinct matrix effect that can be influenced during juice processing. This study suggests that berry proteins, polysaccharides like pectin, and native enzymes play an important role during the clarification process. On the other side, high macromolecule extraction from the grape

material through excessive shearing can have a negative effect, especially in ripe and overripe grape material. There is no significant difference between a powder or liquid formulation of enzymes as far as the clarification performance is concerned. However, the use of mixed enzymes for clarification proved to be effective. The approach of using an enzyme with various different activities and side activities, pairing it with a robust preparation that works under a wide range of conditions, seems to work in the majority of juices throughout the different ripening stages of the season. The winemaking strategy and use of technical enzymes should, therefore, be adapted according to the incoming crop and the overall processing setup. Besides adjusting the mechanical forces to the level of ripeness, avoiding native fermentation prior to clarification should be prioritized.

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