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Master Thesis in Italian Food and Wine

Use of Transglutaminase to Agglomerate Proteins, Inducing a Reduction in the Amount of Bentonite Needed to Ensure Protein Stability in Wine

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Abstract

In the wine industry, haze development is a major concern. In reality, turbidity and cloudiness in drinks, particularly wine, signify substandard quality, prompting buyers to reject them. As a result, a returned product could result in considerable financial losses for a winery. Therefore, it is critical to stabilize the wine before bottling. The type and formation of haze have an impact on the measurement method used for stabilization. Bentonite appears to be the only choice for stabilizing wine and preventing haze growth after bottling in the case of protein haze. On the other hand, many scientific researches have shown that the bentonite itself has negative on wine aroma. In this context our research has examined the hypothesis of bentonite reduction by using transglutaminase. We have followed three different experiments to evaluate first the initial quantity of protein in the base wine and then to quantify protein after treatment with the transglutaminase and bentonite with the BCA method. Then, we performed the Modified Sommer Assay which is based on a set of spectroscopic color measurements, which not only give a measure of wine color but also give an insight into the contributing elements such as anthocyanin equilibria and phenolic composition. Lastly, we performed the FolinCiocalteu method which is used to quantify total phenolics of either grape juice or wine and then. Based on all the obtained data, a preliminary evaluation of the results seems to indicate that the hypothesis is confirmed. We had almost more than 60% bentonite reduction.

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Literature Review

Haze development is a big issue in the wine industry. In fact, turbidity and cloudiness in drinks especially in wine indicates that they have low quality, which led customers to reject. Consequently, a product which is returned might result in significant financial losses for a winery [1]. So, it is vital before bottling wine get stabilized to stabilize. The formation and sort of haze effects the choice of measurement for stabilizing. In terms of protein haze, bentonite emerges to be the sole option for stabilizing wine and preventing haze development after bottling [1]. This clay mineral is known by its ability to operate as a cation exchanger. Positively charged molecules can be adsorbed on its surface [2, 3].

Montmorillonite accounts for at least 75% of the composition of bentonite. Montmorillonite has a layered structure composed of aluminium hydrosilicate platelets [4, 5]. Different cations, such as Ca_2, Na, or K, are complexed in the interlayer area and can impact the interlayer distance during swelling and adsorption [6, 7, 8]. On the other hand, bentonite is not a specialized absorber. Following of this, not only will haze-related proteins be eliminated, nevertheless,

chemicals beneficial to wine fragrance and color may also be adsorbed [9]. The effect on protein removal is not thoroughly studied; there are just a few studies that show bentonite adsorption of wine proteins [10, 11, 12, 13]. The effects of various wine components on haze are examined. Proteins appear to be a major cause of turbidity. Proteins, because to their structure and charge characteristics, can form massive complexes with other wine components. [14, 15, 16]. Wine contains proteins originating from many sources. During fermentation, yeast generates mannoproteins as a result of cell lysis. These are large glycosylated compounds. A protein content of 19% that have been regarded as being proper for wine, texture and stability, for example [17, 18, 19]. A variety of protein-based fining agents, such as casein, isinglass, and ovalbumin, are also used to enhance wine aroma and mouthfeel. Although, they are mostly eliminated entirely prior to bottling [20, 21, 22]. The majority of the protein in wine comes from grapes. The bulk of them are pathogenesisrelated (PR-proteins), with high stability against proteolytic digestion and a wine pH of 3e4 [10, 23]. Some PRproteins, including as thaumatin-like proteins (TLP), chitinases, and b-glucosidases, are thought to be hazerelated. [24, 25, 26, 27, 28, 29]. TLP isoforms and chitinases are the majority of the proteins in wine. These proteins have molecular weights ranging from 20 to 30 kDa and isoelectric points (pI) ranging from 3 to 5 [30, 11, 31, 32]. As a result, the discovery and characterisation of such proteins, particularly their identification and characterisation, are critical research topics for creating novel methods of removing them to minimize turbidity. Chitinases and TLPs have previously been demonstrated to have heat stability and haze generating properties, as well as different TLP isoforms. [26, 30] ascribe differing unfolding qualities, aggregation features, and electrophoretic mobilities to structural changes in one loop and the amino acid composition in the flanking regions, as well as hydrophobicity discrepancies [30].

1.1 Bentonite Definition

Bentonite is a commonly used technical aid in winemaking for removing or reducing the accumulation of unwanted constituents. It particularly works as a settling aid to breakdown proteins, decreasing the likelihood of protein haze in wine, which might endanger market acceptance. Because of its mineral character, bentonite treatment agent has a significant influence on the elements makeup of the final wine. [33, 34, 35].

Bentonite is the commercial name for a porous clay substance mostly consisting of montmorillonite. In addition to montmorillonite, bentonite can contain accessory minerals such as quartz, chalcedony, dolomite, calcite, feldspars, analcites, and pyrite. Activation treatment, which is often performed on natural Ca bentonites (high Ca2+/Na+ ratio), can change the composition of bentonites.

1.2 Bentonite Activation Therapy

The amplification method involves treating the wet mud with solid Na_2CO_3 at a temperature of 80 degrees Celsius in order to reach properties comparable to natural Na bentonites (high Na+/Ca2+ ratio), which have increased potential of protein binding [36, 37]. The cationic exchange characteristics of these clays play a significant role in the adsorption of positively charged proteins and other soluble cationic components by bentonites in wine. Competition from other cations in the solution matrix, as well as the pH and ethanol concentration of the solution, all impact protein adsorption. [36] K+, Ca2+, Mg2+, Na+, and H+, as well as majority of amino acids, certain peptides, and other cationic fractions, would compete. An analysis followd by Blade and Boulton (10 on many possible influencing factors) revealed that protein adsorption was temperature independent but differed with protein and ethanol contents and pH importance.

1.3 Bentonite Impact Area

The results of bentonite addition to wine have primarily been investigated to determine the degradation of proteins, bioamines, polyphenols, amino acids, and aroma compounds [38, 39, 40]. Some research has also been conducted on the fluctuation of main element concentrations (Na, Ca, Mg) as well as Al, Fe, Cr, Mn and Pb when bentonites are used as fining agents (1, 3, 14, 16, 17).Furthermore, decreases in K, Cu, Zn, and Rb concentrations in wine have been documented [35, 40]. Meanwhile, there is not perfect agreement among published findings, which is most likely due to differences in experimental settings.

Code	Form	Type
B1	Fine powder	Activated Ca
B3	Granule	Na
B4	Granule	Na
B5	Powder	Activated Ca
B8	Elongated filament	Na
B9	Stony granule	Na

Table 1.1: Bentonites used in extraction essays

Despite this, the findings indicated a connection between bentonite properties and elemental release. Considerable increase in micro and trace elements have been identified in treated wine [35, 40]. Furthermore, [34] find out that bentonite treatment can dramatically modify the concentration pattern of rare it lacks earth elements, rendering it inappropriate for use as a fingerprint for wine origins. The composition and structure of bentonites have been examined and linked to their protein adsorption abilities from an oenological standpoint [41, 42, 43, 44, 45].

Regardless, with the exception of rare earth elements, comparable study on the release of micro and nanoparticles trace elements into wine have not been conducted [34, 46]. As a result, an analysis was conducted to assess the leakage of contaminant elements from many bentonites

into wine and to comprehend the consequences of their physical and chemical properties. The different wine pH values modified the efficacy of bentonite clarification. The most essential element influencing adsorption is pH, because it influences the final surface charge of the bentonite and the degree of ionization and speciation of the protein [47]. Lastly, according on this research we realized that bentonite influences the mineral composition of wines. The use of bentonite resulted in a little higher concentrations of a variety range of elements. In comparison, the concentrations of B, K, Cu, Zn, and Rb in wine have significantly reduced. For Pb wine concentrations, different findings were observed, suggesting that bentonite has the capacity to remove it, which is remarkable from a technical viewpoint.

1.4 Bentonite and Wine Aromas

Bentonite not only interacts with proteins, but also interacts with other compounds. Aroma loss after fining is usually regarded as a secondary, generic consequence of bentonite, despite the fact that the reasons and frequency of incidence in white wines are unclear. The percent reduction of numerous odor-active white wine components after bentonite fining was significantly influenced by bentonite dose, bentonite sample, and wine style [48].

Because of its net negative charge at PH of wine. Bentonite electrostatically interacts with positively charged wine proteins, causing in flocculation [49]. Another consideration is the duration of interaction which is also stated that the bentonite fining procedure may have a negative impact on wine sensory qualities [50], which is related to absorption of flavor or fragrance components [50]. Long periods of contact between bentonite and wine during batch fining may have led to this problem. One method of bentonite fining is in-line dosing. Adding bentonite to wine is one possibility while it is pushed through one tank to another through a hose (or elsewhere). With enough contact hours, the bentonite may absorb and remove the haze-forming proteins from the wine, as a result, it is heat stable. Filtering or centrifugation would be employed to remove the bentonite from the wine [51]. "In line dosing for bentonite fining

of wine or juice: Contact time, clarification, product recovery and sensory effects." Australian Journal of grape and wine research 12.3 (2006): 221-234.).

Bentonite's adsorption capabilities in wine are mostly attributable to cation activity of exchange. The exchangeable cation's function is used to classify bentonites (Na bentonite, Ca bentonite). These exchangeable cations alter the bentonite's interlayer spacing and characteristics of edema, altering the intercalation of water into the inner layers [52]. The impact of bentonite towards proteins was likewise affected by the varying ethanol concentrations [36].

The European Community's list of permitted additives, adjuvants, and fining agents in winemaking (EC, Regulation of the Council n. 1493/99; EC, Commission Regulation n. 1622/00) contain clarifying products, the most important of which is bentonite. Natural clay is more often used in winemaking than other "earths" like as kaolin and Spanish earth. The most fascinating technical characteristics of this montmorillonite-type phyllosilicate are its potential to operate as a settlement aid to clear juice and wine and to eliminate proteins, As a result, the risk of protein haze is reduced in wine. The chemical, physical, and enological properties of bentonites have been examined in depth [41, 42, 45].

This study shows how several Bentonite and yeast husks are two winemaking techniques, might impact on the final micro- and trace element content of wine. It was possible to show that these therapies, which were not employed on purpose to alter mineral composition, lead to an increase or decrease in the final concentration of specific mineral components Such adverse effects may be taken into account in order to reduce the need for specific metal depletion treatments in wine. Furthermore, when the mineral composition is utilized to determine the geographic origin of a wine, these activities must be considered and avoided utilizing extensive sample and databases. (G. NICOLINI, R. LARCHER, P. PANGRAZZI and L. BONTEMPO U.O. Enologia e Chimica Agraria, Istituto Agrario, S. Michele all'Adige (Trento), Italia)

1.5 Useful Methods to Measure Bentonite Effects

We used two heat-unstable Chardonnays. Chardonnay A was not subjected to any lees aging following alcoholic fermentation (AF) or malolactic fermentation (MLF). Following the completion of AF, Chardonnay B was aged for six months on yeast lees. MLF happened during this time period. To increase wine protein content, contact with wet lees was pressed to an 18/100 (v/v) ratio.

Superbenton, Top Gran, and an experimental clay were purchased as activated sodium bentonite samples (Dal Cin Gildo S.p.A., Sesto S. Giovanni, Milan, Italy). The montmorillonite content of Superbenton and Top Gran ranged from 85 to 89 percent. With average sizes of 63 m and 1 mm, respectively, Superbenton was a powder while Top Gran was granular. Commercial wine was not clarified using the experimental clay. It was a 90 to 95 percent montmorillonite powder with an average diameter of 180 m [48].

Total protein was determined using a previously established method. [53] with bovine serum albumin (BSA) as a reference. Following protein purification, the following analysis was performed: To 100 mL of wine, 400 mL of pure ethanol was poured.

The samples were centrifuged after 72 hours of precipitation (20 min at 5000 rpm). The precipitate was floated in water and dialyzed in tubes with a molecular weight cut-off of 3500 Da (Membrane Filtration Products, San Antonio, TX). After resuspension in 10 mL water, the dialyzed samples were lyophilized and the protein content was measured [48].

The efficiency of bentonite clarifying was affected by the initial protein content (Table 3): As Wine with less beginning protein showed a higher proportion of protein removal, as previously found [54]. The adsorption of the protein-bentonite system isotherm demonstrates higher adsorption at low solute concentration [54, 36, 55]. In addition, the presence of polysaccharides and mannoproteins generated by yeasts during yeast lees aging was responsible for the low percentage of protein removal reported in wine B. Yeast cell wall polysaccharides and glycosylated cell wall-derived yeast proteins rise after lengthy yeast lees age of the wine [56], a tech-

nique required by a few particular wine types and utilized in wine B.

The effectiveness of bentonite treatments on fragrance components in white wine was determined by the chemical composition and initial concentration of the number and kind of proteins in the wine, as well as the volatile chemicals. Only a few odor-active chemicals were directly adsorbed by bentonite; the majority were eliminated indirectly as a result of deproteinization [48].

1.6 Combination of Bentonite and Wine Protein on Loss of Aromas

Additionally a study observed the combination effect of Bentonite fining and protein on the loss of wine aromas. The results showed that Bentonite has little effect on terpenes on its own loss but removes ethyl esters and fatty acids. When grape proteins were incorporated into the bentonitetreated solution, when pure proteins were used, they tended to accelerate the loss of esters with the longest carbon chains (from ethyl octanoate to ethyl decanoate). The data suggest that hydrophobicity may be one of the driving factors in aroma interactions with bentonite and proteins. Wine proteins, which have a tendency to insolubilize during wine storage, are the primary source of this issue [57, 49, 58]. The majority of wine proteins come from grapes, and several authors have established that the haze-forming proteins are grape specific [59, 25, 11]. These proteins have been linked to the pathogenesis of grapes, and they are permitted to stay for the duration of the winemaking process due to their resistance to proteolysis and stability at acidic pH3.

1.7 Bentonite Effects on Rare Earth Elements (REE)

Although wine is very important economically, wine falsification, incorrect labeling, and blending lead to severe financial shortages. As a result, there is a pressing demand for create finger-

	Protein removal *			
	Chardonnay A		Chardonnay B	
Bentonite (dose)	**(mg/L)**	**(%)**	**(mg/L)**	**(%)**
Experimental clay, 20g/h/L	14.2 ± 2.7 bc	34.2 ± 6.5 bc	20.4 ± 5.4 a	6.4 ± 1.7 c
Experimental clay, 50 g/h/L	16.7 ± 4.0 ab	40.3 ± 9.6 ab	43.9 ± 12.1 b	13.8 ± 3.8 b
Experimental clay, 100g/h/L	17.1 ± 2.7 ab	41.1 ± 6.4 ab	79.0 ± 7.6 a	24.8 ± 2.4 a
Superbenton, 20 g/h/L	10.9 ± 2.7 c	26.3 ± 6.4 c	16.6 ± 6.4 c	5.2 ± 2.0 c
Superbenton, 50 g/h/L	13.5 ± 4.3 bc	32.6 ± 10.4 bc	37.3 ± 15.0 bc	11.7 ± 4.7 bc
Superbenton, 100 g/h/L	17.1 ± 4.0 ab	41.1 ± 9.7 ab	43.0 ± 5.7 b	13.5 ± 1.8 b
Top Gran DC, 20 g/h/L	16.1 ± 2.4 b	38.8 ± 5.7 b	18.5 ± 11.2 c	5.8 ± 3.5 c
Top Gran DC, 50 g/h/L	19.3 ± 4.6 ab	46.6 ± 11.1 ab	33.5 ± 8.6 bc	10.5 ± 2.7 bc
Top Gran DC, 100 g/h/L	22.7 ± 3.6 a	54.6 ± 8.8 a	42.4 ± 3.5 b	13.3 ± 1.1 b
*Values are means ± SD ($n = 6$). within each column, different letters indicate statistically different values according to post-hoc comparison (Turkey's test) at $\alpha = 0.05$.				

Table 1.2: Protein decreases in bentonite-treated wine samples. Absolute concentrations (mg/L) and percentages (%) of the starting protein content are used to express protein removal.

print procedures that are aided by quantitative statistical methodologies in order to establish the provenance of wines. The most considerable constraint of fingerprinting is the representative selection of the group of elements or compounds detected in wine samples, which should ideally be determined solely by soil composition and not on wine technology, transit, or storage. Because they have comparable chemical characteristics, rare earth elements (REEs) would be perfect candidates for fingerprinting [46].

The filtration of wines by bentonites has the highest danger for introducing REEs and other contaminants. [34] discovered that wine purification with bentonites enhanced the REE contents of the samples shaken for 48 hours by measuring the REE concentrations of young and finished wine samplesRare earth elements (REEs) would be ideal candidates for fingerprinting since they have comparable chemical properties. The filtration of wines by bentonites has the highest danger for introducing REEs and other contaminants [34].

Despite the fact that the origin, kind, and label variation of bentonites on the market is vast, a wide chemical and structural characterization, as well as application instructions, are frequently offered. However, no information on probable mineral leakage into wine is supplied. The need of bentonite quality management in guaranteeing them safe oenological usage is emphasized., the International Organisation of Vine and Wine (OIV) specifies a lowest possible montmorillonite content and highest possible recoverable Pb, Hg, As, Fe, Al, Na, and combined Ca and Mg contents to be determined in a bentonite extraction solution (OIV, 2003) [52].

1.8 Heat-Unstable Protein and Removal With Bentonite and Heat Treatment

Despite multiple studies on grape juice and wine proteins [49], the proteins responsible for wine turbidity are yet unclear. Protein instability does not appear associated well with total protein content, and there appears to be contradictory evidence in the literature about which

proteins (protein fractions) induce haze and silt formation. Using Electrophoresis on Paper [60]. Discovered that grapes and wines included two primary protein fractions, both of which were reduced by heat treatment and the addition of bentonite.

1.9 Protein Stabilization Zirconia and Bentonite

Winemakers for the purpose of protein removal and avoiding turbidity in white wine use bentonite which causes several negative effects, Wine proteins are stabilized during the winemaking process because bentonite absorbs them. These ad hoc processes, on the other hand, have a low selectivity and a significant environmental impact. This has an effect on the wine's quality and implies that some items are lost as a result. For all of these reasons, developing new options that are both commercially viable and protect wine quality is preferred [61]. The stabilizing of wine protein by bentonite is a discontinuous process that requires a significant amount of time throughout the preparation and gravity-settling processes. Before being added to wine in the winemaking process, bentonite must be fully hydrated, and the amount applied must be appropriate to avoid harming the wine's organoleptic characteristics [62, 63]. Previous scientific studies have shown that continuous stabilization of white wines (Chardonnay and Muscat) is possible utilizing a packed column with zirconia as the adsorbent material [64]. Furthermore, the environmental effect of ongoing zirconia stabilization is smaller than that of traditional bentonite treatment since this material's chemical and mechanical resilience (among other physical characteristics) allows it to rebuild [64, 65].

1.10 Amendment of Acid Soils with a Bentonite-Based Waste from Wineries

Spain is the European country with the most vineyard land and the third in wine production (FAOSTAT, 2006) together with France and Italy, they account for half of all global wine pro-

duction. These figures, which show the economic importance of wineries in Spain, are also indicative of the vast volumes of waste and co-products produced. Which approached 1.5Mt in 2005 (FAOSTAT, 2006). European regulation requires that a portion of the waste from vineyards be committed to alcohol distillation (Council Regulation EC 1493/1999 on the common organization of the market in wine, 17 May 1999.), however many small wine merchants do not comply with the regulation and instead dispose of the waste in the environment. It's also tough to keep track of all of these different constituents; there are solids like grape stems, grape marcs, and wine lees, as well as liquids like vinasses. These waste materials can be used in a variety of ways [66]. Their direct application to soil is one of the most efficient methods of disposal or enhancing value [67, 68], especially when considering their input of organic matter and other nutritive ingredients to the soil–plant system [69]. There are several options for repurposing these waste materials [66]. Their direct application to soil is one of the most efficient methods of disposal or enhancing value [67, 68], especially when considering their input of organic matter and other nutritive ingredients to the soil–plant system [69]. In addition to the possibility of using these wastes as soil amendments, they can also be employed to immobilize heavy metals [70, 71, 72] and pesticides [73, 74, 75]. Because of the high organic matter content, they have a high sorption capability in these conditions content can be combined with their low economic and environmental costs for active carbon and peat.

1.11 Bentonite Winery Waste and Soil Amendment

Winemaking is one of the most popular economic agroindustrial activities in Mediterranean countries, but it has led and also has an unfavorable side effect which contributes to the vast number of solid, liquid, and semi-solid winery wastes that remain [76, 77]. For example, every year, the Spanish wine industry generates up to 1.4 million megagrams of solid winery waste and roughly 24 million m3 of wastewater. [76]. Several techniques for repurposing these wastes have recently emerged, including raw materials for the food sector [78], substrates for

plant development [79], regarding the retention of heavy metals and pesticides [80, 81]. Winery waste, on the other hand, is largely employed as a soil supplement since it increases the bulk density and porosity of the soil [82] and soil fertility due to the presence of organic matter and nutrients, such as potassium (K), calcium (Ca), and phosphorus (P), in the wastes [68, 76]. Although the bulk of solid winery wastes are natural, containing more than 80% total organic carbon (C), there is inorganic waste, such as bentonite waste (BW), which originates from the use of bentonite as a clarifying agent during winemaking. As a result, bentonite waste includes more plant nutrients like as K, P, copper (Cu), and organic C, whereas nitrogen (N) content is determined by the yeast biomass accumulated during winemaking. This nitrogen enrichment transforms BW into an agricultural acid soil amendment [83]. Considering its origins, adding BW to vineyard soils looks to be a viable technique for contributing to long-term agricultural sustainability. Vine plants, on the other hand, are particularly sensitive to an overabundance of readily available nitrogen, which causes a drop in grape and wine quality as well as an increase in inorganic N leaching, which negatively affects groundwater quality [84]. Thus, before adding N-rich winery wastes to vineyard soil, it is critical to analyze N mineralization in order to limit any shortfall or excess in soil accessible content. Currently, the specifics of N mineralization dynamics in agricultural soil treated with bentonite waste are unclear [81]. "Nitrogen mineralization dynamics in acid vineyard soils amended with bentonite winery waste." Archives of Agronomy and Soil Science 64.6 (2018): 805818).

According to [85], dewatered wastewater sludge represents for 12% of total organic solid waste produced by vineyards, and its disposal by third-party companies is both expensive and inefficient. As an alternative, anaerobic digestion could be employed to valorize this waste stream. Anaerobic digestion (AD) is a well-established process for treating a wide range of organic wastes (municipal solid wastes, sewage and waste activated sludge, agroindustrial residues, animal effluents, etc.) while recovering bio-energy and lowering their biodegradability. The use of a combination of the traditional activated sludge process (CAS) and AD is common in municipal wastewater treatment facilities, and it lowers sludge disposal costs by reducing sludge

volume. Biogas is a sustainable energy source that may be used inside the same manufacturing process and/or wastewater treatment facility, reducing energy needs [86].

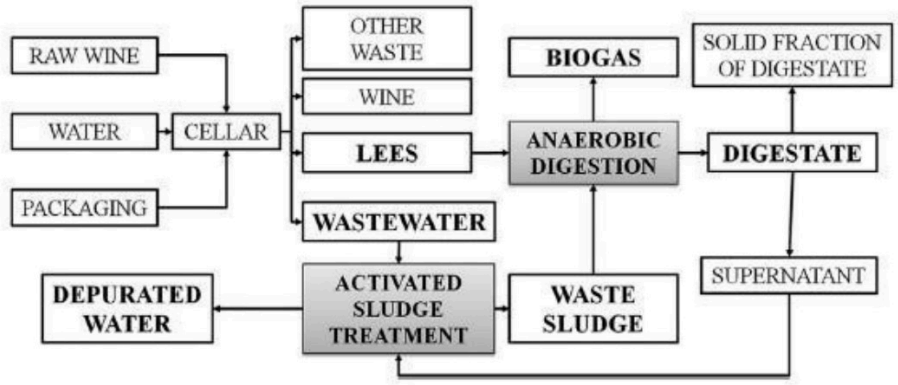

Figure 1.1: Incorporating aerobic fermentation into the production of wine

Winemaking is a large global agroindustry, with around 3.3 million tons of wine produced in 2011 FAOSTAT [87]. As a result, a significant amount of trash is generated during the vinification process. Bentonite waste (BW) is a byproduct of the use of bentonite, a clay mineral that belongs to the 2:1 dioctahedral phyllosilicates group known as smectites and is used as a fining agent because of its high adsorption capacity [87]. Large volumes of winery waste (including bentonite waste) are dumped of in an unregulated way in most soil and watercourses are the key destinations in wine-producing regions. This action is contrary to European Community norms (Official Journal of the European Communities, 2008), which state that winery waste should be directed to distillation or designed to prevent contamination, thus respecting the environment, avoiding soil contamination, and maintaining ground and surface water quality. Winery wastes can be used as an agronomic additive in soils instead of being disposed of in landfills. Winemaking wastes have been studied as an amendment because of their high nutrition and organic matter content [88, 67, 68, 89]. The careful incorporation of these wastes into the soil boosts the levels of organic C and N. [82]. Despite the fact that large bentonite waste doses impeded the establishment of herbaceous species, (Arias-Estevez et al. proved that adding bentonite produced wastes to an acid soil raised its pH, N, K, and P content.

1.12 What Do We Know About Transglutaminase?

Transglutaminase (EC 2.3.2.13) catalyzes an acyl transfer reaction between glutamine residues' çcarboxamide group and lysyl residues' -amino groupIn addition to this crosslinking reaction, it may also catalyze aminolysis of the ç-carboxamide group of peptide-bound glutamine residues, hydrolysis of the ç-carboxamide group of glutamine residues when the amine substrate is low or absent, and hydrolysis and aminolysis of some aliphatic amides [90]. It's been difficult to build a molecular model for transglutaminase's catalysis of the protein cross-linking process since it catalyzes so many diverse processes. Transglutaminase has been shown to catalyze the homologous polymerization of various proteins in a variety of investigations. However, it has yet to be proven that it can catalyze the heterologous polymerization or dimerization of proteins in an experimental setting. [91] showed that the transglutaminase process created high molecular weight polymers when acetylated RS1-casein was treated with a variety of proteins, including â-lactoglobulin and soy 11S and 7S globulins. Myosin cross-linking to soy globulins, casein, and gluten has also been claimed to be catalyzed by transglutaminase [92]. In general, transglutaminase protein substrates are divided into four categories: (1) Gln-Lys-type, with both Gln and Lys residues accessible for crosslinking; (2) Gln-type, with only the Gln residue accessible for reaction; (3) Lys-type, with just Lys residues accessible; and (4) a nonreactive type, with both Glu and Lys residues inaccessible for reaction [93]. This classification is based on the presence of Lys and Gln residues on the protein's surface.

The findings clearly reveal that not all Gln-Lys-type substrate proteins may produce heterologous polymers in the transglutaminase-catalyzed process. The thermodynamic compatibility of the substrate proteins appears to be required for heterologous dimer or polymer formation. Table 1 summarizes the characteristics of the protein substrates used, as well as their ability or incapacity to construct heterologous dimers and polymers with one another.

Heterologous dimers are impossible to form in caseins and albumins, for example. This is owing to the difficulty of these proteins to overlap at the catalytic areas of the enzyme [94].

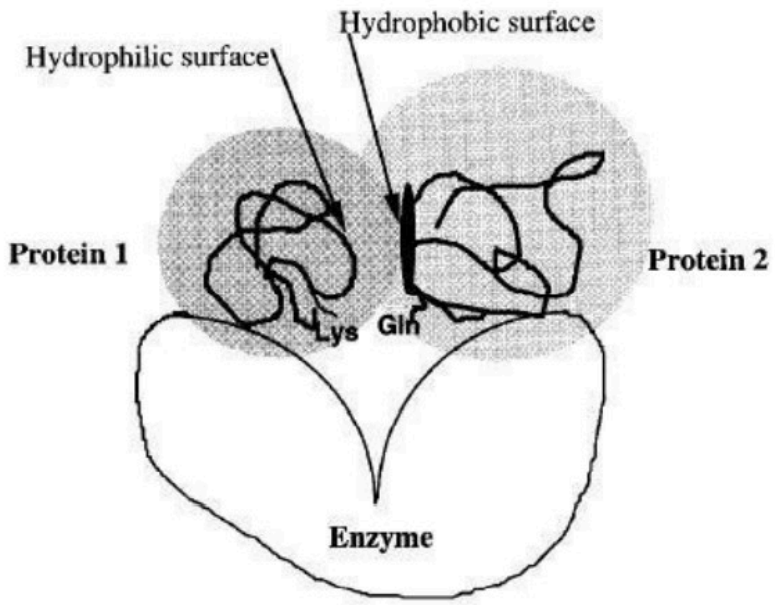

Figure 1.2: Thermodynamic Compatibility of Substrate Proteins

"Thermodynamic compatibility of substrate proteins affects their cross-linking by transglutaminase." Journal of Agricultural and Food Chemistry 44.5 (1996): 12111217.)

TRANSGLUTAMINASE (TGase) has been discovered to catalyze the integration of primary amines through certain proteins and polypeptides via a replacement process, resulting in changed protein and ammonia [95] [96]. It has also been demonstrated that the process takes place by calcium-dependent acyltransfer between the ycarboxyamide group of protein-bound glutaminyl residues and various primary amines [91]. Transglutaminase is an enzyme that bonds protein molecules together. Protein properties like as gelation ability, thermal stability, water-holding capacity, and so on are all affected by crosslinking. The use of a transglutaminase isolated from Streptoverticillium sp. in the food industry has been validated. Seafood, surimi, pork, noodles/pasta, dairy products, baked goods, and other foods now include transglutaminase. (Food Research and Development Laboratories, 1-1, Suzuki-cho, Kawasaki 210-8681, Japan). Transglutaminases have been found in a variety of mammal tissues, fish, plants, and microbes in nature [97, 98]. The microbial transglutaminase is effective at a variety of tem-

peratures and sustainable between pH 5 and 9, which is also the pH range used in most food preparation [99, 100].

Yogurt production appears to be the most advanced field of dairy product processing employing transglutaminase these days. Several studies have been reported on the impact of transglutaminase on yogurt [101, 102, 103]. Several studies on the gelling and emulsifying capabilities of transglutaminase-treated milk proteins have been undertaken. Dickinson and Yamamoto discovered that milk protein-stabilized emulsion gels are crosslinked with transglutaminase [104].

The ability to modify functional qualities in milk caseins and soybean globulins using TGase generated from guinea pig liver [93] or bovine plasma (Kurth, L., and P. J. Rogers 1984) was established in the early 1980s. Crosslinking of food proteins of different sources and incorporation of amino acids or peptides into food proteins to address nutritional deficiency were demonstrated in both experiments [105] (Motoki, M., and K1 Seguro.) Using the guinea pig enzyme, we explored the possibility of dietary protein modification for industrial use [91, 106].

1.13 Influences of Transglutaminase Protein Crosslinking with Casein

The use of rennet to coagulate milk is an important stage in the production of cheese. Rennet is a proteolytic enzyme complex that consists of chymosin (EC 3.4.23.4) and catalyzes the hydrolysis of k-casein in milk by cleaving the Phe105–Met106 link. TG is a transferase that generates both inter- and intramolecular connections in and across diverse proteins by crosslinking the amino acids of protein bound glutamine and lysine [99, 107]. Among dairy proteins, the casein fraction is a good TG substrate [108, 92].

1.14 Crosslinking Transglutaminase with Whey

Transglutaminase was tested for its capacity to crosslink pure and mixed whey protein compositions, whey-based associated proteins, as well as other dairy proteins at pH 7.5 and 37'C for 4 hours. After 4 hours, transglutaminase crosslinked all protein substrates [109] Figure 1.3. The loss of protein bands, the formation of new protein bands, and the accumulation of inflexible protein polymers near the gel origin all showed crosslinking.

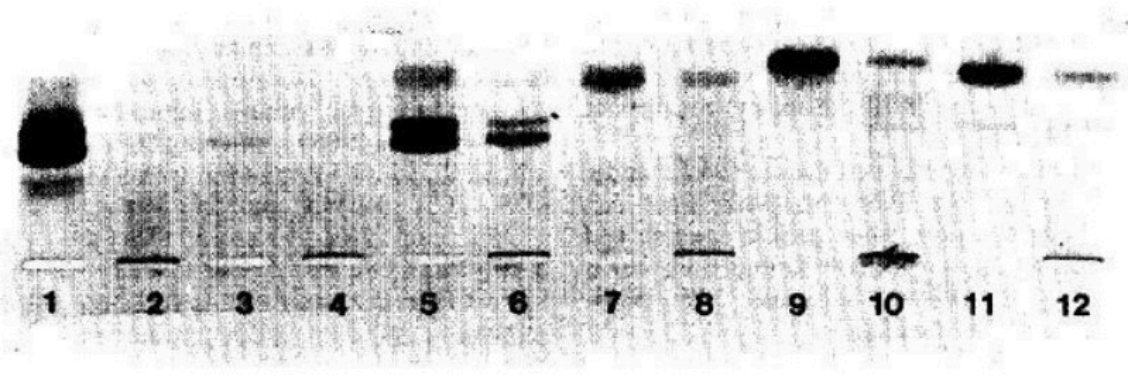

Figure 1.3: The SDS-PAGE of dairy proteins subjected to transglutaminase activity. Lanes 1 = whole casein, 3 = α-casein, 5 = NDM, 7 = whey powder, 9 = α-lactalbumin, 11 = β-lactoglobulin. Lanes 2, 4, 6, 8, 10, 12 are the respective substrates after reaction with transglutaminase.

1.15 Wine Protein Haze Mechanism

Protein synthesis and sugar buildup in grape berries rise rapidly after veraison [110, 111, 112]. Because only soluble proteins are removed during the winemaking process, protein diversity in musts and wines is substantially smaller than in grapes [113, 111]. In addition, the protein content of wines is always lower than that of matching musts. Proteolytic activity, polyphenol precipitation, and adverse conditions associated with low pHs are all factors in this decrease. Protein content in white wines ranges from 10 to 500 mg 11 due to the growing ethanol level [11]. Grape varietal, ripeness, and the winemaking method are all important factors in determining the ultimate protein concentration. These proteins have a molecular mass of 9 to

66 kDa and an isoelectric point of 3 to 9 [114, 59, 49]. Most of them, identified as being chitinases and thaumatin-like proteins, are found within the range 20–30 kDa and are acidic [59, 49, 111, 112, 115] [11]. Protein content in white wine ranges from 10-500 mg/L on average [57]. Grape and wine proteins of white wine varietals [57, 9]. Removal of specific protein components by chitin enhances protein stability in a white wine [9].

Protein haze is a visual issue in white wines that may be avoided by eliminating grape proteins which have passed the winemaking process. The haze-forming proteins are associated to grape pathogenesis and are highly resilient throughout the winemaking process; nevertheless, a few of them precipitate over time and at high temperatures. For many customers, wine transparency, particularly in white wines Figure 1.4, is important, as it is one of the characteristics most easily altered by poor shipping and storage circumstances. Various tests have been developed to evaluate wine stability/instability in terms of protein haze [116]. These assays use a variety of methods to cause protein aggregation and precipitation. Heat stability studies based on heat-induced precipitation are the most common. As a result, guaranteeing wine stability prior to bottling is a critical step in the winemaking process and a significant challenge for winemakers [1]. "Wine protein haze: mechanisms of formation and advances in prevention." Journal of agricultural and food chemistry 63.16 (2015): 40204030). The most prevalent causes of hazy wine and the appearance of precipitates are three: microbiological instability, tartrate instability, and protein heat instability [117]. Sulfur dioxide treatment and filtration are used to provide microbial stability ahead to bottling [118] tartrate stability is obtained using cool stabilization, ion exchange resins, or electrodialysis [119].

The two main types of pathogenesisrelated (PR) proteins that persist in wine are chitinases (PR3) and thaumatinlike (TL) proteins (PR-5), both of which are normally resistant to the winemaking process but are implicated in hazing during wine storage [58, 120, 121]. Grape PR proteins [122] Preventing protein haze in bottled white wine. Aust J Grape Wine Res 11: 215–225) are constantly produced in healthy plants and are expressed even more in reaction to biotic or abiotic stressors [123]. Structure of thaumatin in a hexagonal space group: com-

Figure 1.4: Wine that is cloudy and clear due to protein aggregation

parison of packing contacts in four crystal lattices. Acta Crystallogr Sect D Biol Crystallogr 60: 83–89). The structural integrity of grape PR-proteins such as thaumatin-like proteins (TLPs) and chitinases can alter under specific situations, when wines are exposed to warmer temperatures during storage or shipping, for example. According to comparative studies, chitinases are more heat unstable and prone to gather than TLPs [26, 27]. Furthermore, heat unfolded chitinases cannot refold to their original state, but TLPs may [26]. Protein unfolding occurs as a result of these alterations, revealing amino acid side chains that are normally hidden in the protein's core. The newly formed side chains are then free to form aggregates with nearby proteins or other wine components, resulting in haze or precipitates in the bottles [27]. Heating and reduction affect the reaction with tannins of wine protein fractions differing in hydrophobicity. Anal Chim Acta 660: 110–118). Interestingly, it has recently been revealed that other grape TLPs do not exhibit this behavior [124, 11, 125], and these findings, together with data on the existence of both TLPs and chitinases in wine hazes [122, 25], suggest that some TLPs might lead to haze synthesis. Yeast can also affect the protein composition of wine. They accomplish so by creating biological components like mannoproteins, which help to keep wine

from becoming hazy [19, 126, 18] or indirectly by the secretion of extracellular proteases that may aid in the breakdown of berry proteins [127, 128]. However, it has been demonstrated that berry-derived proteins in wine have a great resistance to the activity of the fermenting yeast [58]. Increased temperatures that wines can be exposed to during storage or transit are thought to be linked to the development of protein haze in wine, which can affect the consistency of PR proteins, resulting in their aggregation into granules visible to the naked eye [124]. As a result, PR proteins must be removed from white wines through the use of bentonite fining. Bentonite, a clay cation exchanger that attaches proteins and precipitates them out of wine, is frequently used in industrial winemaking to maintain protein stability. Proteinbound bentonite settles to the bottom of wine tanks as lees, accounting for around 31% of the original wine volume [129]. PR protein production in vines occurs largely in grape skins [115, 122] and is controlled in a temporal and tissue-specific manner. The both grape activity of the VvTL1 gene and the contents of the corresponding main thaumatinlike protein rose rapidly at the commencement of berries softening (veraison) and maintained through berry ripening in V. vinifera cv. Muscat Gordo Blanco [121]. Protein synthesis and sugar buildup in grape berries rise rapidly following veraison [110, 111, 112].

1.16 Haze Forming of Protein

Chitinases and thaumatin-like proteins are the most prevalent groups of haze-forming proteins found in grapes (Vitis vinifera) juice and white wines (TLPs). [130, 112, 27, 120] these proteins are tiny (35 kDa) and compacted, with globular structures, [30] are positively charged at wine pH, and can withstand low pH in juice and wine. [113, 122] various proteins, like βglucanases, have also been believed to lead to haze formation [25, 11], albeit they are far less prevalent in wine than chitinases and TLPs and have received less attention.

Wines include a variety of nitrogenous compounds, including proteins, in variable concentrations. Because the density of these polymers varies from 15 to 300 mg/l, they do not

contribute significantly to the nutritional value of wines [113, 122]. On the other hand, the presence of a residual amount of unstable protein in wines is a major cause of concern for wine-makers. Slow denaturation of wine proteins, owing to poor storage conditions, is thought to produce protein aggregation and flocculation into a hazy suspension, resulting in a haze or deposit in the bottled wine. The unpleasant fog has no effect on the wine's aromatic and gustatory qualities. Translucency, on the other hand, is important to wine quality since it is the first thing a client observes about wine, and consumers will reject wines with murky precipitates regardless of how good they taste [113]. Protein precipitation in wines diminishes their economic potential and suggests that they have been unstable and so inappropriate for sale for all of these reasons [57, 49, 58]. Protein is undoubtedly necessary for the development of haze in wine, and it appears that the higher the total protein level in the wine, the more likely it is to become unstable [11]. Despite proteins from Saccharomyces cerevisiae [131, 18] and Botrytis cinerea [132, 133] have been found in wines, the bulk seems to come from the grape pulp [113]. Wine proteins are a blend of compounds originating from both grape berries and yeast [49].

1.17 Other Wine Factors Contribute to Haze

The effect of wine pH on protein stability varies based upon on protein typeChanging the pH of wine from 2.5 to 4.0 at room temperature was adequate to disrupt chitinases' normal state, exposing hydrophobic receptors and encouraging protein aggregation [125]. TLPs and invertases, chitinases, on the other hand, remained stable under the same conditions, demonstrating their relative instability and haze-forming potential when compared to stable TLP isoforms. Polyphenols additionally can lead to the agglomeration and precipitation of wine proteins, which might be owing to the creation of hydroxyl group or, more likely, hydrophobic interactions [134, 135, 136, 137].

1.18 Tannin in Wine

Tannins are water-soluble phenolic compounds with molecular weights ranging from 500 to 3000 Da with a strong proclivity to bind proteins, particularly those with a high proline concentration [138, 139, 140]. Tannins contributed to the wine's astringency [141] by precipitating salivary proteins [142, 143, 144]). Furthermore, tannins are responsible for a variety of phenomena, including the formation of protein haze in alcoholic beverages [136, 137], and this property has been utilized to remove protein from wines [145, 146]. Although wine proteins' reactivity with endogenous grape tannins has been extensively studied, a full description of the behavior of specific wine protein components is absent [136, 137, 145, 146, 147]. Hydrophobic bonding may be the major mode of interaction between condensed tannins and proteins, according to various studies. The interaction of tannin–protein complexes was investigated, and it was discovered that hydrophobic contact, rather than hydrogen bonding, was the dominant mechanism, as previously supposed. Siebert et al. confirmed this comment [148], who showed that hydrophobic interactions are more important than hydrogen bonding in determining the reactivity of proteins and polyphenols.

Purifying proteins and tannins from the same wine and assessing their reactivity is a realistic technique for identifying the still-unknown chemical–physical mechanism of white wine hazing, according to this study [120]. The proteins that cause haze in wines have been identified as PR-proteins generated from grape fruit. Grape TL-protein protein fractions, in particular, have the most volatile protein fractions. Our findings corroborate these findings, revealing that, at least under certain conditions, TL-proteins are the most reactive when combined with wine tannins.

		Cross-linking with						Thermodynamic compatibility with					
Protein	**Type of substrate**	**CN**	**suCN**	**LA**	**LG**	**BSA**	**OV**	**CN**	**suCN**	**LA**	**LG**	**BSA**	**OV**
β-casein (CN)	Gln-Lys	+	+	+/-	-	+	-	+	+	+/-	-	-	-
Succinylated β-casein (suCN)	Gln	+	-	+/-	+			+	+	+/-	+		
α-lactalbumin (LA)	Gln-Lys	+/-	+	+		+	-	+/-	+	+	+	+/-	
β-lactoglobulin (LG)	Gln-Lys	-	+	+	+		-	-	+		+		-
BSA	Gln or Lys	-	+	+	-	-	-	-	+	+	-	+	
Ovalbumin (OV)	Gln or Lys	-	+	-	-	-	-	-	+/-	-	-	-	

Table 1.3: A list of the protein's characteristics, including whether it can create heterologous dimers and polymers with other proteins or not.

2

Aim of the Thesis

In this chapter the main impacts of bentonite on environment and wine will be explained. As the previous chapter being mentioned, there are several scientific researches which have been shown with the aim of bentonite impacts such as environmental, wine aroma, REE (Rare Earth Elements) impact.

2.1 Research Question

Whether using transglutaminase as the first additive to the wine basically to glutamate to protein that are present and the use of very small amount of bentonite if that's sufficient, do we have to still continue using that or not?

Based on the scientific researches and experiments Bentonite appears to be the only choice for stabilizing wine and preventing haze growth after bottling in the case of protein haze [1]. Bentonite is a typical winemaking technical tool for eliminating or minimizing the accumula-

tion of undesirable elements. It works especially well as a settling aid for breaking down proteins, reducing the risk of protein haze in wine, which could jeopardize market acceptance. Bentonite treatment agent has a considerable impact on the elements makeup of the final wine due to its mineral nature. [35, 33, 34]. Bentonite is a general-purpose absorbent. Not only will haze-related proteins be removed as a result, but compounds that are helpful to wine smell and color may also be adsorbed [50]. [34] discovers that bentonite treatment can drastically alter the concentration pattern of rare earth elements, making it unsuitable for use as a wine origins fingerprint. The content and structure of bentonites have been examined and linked to their protein adsorption capacities using an oenological approach [41].

Bentonite interacts with a variety of different compounds in addition to proteins. Aroma loss after fining is usually regarded as a secondary, generic effect of bentonite, despite the fact that the causes and frequency of occurrence in white wines are uncertain. The percent reduction of many odoractive white wine components after bentonite fining was considerably influenced by bentonite dose, bentonite sample, and wine style [48].

Winery waste, on the other hand, is frequently used as a soil supplement because it improves soil bulk density and porosity [82], as well as soil fertility, due to the presence of organic matter and nutrients such as potassium (K), calcium (Ca), and magnesium (Mg) [82]. In the wastes, there is calcium (Ca) and phosphorus (P) [68, 76].

Although the bulk of solid winery wastes are natural, containing more than 80% total organic carbon (C), there is inorganic waste, such as bentonite waste (BW), which originates from the use of bentonite as a clarifying agent during winemaking. As a result, bentonite waste contains higher plant nutrients like as K, P, copper (Cu), and organic C, whereas nitrogen (N) concentration is determined by yeast biomass accumulated during winemaking. As a result of the nitrogen enrichment, BW is changed into an agricultural acid soil amendment [83]. Given its roots, adding BW to vineyard soils appears to be a potential technique for assisting in long-term agricultural sustainability.

2.1.1 What do we know about Transglutaminase?

Transglutaminase (EC 2.3.2.13) catalyzes an acyl transfer reaction between glutamine residues' çcarboxamide group and lysyl residues' -amino group. Transglutaminase has been shown to catalyze the homologous polymerization of several proteins in a number of investigations. However, it has yet to be proven in an experimental setting that it can promote protein heterologous polymerization or dimerization.

3

Material and Methods

3.1 Wine Used and Ortega Grape

England is not the traditional wine country but the last decade has become increasingly popular with its outstanding sparkling wines made accordingly to the traditional method. England, it may not seem but is one of the oldest wine-producing countries in the world since Roman times and documented in a document from 731 and later in 1152 King Henry II (11331189) came into possession of Gascony and large parts of western France, including Bordeaux by marriage to Eleonora of Aquitaine where a long and successful relationship has been established between Bordeaux and England. As everybody knows the English weather is not so favorable and sunny, that's why most of the vineyards are located in the south part of the country where the climate is slightly warmer and drier. The English climate is tempered by the Gulf Stream, a major Atlantic Ocean current that carries warm water from the Caribbean to the southern coasts of England and Wales. Also, the global warming has helped the English wine industry and winegrowers to

overcome the difficult grape ripening process which has become much easier, but still, most of the grapes planted are early ripening and suitable for cool climate. Because of all these factors, the enrichment of the grape must with sugar was a common practice in England, but since the 1980s the sugar levels are gone up,and that is not a necessity any more.

There are around 20 wine-growing areas in the United Kingdom, but the most prominent and important are Sussex, Kent and Surrey, and all of them are related and famous for the sparkling wine production. There are major champagne houses investing in the south of the country seeing England as one of the future fizz producing nations. The white wine grape varieties are dominating the plantings as the most used ones are Chardonnay, Pinot Noir and Pinot Meunier for the production of the sparkling wine as well as the hybrid grapes Bacchus, Seyval Blanc, Ortega, Reichensteiner, Madeleine Angevine and Müller-Thurgau. Overall the country is experiencing a renaissance in the wine-growing and production and is starting to rival other well-established sparkling producing areas such as Champagne, Cava and Prosecco.

Plumpton Estate Ortega 2015 - A clean, fresh and youthful white wine, with lots of peach and melon notes coming to the fore, plus a creamy vanilla undertone with a hint of honey. A rich and well-rounded wine and a fantastic match for Asian cuisine.

Ortega is a Müller-Thurgau and Siegerrebe cross. Grown first on the Mosel, it was planted first in England in the early 1970s. It ripens early and is known for its low acidity and high sugar levels. This makes it suitable for dessert wine production (Denbies are known for their use of Ortega in their sweet wines), although it is more widely used in dry still wines, both as a varietal and in blends.

Fruity and aromatic, it can also have a notable spicy quality. Biddenden in Kent were one of the first to plant this variety and Westwell, also in Kent, have made good use of it, occasionally employing wild yeasts and amphora for fermentation purposes.

3.2 Experiments and Methods

Back to the topic as we reviewed in previous chapter, the negative effects of bentonite has been undeniable. The Hypothesis of inducing bentonite reduction by transglutaminase has been achieved after several experiments in order to qualification of protein (BCA method), for tannin assay (BSA method) and modified Sommers assay for colour measurement.

3.3 Structure and Purpose of Experiments

We have 2 treatments and one control of the same wine (Ortega) and replicate these treatments (3 times of each) in demijohns. We add to the control the standard amount of bentonite (1.5 gr/L) at the normal rate without adding transglutaminase, then we have the first treatment which contain the dose of transglutaminase 250mg/L, then 500mg/L of transglutaminase for the second treatment and stir them up. After 10 days while we stored the samples at 10 Celsius degree, we add for the first treatment (250mg/l) of transglutaminase 0.5 gr/lit of bentonite and for the second treatment 500 ppm transglutaminase we add 0.5 gr/L dose of bentonite. In the end of the day we want to see after clarifying everything by bentonite, we test and see all the samples of wine are protein stable.

3.4 Protein Assay (BCA method)

The Thermo centficTM PierceTM BCA Protein Assay Kit is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu+2 to Cu+1 by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu+1) using a unique reagent containing bicinchoninic acid.

- BCA protein assay instructions Equipment reagents
- 1.5ml Eppendorf tubes

- 10 mm path length cuvettes – Semi Micro
- 100-1000 µl micropipette
- 20-200 µl micropipette
- 2-20 µl micropipette
- UV/Vis Spectro –set at 562 nm
- Centrifuge with high speed rotor
- Orbital shaker
- Protein reaction solution– 10
- Washing solution – 1M KCl solution
- BCA reagent A B
- Bovine Serum albumin (BSA)

Procedure – Sample preparation

1. To a clean eppendorf tube a. Pipette 1000 µl wine b. Pipette 10 µl protein reaction solution (10% SDS) c. Cap and mix well
2. Allow protein reaction solution to react a. 2 hours room temp or 5 minutes in boiling water bath
3. Add 250 µl washing solution 1 M KCl, then cap and mix well a. Allow samples to mix for 2 hours on an orbital shaker at room temp
4. Cool samples in freezer for 5 minutes
5. Centrifuge tubes 14000 rpm for 5 minutes
6. Decant and discard supernatant – Protein pellet to remain in bottom of tube.
7. Pipette 1000 µl washing KCl into eppendorf tube, then cap and mix well
8. Centrifuge tubes 14000 rpm for 5 minute
9. Repeat steps 6,7,8 until pellet becomes white and free from interference
10. Decant and discard supernatant a. Protein pellet to remain in bottom of tube. b. Remove any excess liquid with pipette or paper

11. Add 1000 µl deionised water to eppendorf then cap and mix until protein pellet is dissolved Use orbital shaker or ultra-sonic bath if required, ensure protein is fully dissolved.

Procedure – Protein measurement

1. Blank spectro with cuvette of de-ionised water
2. Transfer 50 µl of protein sample to Semi Micro Cuvette
3. Add 1000 µl of reagent AB mix (mixed 50:1 just prior to use)
4. Cover with parafilm and mix well.
5. Wait 30 mins in 37 Deg C for colour to develop and stabilise
6. Allow to cool for 5 minutes
7. Ensure all measurements are taken promptly
8. Measure absorbance of sample at 562 nm
9. Reagent A and B should only be mixed together immediately prior to use

Procedure – Calibration curve

1. Blank spectro with a cuvette of de-ionised water
2. Prepare BSA standards directly in eppendorf a. Pipette the required amount of deionised water into eppendorf b. Pipette the required amount of BSA solution into eppendorf c. Cap and Mix well
3. Transfer 50 µl of each BSA solution into semi-micro cuvette
4. Add 1000 µl of reagent AB mix (mixed 50:1 just prior to use)
5. Cover with parafilm and mix well
6. Wait 30 mins in 37 Deg C for colour to develop and stabilise
7. Ensure all measurements are taken promptly
8. Blank spectro with cuvette of de-ionised water
9. Measure absorbance of sample at 562 nm

3.5 Modified Sommer Assay

Somers and Evans (1974, 1977) established a set of spectroscopic colour measurements, which not only give a measure of wine colour but also give an insight into the contributing elements such as anthocyanin equilibria and phenolic composition. The original Somers assay is a four part assay, where the wine is analysed in its original state and is then treated with excess SO2, excess acetaldehyde, and hydrochloric acid to investigate the anthocyanin equilibrium of the wine. First, the absorbance of the young red wine sample is read at 420 (yellow/orange pigments) and 520 nm (red pigments) in its original state (with regard to wine pH and SO2 concentration), and from these values, the wine colour parameters wine color density and hue are calculated. The second reading is taken after the addition of excess SO2 allowing for the measurement of colour (A520) resulting from the SO2-resistant pigments present in the wine. Third, the original wine is treated with excess acetaldehyde, which permits the estimation of coloured anthocyanins at wine pH. Finally, the wine is diluted with 1 M hydrochloric acid, lowering the pH and converting all anthocyanins and many other pigments into their coloured forms. The acidified solution is then monitored at A520 and A280 to give an indication of the concentration of total red pigments and total phenolics, respectively. The main modification to the original method, reported in [149], is the standardisation of the wine pH to pH 3.4 and the alcohol concentration to 12% v/v using a buffer solution prior to the initial analysis.

1. **Prepare HCI treatment:** To a clean test tube pipette 100 micro liter of clarified sample and pipette 5ml of 1M HCI. Mix test tube well. Store at room temperature in the dark and allow3 hours reaction time. Afterward, set spectrophotometer to 820nm. Blank machine using cuvette with water. Measure HCI treatment absorbance A280. Set spectrophotometer to 520nm. Blank machine using cuvette with water. Measure HCI treatment absorbance A520.

2. **Prepare ethanal treatment:** To a clean test tube pipette 1mL of clarified sample and pipette 10ml of wine buffer. Add 10 micro liter ethanal and mix test tube well. Store at room temperature for one hour. Then set spectrophotometer to 420nm. Blank machine using cuvette with water. Measure ethanal treatment absorbance A420nm. Set spectrophotometer to 520nm. Blank machine using cuvette with water. Measure ethanal treatment absorbance A520.

3. **Prepare sodium metabisulphite treatment:** To a clean test tube pipette 1ml of clarified sample and pipette 10ml of Wine buffer. Add 10 micro liter SMS and Mix test tube well. Store at room temperature for one hour.

4. **Prepare buffer treatment:** To a clean test tube pipette 1ml of clarified sample and pipette 10ml of Wine buffer. Mix test tube well and read immediately.

5. Fill cuvette ¾ full with buffer treatment.

6. Set spectrophotometer to 420nm. Blank machine using cuvette with water. Measure buffer treatment absorbance A420. Set spectrophotometer to 520nm. Blank machine using cuvette with water. Measure buffer treatment absorbance A520.

7. After reaction time cuvette ¾ full with sulphite treatment.

8. Set spectrophotometer to 520nm. Blank machine using cuvette with water. Measure sulphite treatment absorbance A520.

9. After reaction time fill ¾ cuvette full with ethanal treatment.

10. Set spectrophotometer to 420nm. Blank machine using cuvette with water. Measure ethanal treatment absorbance A420nm. Set spectrophotometer to 520nm. Blank machine using cuvette with water. Measure ethanal treatment absorbance A520.

11. After reaction time fill cuvette ¾ full with HCI treatment.

12. Set spectrophotometer to 280nm. Blank machine using cuvette with water. Measure HCI treatment absorbance A280. Set spectrophotometer to 520nm. Blank machine using cuvette with water. Measure HCI treatment absorbance A520.

3.5.1 White juice/wine colour

1. Fill cuvette ¾ full with sample.

2. Set spectrophotometer to 280nm. Blank machine using cuvette with water. Measure absorbance A280. Set spectrophotometer to 320nm. Blank machine using cuvette with water. Measure absorbance A320. Set spectrophotometer to 420nm. Blank machine using cuvette with water. Measure absorbance A420.

3.5.2 FC Method

Plant phenolics present in fruit and vegetables, and that are particularly rich in red wine, have received considerable attention because of their potential antioxidant activity. Human consumption of antioxidants has many alleged health benefits, including protection against cardiovascular diseases, and, most recently, cancer. Red wines contain a variety of polyphenolic antioxidants. Five samples of commercial red wines from Spain and four phenolic compounds of red wine: gallic acid, trans-resveratrol, quercetin and rutin, have been studied. The total phenolics content and the total antioxidant activity (TAA) of wines was determined. The total phenolic content, determined according to the Folin-Ciocalteu method, varied from 1800 to 2300 mg/L, expressed as gallic acid equivalents (GAE). The antioxidative effects of wine phenolics were determined using a system based on the inhibition by antioxidants of the absorbance of the radical cation. The relationship between antioxidant activity of phenolic compounds, as hydrogen donating free radical scavengers, and their chemical structures was studied. Furthermore, the total antioxidant activity of the wines investigated was well correlated with phenol content. Thus, the results confirm that red wine polyphenols are, in vitro, significant antioxidants.

This method are to extract total phenolic of either juice or wine and then to measure them by spectrophotometer. The pros of Foley method is that you can measure all mono or dye hydroxylated phenolic's however the cons are that things such as fructose and so2 may interfere with your results and the spent reagent is very hazardous and must be dealt with appropriately. The reagents that you will need for this lab include Gallic acid which you will have to make folding reagent which is purchased and ready to go and finally sodium carbonate which you also have to make.

1. Gallic acid stock solution: In a 100-ml volumetric flask, dissolve 0.500 g of dry Gallic acid in 10 ml of ethanol and dilute to volume with DI water.

2. To prepare a calibration curve: add 0, 1, 2, 3, 4, 5, and 100 ml of the above phenol stock solution into 100 ml volumetric flasks, and then dilute to volume with DI water. These

solution will have phenol concentrations of 0, 50, 100, 150, 250, and 500 mg/L Gallic acid, the effective range of the assay.

3. From each calibration solution pipette 20 micro liter into separate cuvettes and to each add 1.58 mL water, and the add 100 micro liter of the Folin-Ciocalteu reagent, and mix well. Wait between 30 sec and 8 minutes, and then add 300 micro liter of the sodium carbonate solution at 40c for 30 minutes.

4. Determine the absorbance of each solution at 765 nm against the blank (the "0 mL" solution) and plot absorbance vs. concentration.

The equipment: includes seven 10 mL glass tubes or similar size with the rack, 7 cuvettes that are 1 cm, seven 100 mL flasks sharpie to label our glass the Gallic acid plus sodium carbonate and 5 Gallic acid standards. Also we need the relevant size of volumetric pipet and DI water bottle. When preparing Gallic acid use an analytical balance if you can because we will be weighing out 0.500 grams of dry Gallic acid which will then be dissolved in 10 mL of ethanol and transfer into a 100 mL volumetric flask then dilute to volume with DI water this is a 5gr per liter ratio. To prepare a serial dilution of Gallic acid from the stock solution we will need the volumetric pipette and our volumetric flasks. We have had a small amount to prime the volumetric pipette and dispense to the waste container. Repeat these steps to each of the volumetric once they are primed we have to make sure that fill our volume in volumetric pipette slightly above to the dispense line. Once these complete fill to volume with DI water and repeat these steps for all standards. We will need only a few mL of the Foley reagent, so we put it in a small beakers.

For the wine sample we will micropipette 20 micro liter of the wine into the glass tube. For all glass tubes which includes our wine sample and standards we will need to add 1.58 mL of water, 100 micro liter of Folin reagent then we will need to mix it well and wait 8 minutes and after that we should add 300 micro liters of sodium carbonate solution and shake to mix, once this is done place the rack of tubes in the 40 degrees Celsius oven for 30 minutes.

While our standards and wine samples are in the oven for 30 minutes head over to the spectrophotometer and turn it on as it does take quite some times to start up. Once it is ready

set it at 765 nm and then remove samples from the oven after they have reached their 30 minutes waiting period. Transfer the whole amount into the 1.0 centimeter cuvette. We will need set the blank first.

4

Experimental Results

When an analysis of variance (ANOVA) gives a significant result (p-value<0.05), this indicates that at least one group differs from the other groups. This means that adding a new ingredient has affects the amount of protein. (Therefore, for all experiments, you should look at the p-value, and if it is less than 0.05, you should say that adding that ingredient was effective.

ANOVA is a statistical method used to determine whether the means of two or more groups differ from one another significantly. ANOVA compares the means of various samples to examine the influence of one or more factors.

Based my experience I used this statistical method to shows if usage of transglutaminase effects on bentonite usage and also the effects of both substances on wine in each of experiments.

To get the meaningful structure I had to determine specific codes for the each factors that used in my experiments. Since in each of the tests, four separate tests were performed with different concentrations of Transglutaminase and Bentonite, hence, a set of codes has been specified for each experiment.

For instance, Group 1 represent the experiments First treatments with transglutaminase and bentonite with 250mg/l, group 2 represent the second treatment with 500mg/l bentonite. Group 3 represent the control and finally group 4 represent samples before treatment.

Table's explanation:

Following our all experiments results, I had to set the raw data through the tables' sheet. For instance, the tables for Modified Sommer Assay which are divided through the 5 different experiments show our observation after reading with different spectrophotometers calibration. As we read the concentration of substances before treatment and then after treatments with different amounts of bentonite (0.5 gr/L) also Transglutaminase (250mg and 500 mg), we collected various numbers after reading with a spectrophotometer. For plotting the standard curves we had to get an average of the numbers for each section and we used the same method of mediation to make the tables and plot the standard curves for all the tests.

4.1 Modified Sommer Assay

4.1.1 Prepared Buffer

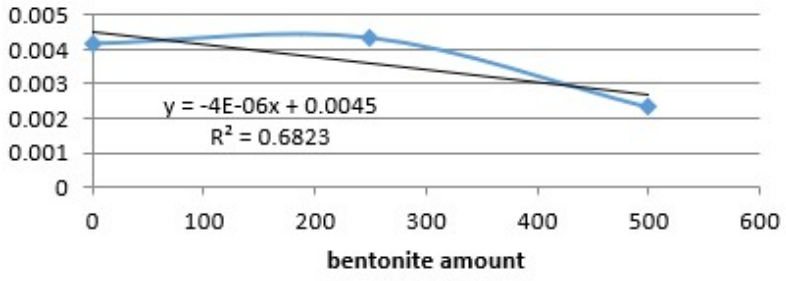

Figure 4.1: Modified Sommers Prepared Buffer after treatment with bentonite 420nm

	Modified Sommers Prepared Buffer before treatment 420nm	520nm
	0.004	0.001
	0.004	0.001
	0.007	0.004
	Modified Sommers Prepared Buffer after treatment with 250mg/l of Tgase and 0.5g/l of bentonite at 420nm	520nm
	0.003	0.001
	0.006	0.003
	0.004	0.001
	0.002	0
	0.004	0.001
	0.007	0.003
avg	0.004333333	0.0015
	Modified Sommers Prepared Buffer after treatment with 500mg/l of Tgase and 0.5g/l of bentonite at 420nm	520nm
	0.001	0.001
	0.003	0.001
	0.002	0
	0.002	0
	0.003	0.001
	0.003	0
avg	0.002333333	0.0005
	Modified Sommers Prepared Buffer, wine control 420nm	520nm
	0.004	0.001
	0.004	0.001
	0.004	0
	0.003	0
	0.005	0.001
	0.005	0
avg	0.004166667	0.0005

Table 4.1: Prepared Buffer Treatment

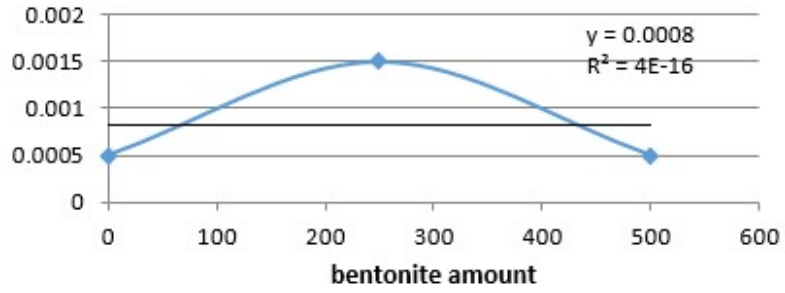

Figure 4.2: Modified Sommers Prepared Buffer after treatment with bentonite 520nm

Wine colour parameters and wine colour density:

1. Following the P>0.05, we can see that the findings of these studies are significant. The quantity of color and density in the first treatment, which comprises 250% Transglutaminase and Bentonite, is higher than in the second treatment and the control, as can be seen in this test. As you can also see, this amount was very high in the sample prior to treatment.

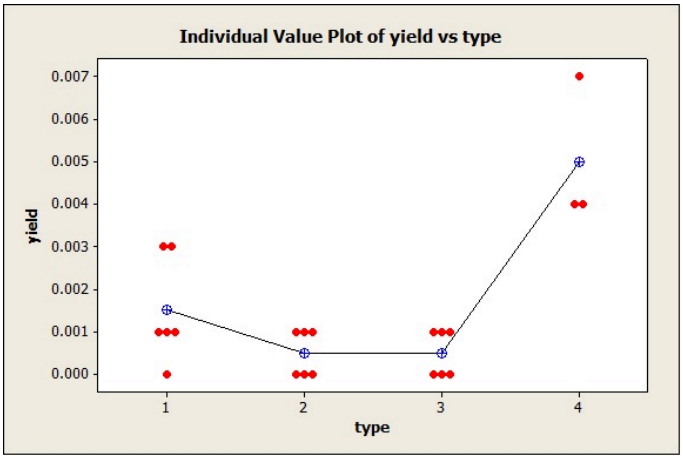

Figure 4.3: ANOVA analysis of prepared buffer treatment (P=0.000)

2. Based on the P>0.05 for reading the 420 nm samples in the same experiment, we can infer that there is still a discernible trend. As a result, the first treatment shows more color than the control sample, while the second treatment has the lowest concentration.

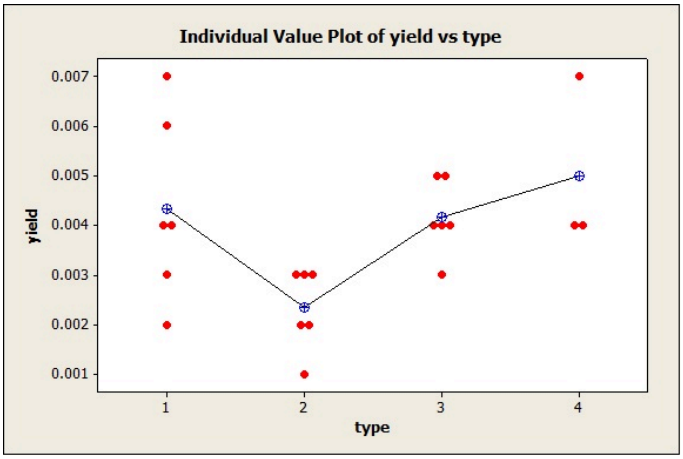

Figure 4.4: ANOVA analysis of prepared buffer treatment (P=0.030)

4.1.2 White juice and wine colour

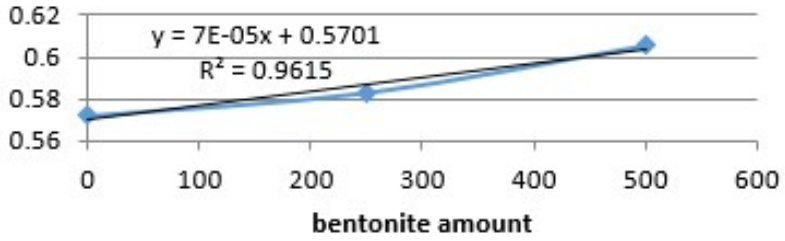

Figure 4.5: Modified Sommer White juice/wine colour after treatment with bentonite 280nm

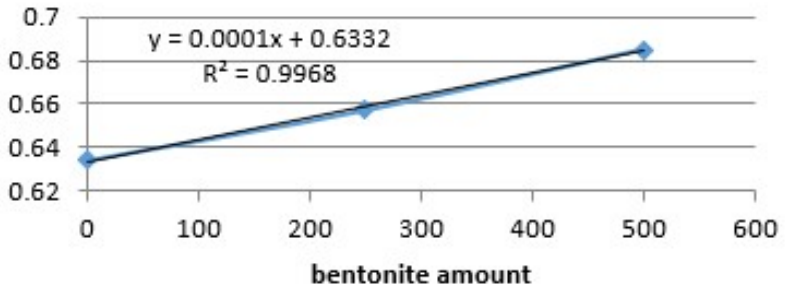

Figure 4.6: Modified Sommer White juice/wine colour after treatment with bentonite 320nm

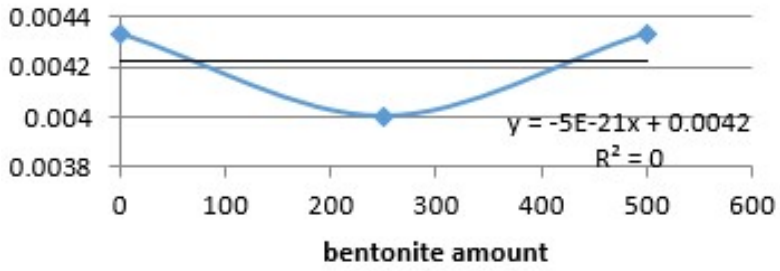

Figure 4.7: Modified Sommer White juice/wine colour after treatment with bentonite 420nm

The result of white juice and wine color420nm: Considering the P>0.05 we observe that we have the significant results following this experiments while with the other spectrophotometer result with 280 and 320nm we do not have a meaningful outcomes based on their P-value.

As the graph shows the first and second treatment and also the control trend are almost in the same level while the sample before treatment is drastically higher than them.

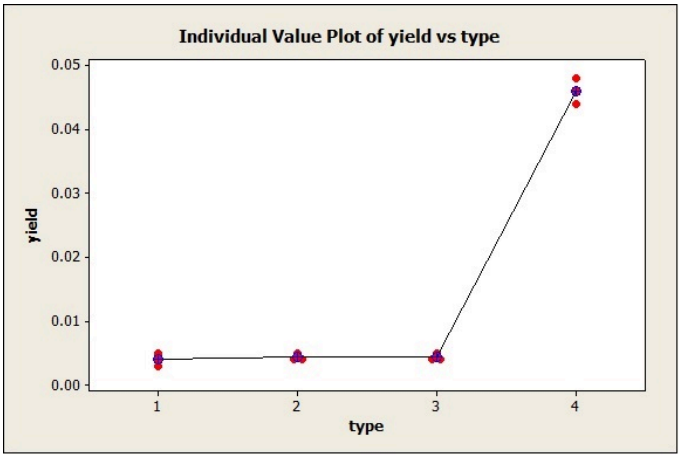

Figure 4.8: ANOVA analysis of white juice and wine colour

	Modified Sommer White juice/wine colour before treatment 280nm	320nm	420nm
	0.73	0.622	0.048
	0.28	0.581	0.046
	0.71	0.598	0.044
	Modified Sommer White juice/wine colour after treatment with 250mg/l of Tgase and 0.5g/l of bentonite at 280nm	320nm	420nm
	0.545	0.596	0.005
	0.588	0.698	0.003
	0.616	0.677	0.004
avg	0.583	0.657	0.004
	Modified Sommer White juice/wine colour after treatment with 500mg/l of Tgase and 0.5g/l of bentonite at 280nm	320nm	420nm
	0.594	0.654	0.004
	0.646	0.724	0.005
	0.577	0.677	0.004
avg	0.605666667	0.685	0.004333
	Modified Sommer White juice/wine colour, wine control 280nm	320nm	420nm
	0.611	0.682	0.004
	0.603	0.659	0.005
	0.502	0.561	0.004
avg	0.572	0.634	0.004333

Table 4.2: White juice and wine colour

4.1.3 Sodium Metabisulphite

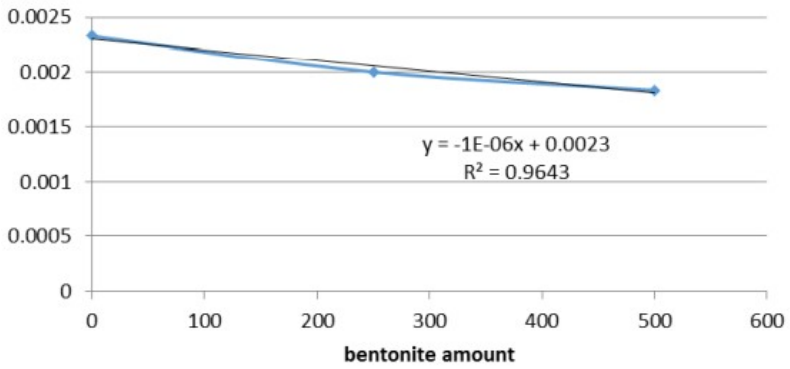

Figure 4.9: Modified Sommers prepare sodium metabisulphite after treatment with bentonite 520nm

	Modified Sommers prepare sodium metabisulphite before treatment 520nm
	0.002
	0.002
	0.003
	Modified Sommers prepare sodium metabisulphite after treatment with 250mg/l of Tgase and 0.5g/l of bentonite at 520nm
	0.001
	0.001
	0.002
	0.003
	0.001
	0.004
avg	**0.002**
	Modified Sommers prepare sodium metabisulphite after treatment with 500mg/l of Tgase and 0.5g/l of bentonite at 520nm
	0.001
	0.002
	0.002
	0.002
	0.002
	0.002
avg	**0.001833333**
	Modified Sommers prepare sodium metabisulphite, wine control 520nm
	0.003
	0.002
	0.002
	0.002
	0.002
	0.003
avg	**0.002333333**

Table 4.3: Sodium Metabisulphite

Sodium metabisulphite: So2 resistant pigments present in the wine: Following our reading with spectrophotometer at 280nm we do not have meaningful effects of adding bentonite and Transglutaminase on in this experiment. We can see that the amount of color resistant to SATO in the control and the sample before treatment is almost the same, while in the first treatment, this amount is more than the second treatment. In total, the amount of pigment in the control and sample before treatment is higher than the first and second treatment.

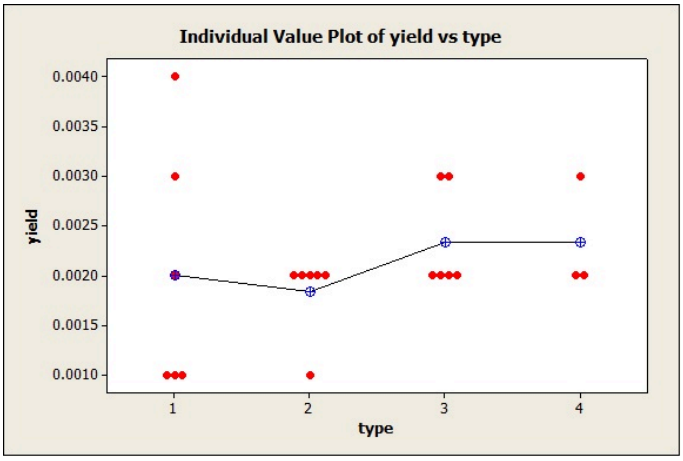

Figure 4.10: ANOVA analysis of Sodium metabisulphite

4.1.4 HCl

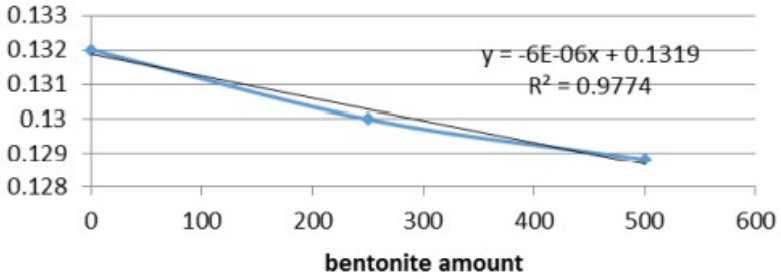

Figure 4.11: Modified Sommers prepare HCl after treatment with bentonite 280nm

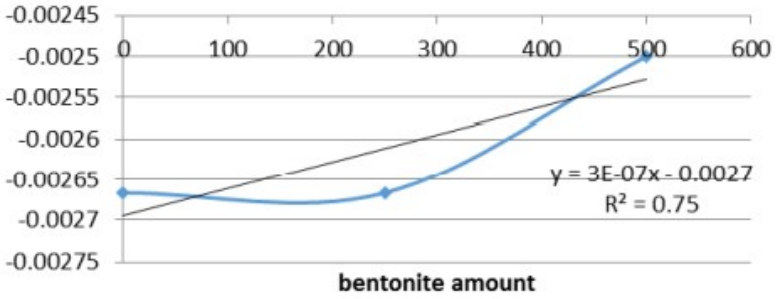

Figure 4.12: Modified Sommers prepare HCl after treatment with bentonite 520nm

	Modifeid Sommers prepare HCI before treatment 280nm	520nm
	0.14	0.001
	0.136	0.001
	0.139	0
	Modifeid Sommers prepare HCI after treatment with 250mg/l of Tgase and 0.5g/l of bentonite at 280nm	520nm
	0.131	-0.002
	0.132	-0.003
	0.134	-0.002
	0.13	-0.003
	0.126	-0.003
	0.127	-0.003
avg	0.13	-0.00267
	Modifeid Sommers prepare HCI after treatment with 500mg/l of Tgase and 0.5g/l of bentonite at 280nm	520nm
	0.131	-0.001
	0.13	-0.003
	0.126	-0.003
	0.124	-0.003
	0.13	-0.003
	0.132	-0.002
avg	0.128833333	-0.0025
	Modifeid Sommers prepare HCI, wine control 280nm	520nm
	0.129	-0.003
	0.133	-0.002
	0.134	-0.002
	0.132	-0.002
	0.132	-0.005
	0.132	-0.002
avg	0.132	-0.00267

Table 4.4: HCI

Converting all anthocyanin and other pigment into their colour form (HCI)

1. We can see from the P>0.05 that the experiment39;s findings are significant. The amount of anthocyanin and color in the sample before treatment, in the first and second treatments, and in the control, respectively, increased somewhat when the treatments were read at 520nm in this experiment.

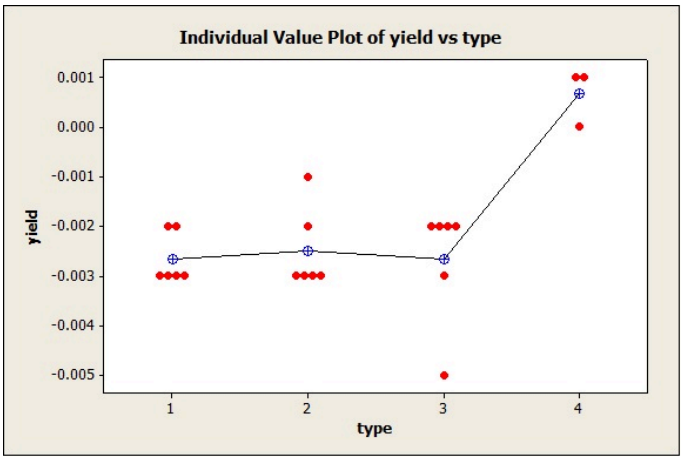

Figure 4.13: ANOVA analysis of HCI treatment with bentonite 520nm

2. Based on the P>0.05 we observe that we have the significant results following this experiments when we read at 280nm, This test is similar to the previous one, with the difference that the second treatment has a lower amount of anthocyanin and pigment than the first and second treatments.

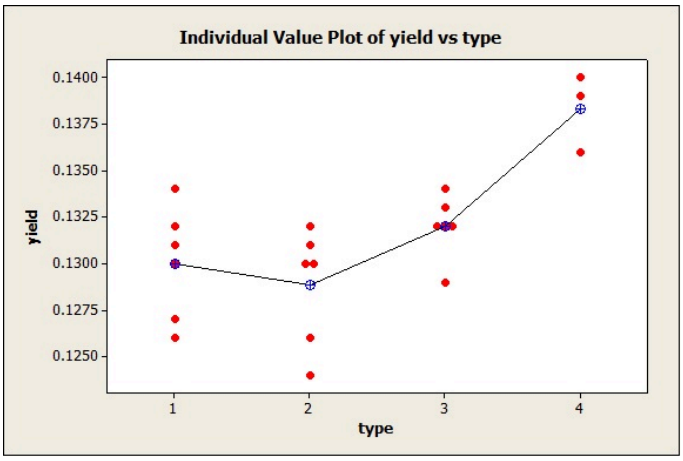

Figure 4.14: ANOVA analysis of HCI treatment with bentonite 280nm

4.1.5 Acetaldehyde

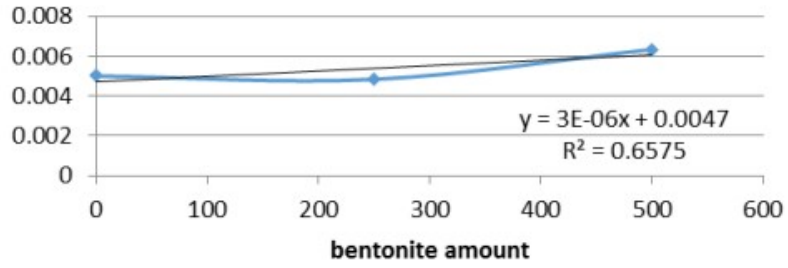

Figure 4.15: Ethanal assey after treatment with bentonite 420nm

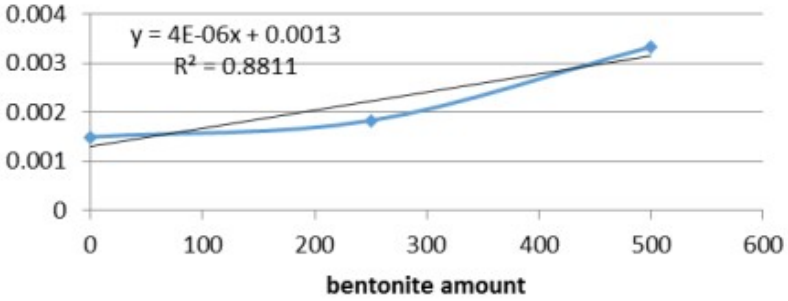

Figure 4.16: Ethanal assey after treatment with bentonite 520nm

	Ethanal assey before treatment 420nm	**520nm**
	0.004	0.002
	0.006	0.002
	0.003	0.003
	Ethanal assey after treatment with 250mg/l of Tgase and 0.5g/l of bentonite at 420nm	**520nm**
	0.005	0.002
	0.005	0.002
	0.006	0.003
	0.004	0.001
	0.005	0.002
	0.004	0.001
avg	**0.004833333**	0.001833
	Ethanal assey after treatment with 500mg/l of Tgase and 0.5g/l of bentonite at 420nm	**520nm**
	0.004	0.001
	0.009	0.006
	0.004	0.001
	0.004	0.001
	0.013	0.01
	0.004	0.001
avg	**0.006333333**	0.003333
	Ethanal assey, wine contorl 420nm	**520nm**
	0.005	0.002
	0.005	0.001
	0.005	0.001
	0.004	0.001
	0.006	0.002
	0.005	0.002
avg	**0.005**	0.0015

Table 4.5: Acetaldehyde

Excess Acetaldehyde

1. Following our result for both reading with spectrophotometer 420nm and 520nm we do not have meaningful effects of adding bentonite and Transglutaminase on Acetaldehyde. In this experiment which is observed with 420nm spectrophotometer, the amount of acetaldehyde in the first treatment and control as well as in the sample before treatment is almost the same, while it is more observed in the second treatment. In general, the amount of acetaldehyde in the sample before treatment is lower than previous one.

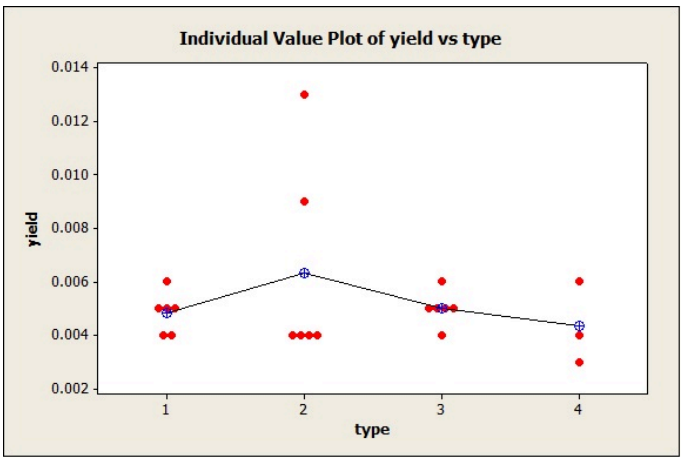

Figure 4.17: ANOVA analysis of Acetaldehyde with bentonite 420nm

2. The amount of acetaldehyde when we used 520nm spectrophotometer in the second treatment is more compared to the other treatments, and also in the sample before the treatment, the amount of acetaldehyde is more than the other treatments.

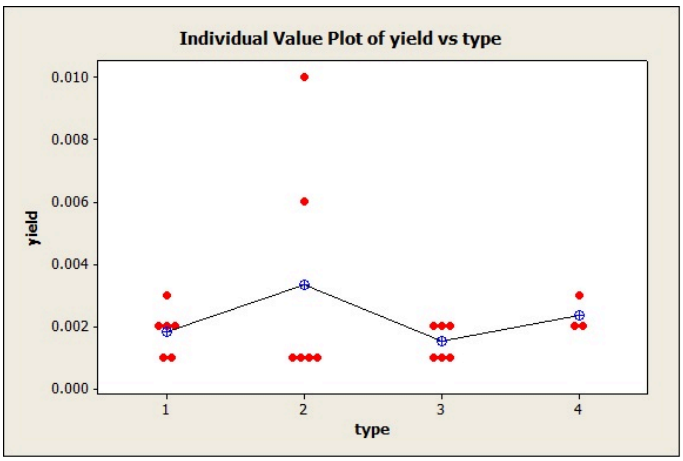

Figure 4.18: ANOVA analysis of Acetaldehyde with bentonite 520nm

4.2 BCA

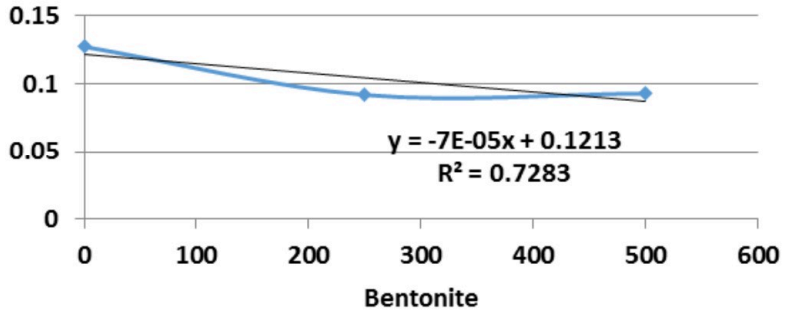

Figure 4.19: BCA experiment (treatment with bentonite and Tgase in 562nm)

The result of BCA 562: As we can see we have a significant result based on P-value for the BCA experiment. In this experiment the first treatment, second treatment and the control gradually increased respectively and we can observed that the sample before treatment has the higher trend compared to the other treatments.

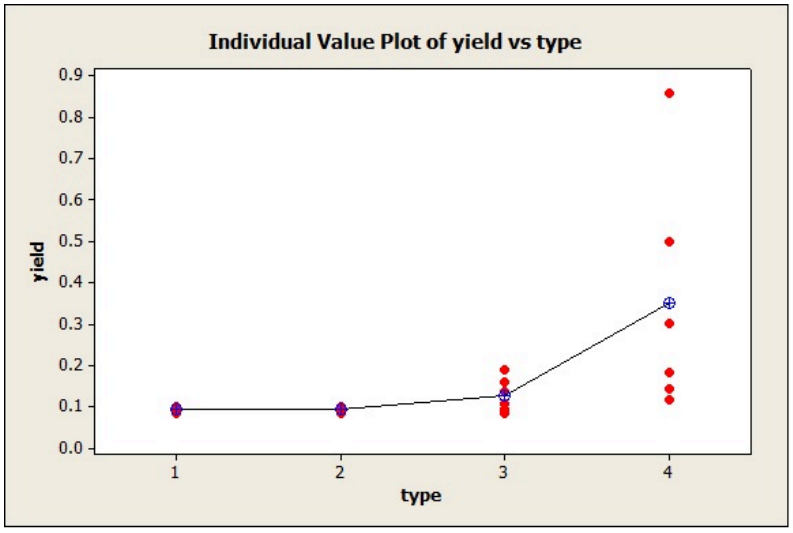

Figure 4.20: ANOVA analysis of BCA

	BCA before treatment with Tgase and control with bento 562nm
	0.858
	0.5
	0.3
	0.184
	0.142
	0.115
	BCA after treatment with Tgase and contorl with bentonite 250mg/l of Tgase and 0.5g/l of bentonite at 562nm
	0.089
	0.084
	0.1
	0.094
	0.091
	0.093
avg	**0.091833333**
	BCA after treatment with Tgase and control with bentonite 500mg/l of Tgase and 0.5g/l of bentonite at 562nm
	0.09
	0.082
	0.098
	0.095
	0.093
	0.099
avg	**0.092833333**
	BCA wine control 562nm
	0.108
	0.082
	0.135
	0.188
	0.158
	0.093
avg	**0.127333333**

Table 4.6: BCA

4.3 FC Method

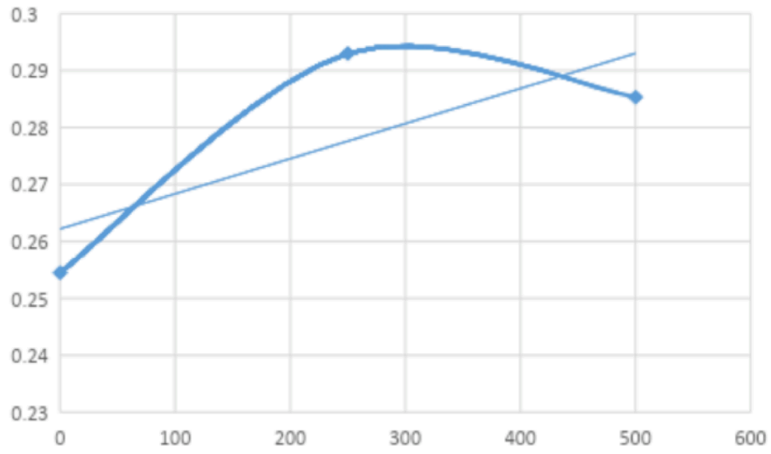

Figure 4.21: FC Method

FC method result: For this experiment we have set 5 different codes and compare all the samples before treatments and after treatments. The codes has defined from number 1 to 5 that each of codes shows respectively code 1 foe total poly phenol (Galic acid) before treatment, code 2 is defined for total poly phenol (Galic acid) wine control, code 3 is for Total polyohenols after tretment with 250mg/l bentonite, code number 4 is for Total polyohenols after tretment with 500mg/l bentonite and code number 5 is for Total polyohenols wine control.

Considering the p value which shows pgt;0.05 we understand that after treatment with Tgase the total polyphenols have been effected by it compare to before treatment. Wine control which is included by normal rate of bentonite and galic acid before treatment contain much less amount of polyphenols afterward when we add transglutaminase the amount of polyphenols sharply increased.

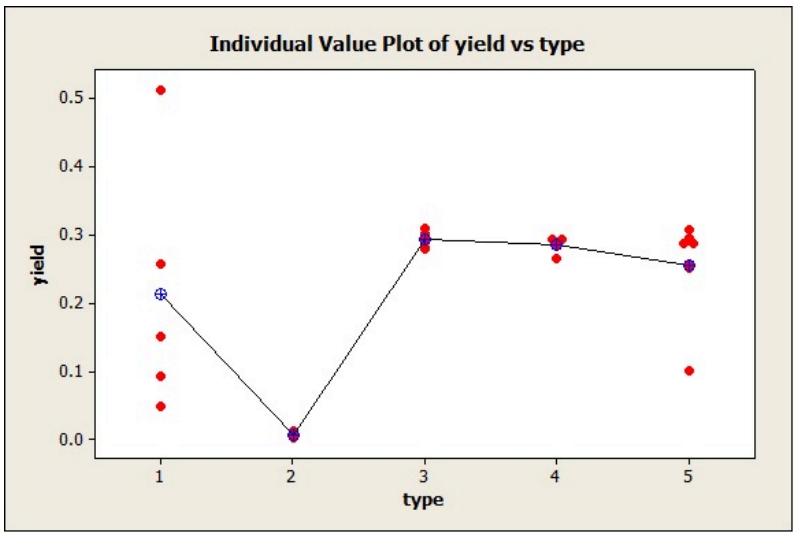

Figure 4.22: ANOVA analysis of FC method

	Total Polyphenol (Galic acid) before treatment	765nm
	50 ML	0.046/0.051
	100 ML	0.094/0.091
	150 ML	0.149/0.152
	250 ML	0.254/0.260
	500 ML	0.508/0.515
	Total polyohenols after treatment with 250mg/l tgase and 0.5g/l of bentonite at 765nm	**765nm**
	50	0.003/0.011
	100	0.012/0.012
	150	0.004/0.001
	250	0.005/0.002
	500	0.004/0.004
	Total polyohenols after treatment with 500mg/l tgase and 0.5g/l of bentonite at 765nm	
	0.31	
	0.282	
	0.294	
	0.279	
	0.291	
	0.302	
avg	**0.293**	250
	Total polyohenols after tretment with 500mg/l bentonite 765nm	
	0.287	
	0.293	
	0.29	
	0.283	
	0.293	
	0.266	
avg	**0.2853333333**	500
	Total polyohenols wine control 765nm	
	0.1	
	0.287	
	0.307	
	0.287	
	0.25	
	0.296	
avg	**0.2545**	0

Table 4.7: FC Method

5

Conclusion

After all the experiments the preliminary evaluation of the results seem to indicate that the hypothesis is confirmed.

Haze development is a big issue in the wine industry. In fact, turbidity and cloudiness in drinks especially in wine indicates that they have low quality, which led customers to reject. Consequently, a product which is returned might result in significant financial losses for a winery [1]. So, it is vital before bottling wine get stabilized to stabilize. The formation and sort of haze effects the choice of measurement for stabilizing. In terms of protein haze, bentonite emerges to be the sole option for stabilizing wine and preventing haze development after bottling [1]. This clay mineral is known by its ability to operate as a cation exchanger. Positively charged molecules can be adsorbed on its surface [2, 3].

Not only does bentonite interact with proteins, although it also interacts with other compounds. Aroma loss after fining is usually regarded as a secondary, generic consequence of bentonite,despite the fact that the reasons and frequency of incidence in white wines are un-

clear. The percent reduction of numerous odor-active white wine components after bentonite fining was significantly influenced by bentonite dose, bentonite sample, and wine style [150].

Wine is an important economic commodity; regardless, incorrect declaration wine fabrication, wine declaration and blending of the wines cause a serious financial shortage. As a result, there is a pressing demand for create fingerprint procedures that are aided by quantitative statistical methodologies in order to establish the provenance of wines. The most considerable constraint of fingerprinting is the representative selection of the group of elements or compounds detected in wine samples, which should ideally be determined solely by soil composition and not on wine technology, transit, or storage. Because they have comparable chemical characteristics, rare earth elements (REEs) would be perfect candidates for fingerprinting. [46]. Winemaking is one of the most popular economic agroindustrial activities in Mediterranean countries, but it has led and also has an unfavorable side effect which contributes to the vast number of solid, liquid, and semi-solid winery wastes that remain [76, 77]. For example, every year, the Spanish wine industry generates up to 1.4 million megagrams (Mg) of solid winery waste and roughly 24 million m3 of wastewater [76]. Several techniques for repurposing these wastes have recently emerged, including raw materials for the food sector [78], substrates for plant development [79], regarding the retention of heavy metals and pesticides [80, 81]. Winery waste, on the other hand, is largely employed as a soil supplement since it increases the bulk density and porosity of the soil [82] and soil fertility due to the presence of organic matter and nutrients, such as potassium (K), calcium (Ca), and phosphorus (P), in the wastes [68, 76]. Although the bulk of solid winery wastes are natural, containing more than 80% total organic carbon (C), there is inorganic waste, such as bentonite waste (BW), which originates from the use of bentonite as a clarifying agent during winemaking.

Based on Anova and Tukey statistical analysis for the results of experiments we realized that the effects of Transglutaminase on Bentonite is feasible and significant regarding methods of protein stability and some other factors such as colour density which is a part of Modified Sommer Assay and polyphenols.

In Wine colour Parameters and wine colour density within both dosage of Tgase and Bentonite 250mg/L and 500ml/L we have a significant reduction of Bentonite usage.

For the experiment of Excess Acetaldehyde and Sodium metabisulphite So2 resistant pigments present in the wine we do not have a significant effects of Tgase and Bentonite before treatment of base wine and after treatments with Tgase.

In the experiments of converting all anthocyanin and other pigment into their colour form (HCI) also we have a significant and meaningful of Tgase treatment on Bentonite.

In white juice and wine color experiment we observed that the effect of Tgase treatment is significant on Bentonite.

The statistical result of BCA experiment shows that we have a significant effect of Tgase on Bentonite regarding protein qualifying and stability.

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