

AN ABSTRACT OF THE THESIS OF

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Title: Characterization and Removal of Unstable Proteins
from Grape Juice and Wine.

Abstract Approved: _____
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Improved sensitive techniques were developed for the determination of soluble proteins in grape, juice and wine. These techniques which permitted direct sample analysis without prior dialysis or concentration, were used for: 1) characterization of heat unstable proteins, 2) evaluating the effect of bentonite fining and ultrafiltration (UF) on the soluble proteins and heat unstable proteins in Gewurztraminer and Riesling juices and wines. Protein molecular weights (MW), isoelectric points (pI) and glycoproteins were determined by using LDS-polyacrylamide gel electrophoresis and 2-dimensional isoelectric focusing (IF)-LDS electrophoretic techniques with silver staining as well as protein blotting for glycoprotein detection. Relative concentrations of proteins in stained gels were determined by laser scanning densitometry. Bentonite fining tends to remove higher pI (5.8-8.0), intermediate MW (32,000-45,000) protein fractions first. In general, it is necessary to remove the lower pI (4.1-5.8), lower MW (12,600 and 20,000-30,000) fractions and glycoproteins to

"protein stabilize" wines to heat testing. Unstable proteins precipitated by heat test were recovered and analyzed. These proteins were mainly of low MW (<30,000) and primarily glycoproteins. Protein fractions with MW of greater than 14,000 were more heat sensitive than lower MW fractions. Wines and juice were ultrafiltered with Romicon and Millipore systems operated with membranes of "nominal MW cut-off (MWCO)" of 10,000-100,000 daltons. A progressive increase in membrane retention of soluble protein was observed with decreasing MWCO, up to 99% of wine protein being retained with membranes of 10,000 dalton MWCO. However, certain high MW protein fractions also remained in permeates even with 10,000 MWCO membranes. In the order of 3-20 mg protein/L frequently remained in UF wine permeates, this correlated with the periodic detection of heat instability and of a low bentonite requirement for "protein stability" as determined by sensitive heat testing. "Protein stability" could be obtained with MWCO of 10,000 and 30,000; however, when not obtained, reductions in the order of 80-95% in "bentonite demand" were achieved. Protein stabilization of wines by UF is similar to that by bentonite fining in that it is necessary to remove the lower MW (12,600 and 20,000-30,000), lower pI (4.1-5.8) fractions, and glycoproteins to stabilize wines to heat testing. UF membranes were more effective at retaining wine proteins than juice proteins. UF juices were more susceptible to heat induced haze formation than UF wines.

Characterization and Removal of Unstable Proteins
from Grape Juice and Wine

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CONTRIBUTION OF AUTHORS

B.T. Watson and J.H. Flores contribution to the third manuscript was the processing of grape juice and wine, and the ultrafiltration of grape juice and wine samples for this study respectively.

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Characterization and Removal of Unstable Proteins from Grape Juice and Wine

.. Introduction

A knowledge of the proteins present in grapes, grape juices and wines is important to grape juice processors and winemakers. Of particular concern is the presence of unstable soluble proteins which can come out of solution and form hazes and sediments. Although there have been numerous investigations of proteins present in grapes, juices and wines, there remains an incomplete understanding of the nature of proteins involved in turbidity formation. In part, this has been due to limitations in analytical techniques.

Part I of this study is concerned with the development of improved techniques for the isolation and characterization of the soluble proteins in grape, grape juice and wine. Parts II & III are concerned with the application of these techniques for the characterization and removal of heat unstable proteins in grape juice and wine by bentonite fining, heat treatment, and ultrafiltration.

Literature Review

Characterization of grape and wine proteins

The soluble proteins in grapes and wines are globular in nature, and are mainly albumins (Koch and Sajak, 1959; Hennig, 1970). Unique electrophoretic patterns of the protein components have been obtained for various wines from different varieties of grapes (Koch and Sajak, 1959; Ebermann et. al., 1972; Yokotsuka et. al., 1977; Luis, 1983).

The molecular weight (MW) of the protein fractions is reported to be distributed over a wide range. The highest molecular weight reported is greater than 1,000,000 in Australian wines. The higher MW fractions only account for a very small percentage of the total proteins (Heatherbell et. al., 1985; Lee, 1986). To account for the high MW of some wine protein fractions, it might be necessary to consider previous suggestions that proteins of must and wine are actually complexed with tannins (Somers and Ziemelis, 1973), polysaccharides, and pectin (Usseglio-Tomasset and Stefano, 1977).

The isoelectric points (pI) of the protein fractions have been reported to range from pI of 2.5-8.7 (Lyubarevich et. al., 1975; Anelli, 1977; Yokotsuka et. al., 1977; Luis, 1983; Mesrob et. al., 1983; Heatherbell et. al., 1985; Lee, 1986). However, acidic components are predominant (Molnar,

1975; Anelli, 1977; Luis, 1983). This is consistent with the observation that the predominant amino acids in both juice and wine are aspartic acid, glutamic acid, threonine, serine and alanine (Pavlenko, 1976; Anelli, 1977; Yokotsuka et. al., 1977).

The presence of carbohydrates in grape proteins is reported for the White Riesling, Muller-Thurgau and Muskat Alexandriiskii varieties (Koch and Geiss, 1955; Weiss et. al., 1960; Lyubarevich et. al., 1975). The Schiff reagent has been used to test for the presence of sugar residues in protein fractions (Lyubarevich et. al., 1975).

Extraction of grape proteins

The isolation of grape proteins has typically been a difficult task, because of the presence of endogenous phenolic compounds, phenol oxidases, and peroxidase (Loomis and Battaile, 1966; Loomis, 1974; Matheis and Whitaker, 1984). During conventional extraction procedures, some proteins may be precipitated by phenols and oxidases, whereas others may be degraded and modified without precipitation. In addition to the covalent attachment of quinones through sulphhydryl and amino groups, the unoxidized phenols can also interact with proteins by hydrogen, hydrophobic, and ionic bonding (Loomis, 1974).

The use of liquid nitrogen in the preparation of liquid nitrogen powder (LNP) has been used as a first step in

isolation of proteins (Wesche-Ebeling, 1984). Liquid nitrogen lowers the temperature to prevent any reaction occurring before protein extraction.

Phenol-complexing agents which aid in the separation of proteins from phenols have been recommended by Loomis (1966, 1969, 1974, 1979). These include polycaprolactam powder (Sanderson, 1964), polyethylene glycol (Badran and Jones, 1965; Benjamin and Montgomery, 1973), polyvinylpolypyrrolidone (PVPP) (Loomis and Battaile, 1966; Anderson and Sowers, 1968), Amberlite XAD resins (Loomis, 1974; Gray, 1978; Loomis et. al., 1979), and anion exchange resins (Lam and Shaw, 1970; Gray, 1978). These absorbents remove phenolic compounds through the formation of hydrogen, hydrophobic and ionic bonds (Loomis and Battaile, 1966; Fields and Tyson, 1973; Olsson et. al., 1976; Loomis et. al., 1979). There has been some debate over the effectiveness of these phenolic scavengers since it has been speculated that protein may also bind to the resin (Walker and Hulme, 1965; Jone et. al., 1965; Lam and Shaw, 1970). However, since all plant materials contains different types and quantities of phenolic compounds there is no single compound or combination of compounds that are universally applicable (Rhodes, 1977).

Therefore, each plant system must be examined individually to optimize the protein extraction and minimize protein-phenolic interactions.

Methods for determination of total proteins in grape juices and wines

Kjeldahl method. The Kjeldahl method depends on converting the protein nitrogen into ammonia. The application of the factor 6.25 to obtain the protein content is arbitrary, as the factor varies between raw materials. This procedure is the most widely used test to determine total nitrogen. However, the values obtained are usually too high, due to the other nitrogenous substances which may be present along with proteins (Ferenczi, 1966; Bayly and Berg, 1967).

Voit method. The Voit method depends on the reaction between phosphomolybdic acid and nitrogenous compounds to form precipitates (Koch and Sajak, 1959). The proteins are selectively removed by washing the precipitates with 95% alcohol, and then determined by the Kjeldahl method. However, the Voit procedure precipitates both proteinaceous and non-proteinaceous materials, and the calculated protein contents are usually too high (Cordonnier, 1966; Kuridze and Mindadze, 1979).

Diemair and Maier method. The modified Diemair and Maier method (Bayly and Berg, 1967) uses phosphomolybdic acid as the precipitant. The proteins are then determined by the Lowry method instead of Biuret method. Greenberg and Shipe (1979) stressed that common protein precipitants are arbitrary and vary in their precipitating capability.

Co-precipitation of phenolics with proteins may cause the high protein values.

UV (E280 : E260) method. The UV method depends on the ratio of UV absorption at 280 nm and 260 nm (Layne, 1957). Phenolics interfere directly with this method. Loomis (1974) reported that negative values of protein in plant extracts could be obtained by this method.

Gel filtration method. Gel filtration methods depend on the separation of proteins by Sephadex G-25 column followed by UV detection at 280 nm. A linear relationship exists between peak height and protein content (Somers and Ziemelis, 1973). Nevertheless, this method is time consuming, and the sensitivity is limited.

HPLC method. An HPLC method used for the analysis of total soluble protein levels in grape, must and wine was first described by Tyson *et. al.* (1981). In this method, the Waters Protein Separation System (PSS) Column I-125 with a mobile phase of 0.01 M ammonium acetate containing 0.01 M sodium dodecylsulfate (SDS) was used for protein separation. Protein was then detected and estimated at 280 nm by using electrophoretically pure bovine serum albumin as the standard protein. Luis (1983) replaced the 0.01 M SDS with 10% glycerol as mobile phase additive to improve the protein specificity and column efficiency. However, poor resolution and sensitivity are still limitations of this method.

Biuret method. The Biuret method depends on the formation of blue copper-protein complexes under alkaline conditions (Gornall et. al., 1949). Unfortunately, copper forms colored complexes with phenols as well and produces falsely high values.

Lowry method. The Lowry method relies on the ability of copper-protein (biuret complexes) to reduce the Folin phenol reagent (Lowry et. al., 1951). Phenolics can reduce the Folin phenol reagent directly and produce falsely high values.

Bio-Rad method. The Coomassie Brilliant Blue G-250 dye protein assay (Bio-Rad method) depends upon the conversion of protein in solution from brownish-orange to an intensely blue color (Bradford, 1976). The test can be used with a variety of proteins and polypeptides with molecular weight greater than 3000 (Sedmak and Grossberg, 1977). Robinson (1979) concluded that when polyphenolic compounds were present the most reliable method for protein determination was this method. Furthermore, this method has been chosen for determination of beer proteins (Hii and Herwig, 1982).

The dye-anion interacts with the amine groups on the proteins under acid condition. Not all proteins have the same proportion of amine groups, and not all amine groups react identically with the G-250 dye. Thus, not all proteins response similarly to binding by G-250 dye (Bradford, 1976; Sedmak and Grossberg, 1977; Van and Hale,

1977; Bearden, 1978; Almog and Berns, 1981). Almog and Berns (1981) recommended therefore that the standard curve be prepared from a protein of similar composition and structure to that of the sample to be measured.

Electrophoresis of grape and wine proteins

Electrophoresis of grape and wine proteins was first reported in 1959 by Koch and Sajak, in which two protein fractions were obtained by paper electrophoresis. Later Moretti and Berg (1965) using disc gel electrophoresis separated wine protein into several fractions. Recently, Heatherbell et. al. (1985) and Lee (1986) have used gel electrophoresis and isoelectric focusing in studies on wine proteins. More information about "electrophoresis of grape and wine proteins" has been reviewed in Section I under "characterization of grape and wine proteins" and "proteins and wine stability".

Proteins and wine stability

Despite the vast literature on protein instability in wine, the nature of the proteins responsible for wine turbidity remains unclear (Heatherbell et. al., 1985). Koch and Sajak (1959), in examining several varieties of grape, obtained two protein fractions by paper electrophoresis. The level of both fractions was decreased by heat treatment and bentonite addition. Moretti and Berg (1965) associated

specific protein fractions with wine turbidity and concluded that only a part of the protein mixture was responsible for protein stability rather than total protein content. Bayly and Berg (1967) further classified the protein fractions according to their heat stability and concluded that removal of protein fractions by addition of bentonite did not occur in equal proportions, but removed the highly charged protein molecules first. Millies (1975) using silica sol/gelatin fining and ultrafiltration to fractionate the wine proteins, concluded that the protein fractions with MW of less than 10,000 did not take part in turbidity formation, fractions between 10,000 and 30,000 were only partly involved and those with MW of greater than 30,000 were the main unstable proteins. Millies also concluded that the binding power of bentonite decreased with increasing MW of fractions, whereas silica sol/gelatin was the most effective at adsorbing the fractions with MW of greater than 30,000. Mesrob et. al. (1983) indicated that the protein-clouding is mainly caused by protein fractions with lower pI and lower MW. Recently, Heatherbell et. al. (1985) characterized the unstable proteins in Oregon wines and concluded that the proteins with lower MW in the range 16,000-25,000 and higher pI in the range 5.2-8.0 are major contributors to protein instability. Lee (1986) reported that protein fractions of MW between 40,000 and 200,000 and pI values between 4.8 and 5.7 are potential

sources of protein instability in bottled wine. Lee also indicated that MW and pI of the proteins in grape and wine are different, and the protein fractions with the higher MW and those are basic are first removed by bentonite.

Protein stability tests

Heat test. The heat tests are probably most comparable to the eventual effects of storage on haze/sediment formation in bottled wine. There are several recommended in the literature. Troost (1961) in Germany has summarised literature reports in which test temperatures ranged from 30 °C to 60 °C for from 15 min to 28 h. Ribereau-Gayon and Peynaud (1961) for French wines recommended that wines heated to 80 °C for 10 min without forming a haze on cooling are heat-stable. Berg and Akiyoshi (1961) for Californian wines recommended heating at 50 °C for 4 days, room temperature for 1 day followed by cooling to -5 °C for 1 day, then bringing to room temperature for evaluation. Pocock and Rankine (1973) for Australian wines recommended 80 °C for 6 h followed by cooling in a refrigerator at 4 °C overnight. The main disadvantage of these tests is that they tend to be time consuming.

Trichloroacetic acid test. The trichloroacetic acid (TCA) test consists of adding 1 mL of a 55% TCA solution to 10 mL of wine, following by heating for 2 min in boiling water and a reaction period of 15 min at room temperature

before observation. Berg and Akiyoshi (1961) indicated that the TCA test can be correlated with protein stability as measured by heat cold test. However, it has not proved satisfactory in industry testing.

Bentotest. In this test, one mL Bentotest reagent is added to 10 mL of filtered brilliant wine and mixed. The solution is then examined under a strong light for haze. The Bentotest reagent is basically a solution of phosphomolybdic acid in HCl. It precipitates the protein in the wine by neutralizing the charge on the protein molecules and also aggregates them by crossing with the heavy molybdenum ion. Although having the advantage of being rapid, the Bentotest tends to be more sensitive than the heat test and may lead to overfining (Rankine and Pocock, 1971).

Colloidal constituents of juices and wines

Grape juices and wines contain colloids, which are mainly composed of polysaccharides, proteins, and polyphenolics (Nilov et. al., 1975; Usseglio-Tomasset and Stefano, 1977). Colloidal phenomena is important to understand, because it is relative to the stabilization of juice and wine. The major sources of the colloidal materials may: 1) originally come from the grapes, 2) come from the grapes infected with Botrytis cinerea or other molds, and 3) be formed by yeasts (Usseglio-Tomasset, 1978;

Wucherpfennig and Dietrich, 1983; Villettaz et. al., 1984). Colloid contents of 167-324 mg/L in white wines and 962.9 mg/L in red wines have been reported (Wucherpfennig and Dietrich, 1983). Usseglio-Tomasset (1978) indicated that the colloids formed by yeasts contain approximately 29% proteins and 71% carbohydrate; and that 43% of the protein is of MW greater than 300,000 which may be due to the protein being complexed with carbohydrate/tannin. Recently, Wucherpfennig et. al. (1984) reported that colloidal material with MW of greater than 10,000 appears during fermentation. When the grapes are infected with Botrytis cinerea (gray rot/noble rot), a beta glucan can be found in the wine. This Botrytis glucan is insoluble in 30% (v/v) alcohol, giving a filament precipitate, and is responsible for clarification and filtration problems (Villettaz et. al., 1984).

Composition of wine protein haze

Protein is the major haze problem reported in wines. For instance, Gortges (1982) analyzed 117 bottles of wine containing haze, and indicated that 44% were due to metal/protein/tannin haze, 20% were crystalline in nature, 22% were due to microorganisms, and 14% were due to other causes. As is well known, the crystalline deposits in wine are caused by an excess of either potassium bitartrate or calcium tartrate (Amerine and Roessler, 1976; Gorinstein

et. al., 1984). Early on Koch and Sajak (1959) reported that heat-formed sediment as well as protein haze in wines mainly contained protein, but also contained pectin (galacturonic acid, galactose, arabinose), tannins and inorganic constituents. Ribereau-Gayon et. al. (1976) described that the protein haze contains 5-12% of nitrogen (of which 50-80% is protein), 1-15% of ash, the remainder being divided between adsorbed phenolics (2-5%) and polysaccharides (12-14%). Usseglio-Tomasset (1978) indicated that protein haze in wine is mainly due to the interactions between proteins, tannins, carbohydrates and pectins.

Four mechanisms have been proposed to account for these reactions: 1) Tannins can compete with proteins for the water of solvation. The phenolic hydroxyl group of tannin through hydrogen bonding dislodges the hydrophilic moiety of the protein remarkably and is sufficient to bring about precipitation. 2) The formation of polymeric compounds due to the oxidation of protein-tannin complex can easily lead to flocculate and form a haze. 3) The formation of salt linkages between phenolic hydroxyl groups of tannins and basic amino acid residues of proteins, causes the positive charge on the protein sol to diminish and a negative charge to predominate. In the presence of even small quantities of metallic salts such as copper, an insoluble complex is formed. 4) The interaction between the aromatic

ring structures of tannins and hydrophobic regions of proteins may be also involved in haze formation (Joslyn, 1953; Ferenczi, 1966; Singleton, 1967; Loomis, 1974).

Removal of proteins by bentonite fining

Bentonite is widely used internationally in the wine industry for protein stabilization of wines. Bentonite is an adsorptive clay colloid used especially as a carrier. The nature and properties of bentonite are described by Rankine (1962), Rankine and Emerson (1963) and Jakob (1975). The main mechanism involved in the adsorption of protein by bentonite is an ion-exchange type reaction. At wine pH, the wine proteins which are positively charged are adsorbed by the clay particles which carry a negative charge (silicate). Electrostatic interaction between charged particles accounts for the secondary adsorptive capacity of bentonite. Taran et. al. (1975) suggested that 3 adsorption zones are established in the course of defecation: 1) kinetic zone: the adsorption occurs on the outer surface of the particles after 10-15 min; 2) diffusion zone: the adsorption occurs in the pores of clay particles after 1.5-2 h; 3) coagulation zone: corresponding to precipitation of colloids by coagulation and sedimentation after 3 h or more.

The factors affecting the efficiency of bentonite treatment include the type of bentonite, level of bentonite

addition, temperature, pH and sample composition. Bentonite which has been pre-swollen under high temperature, low pH, high alcohol level and low tannin content will give the best fining results. Also, amounts of bentonite added have a direct correlation with the quantity of protein removed from wines (Koch and Sajak, 1959; Ferenczi, 1966; Amerine et. al., 1980; Luis, 1983). Bentonite removes many other constituents from wine. Some of these are involved in sensory properties. Bentonite also contributes some other flavor compounds to wine and may influence the quality of wine.

Ultrafiltration of juices and wines:

Crossflow membrane filtration is the separation of the components of a fluid by polymeric semi-permeable membranes through the application of pressure (Paulson et. al., 1984, 1985). The pressure required varies depending on the size of the pores in the membranes. Ultrafiltration (UF) membranes have pore sizes of 10 Angstroms to 0.2 micron, rejecting larger molecules such as proteins and microorganisms while passing most ions. UF polymers currently available include: polysulfone, cellulose acetate-blends, a fluorinated polymer, and Osmonics' patented polymer (aromatic polyamide type). Operating pressure of UF membranes is usually from 20 to 200 psi. Operating temperature can be up to 100 °C and in some

cases membranes are autoclavable. pH range tolerances are usually great, for instance polysulfone membranes are frequently rated as compatible from pH 0.5 to 13.

UF is emerging as a versatile membrane separation process which is increasingly finding application in the beverage industry (Swientek, 1986). Its application as an alternative to conventional processes for clarifying and preserving fruit juices was demonstrated as early as in 1977 by Heatherbell et. al. for apple juice. More recently, there has been increased interest in the application of UF for processing a wide range of fruit juices (Wucherpfennig and Neubert, 1977; Wilson and Burns, 1983; Moslang, 1984; Nagel and Schobinger, 1985; Paulson et. al., 1985) including grape, pear, and berry juices in our own laboratory (Fombin, 1983; Fombin et. al., 1983; Kirk et. al., 1983; Heatherbell et. al., 1983 & 1985). Industrial scale operations for juice processing are in operation in several countries (Moslang, 1984; Anonymous, 1985a; Nagel and Schobinger, 1985; Rosch, 1985; Hackert and Swientek, 1986; Swientek, 1986). The possibility of using membrane ultrafiltration for clarifying and protein stabilizing grape juice and wine has been recognized (Wucherpfennig, 1978; Drioli et. al., 1981; Gaillard, 1984; Poirier et. al., 1984; Miller et. al., 1985; Anonymous, 1985a). Apart from a recent report by Gnekow et. al. (1983) claiming protein stabilization of wine with an industrial scale

operation with California wines, commercial development of UF by the wine industry does not appear as advanced as in the juice industry. However, it is known that commercial scale units are in operation in several countries including France, Italy, Australia, Germany as well as in the U.S..

Isolation and Characterization of Soluble Proteins in Grape, Grape Juice and Wine

Juinn-Chin Hsu¹ and David A. Heatherbell²

ABSTRACT

Improved sensitive techniques for the determination of soluble proteins in Gewurztraminer and White Riesling grape, juice and wine are reported. These techniques: 1) minimize interference from phenolics and oxidase enzymes during extraction of proteins, 2) permit direct determination of proteins in juice and wine samples by electrophoretic techniques without prior pretreatment and concentration, 3) permit the detection of glycoprotein, and 4) permit the simultaneous determination of protein molecular weight (MW) and isoelectric point (pI) by 2-dimensional isoelectric focusing-lithium dodecyl sulfate polyacrylamide gel electrophoresis. Phenolic scavengers, Amberlite XAD-4 & PVPP; and the enzyme inhibitors, PMSF & DECA; are used for protein isolation. One dimensional 7.5-15% sucrose stabilized linear gradient LDS-polyacrylamide slab gel electrophoresis with silver staining permits effective resolution of 3 ug of protein. The sensitivity for protein detection is increased 100 fold (detection at 10 ng level) by using silver staining instead

of Coomassie Blue. Changes in relative concentration of proteins on stained gels were determined by laser scanning densitometry. Two dimensional IF-LDS gel electrophoresis as well as protein blotting for glycoprotein detection were used for further separation and characterization of the soluble proteins in wine. Grapes contain up to 41 protein fractions with MW in the range of 11,200-190,000; juices and wines from these grapes contains up to 25 protein fractions with MW in the range of 11,200-65,000. Each of these fractions were further separated into several fractions on the basis of their pI. A high proportion of wine protein fractions is of low pI (4.1-5.8) and low MW (20,000-30,000). Three protein fractions with MW of 12,600, 25,000 and 28,000 are identified as containing glycoproteins in Gewurztraminer wine. Only two fractions with MW of 25,000 and 28,000 were detected as containing glycoproteins in Riesling wine.

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INTRODUCTION

A knowledge of the proteins present in grapes, grape juices and wines is important to grape juice processors and winemakers. Of particular concern is the presence of unstable soluble proteins which can come out of solution and form hazes and sediments. Although there have been numerous investigations of proteins present in grapes, juices and wines, there remains an incomplete understanding of the nature of proteins involved in turbidity formation. In part, this has been due to limitations in analytical techniques. The isolation and determination of grape proteins has typically been a difficult task, because of the presence of endogenous phenolic compounds, phenol oxidases, and peroxidase (16, 17, 21). During conventional extraction procedures, some proteins may be precipitated by phenols and oxidases, whereas others may be degraded and modified without precipitation. Once extracted and before their determination by electrophoresis, it has been necessary to purify/concentrate proteins by dialysis or ultrafiltration (2, 10, 14, 15, 19, 22, 30, 36). As well as being time consuming, these procedures may also cause modification/denaturation of proteins. These limitations were encountered in recent investigations in our laboratory concerning the nature of unstable proteins and their removal from juices and wines by bentonite, ultrafiltration

and protease enzymes (10).

The present study reports the application of improved sensitive techniques for the determination of proteins in grapes, juices and wines which: 1) minimize interference from phenolics and oxidase enzymes during extraction of proteins, 2) permit direct determination of proteins in juice and wine samples by electrophoretic techniques without prior pretreatment and concentration, 3) permit the detection of glycoprotein, and 4) permit the simultaneous determination of protein molecular weight (MW) and isoelectric point (pI) by 2-dimensional isoelectric focusing-lithium dodecyl sulfate polyacrylamide gel electrophoresis (2-D IF-LDS PAGE).

MATERIALS AND METHODS

Chemicals: All reagents and molecular weight standards used for LDS-PAGE were obtained from Bio-Rad Laboratories (Richmond, CA), except LDS which was purchased from BDH Chemicals Ltd. Silver nitrate used for silver staining was obtained from Sigma Chemical Company. The phenol absorbents, Amberlite XAD-4 (XAD-4) purchased from Rohm & Haas, and polyvinylpolypyrrolidone (PVPP) purchased from Sigma, were cleaned before used (16-18).

Preparation of grape juices and wines: White Riesling and Gewurztraminer grapes from OSU experimental vineyards were harvested between October and November 1984 and processed into juices and wines by conventional procedures in the OSU experimental winery. Settled press juices (overnight settling at 18 °C) and young wines (after 2 rackings) were sampled for analysis of proteins.

Isolation of proteins from grapes, juices and wines: Destemmed intact grapes were frozen in liquid nitrogen and stored at -40 °C until used. For analysis, 50 g of grapes were placed in a Dewar flask (Lab-Line Instruments, Inc., Melrose Park, Ill.) containing liquid nitrogen and then homogenized for 50 sec under liquid nitrogen by using a Waring Blender with a stainless steel container. The

resulting liquid nitrogen powder (LNP) was held under liquid nitrogen in a Dewar flask until used.

For extraction of grape proteins, 3 g of LNP was added to 30 mL of 0.1 M citric acid - 0.2 M sodium phosphate buffer pH 5.0, in presence or absence of: 6 g XAD-4, 3 g PVPP, 5 mM phenylmethanesulfonyl fluoride (PMSF) and 5 mM diethyldithiocarbamate (DECA). The suspensions were mixed gently with a glass rod, set at 4 °C for at least 2 h, filtered through nylon cloth and centrifuged at 12,100 X g for 10 min at 4 °C in a Sorvall RC-5 centrifuge. The supernatants were collected and filtered through a Whatman #1 filter paper to remove floating debris. The filtrates were stored in stoppered glass tubes at 4 °C until used.

For extraction of juice and wine proteins, a combination of XAD-4 and PVPP was used to remove phenolic compounds from juice and wine. Six gram of XAD-4 and 3 g of PVPP were added to 30 mL of juice or wine samples, and the samples treated as described for extraction of grape proteins.

Determination of soluble protein and total phenol: A modified Bio-Rad (Bradford) dye-binding procedure (11) using bovine serum albumin as a standard was used for total soluble protein determination (as bovine serum albumin equivalent, BSAE), with the following modifications: 1) The five-fold diluted filtered dye reagent was prepared fresh

daily. 2) The reaction condition was fixed for 15 min at room temperature. All juice and wine samples were centrifuged at 10,000 X g for 10 min at 4 °C or filtered through 0.45 um membrane before analysis. The method of Singleton and Rossi (29) using gallic acid as a standard was used for determination of total phenols.

LDS-polyacrylamide gel electrophoresis: LDS-PAGE was performed as described by Perdew *et. al.* (26). A Bio-Rad protein 32-cm-slab cell unit with dimensions of 14x32x1.5 cm was utilized to run a sucrose stabilized linear 7.5-15% gradient gel including a 5% stacking gel. LDS was used instead of sodium dodecyl sulfate (SDS), because of its greater solubility in the cold (3).

Bio-Rad molecular weight standards were prepared and used as molecular weight markers (27). The MWs of unknown samples were calculated from regression equation of log MW vs. mobility. To prepare the samples for electrophoresis, two volumes of samples were added to one volume of sample buffer containing 6% LDS, 36% sucrose, 0.15 M Tris-HCl (pH 6.8), 6 mM ethylenediaminetetraacetic acid (EDTA) and 0.06 M dithiothreitol (DTT). Once samples were in sample buffer, they could be run immediately or stored at -40 °C for at least 2 months without change. Forty to 100 ul sample volumes which were calculated to contain 3 ug of proteins were applied to the gels.

Gels were electrophoresed at a constant current setting

of 15 mA per gel at 4 °C until the bromophenol blue tracking dye was 3 cm from the bottom of the gels. Following electrophoresis, gels were Coomassie Blue stained (31), or silver stained (35). Stained gels were scanned with a Zeineh soft laser scanning densitometer (Biomed Instruments, Inc.) interfaced with an Apple IIe computer. Utilizing a software package (Model ER1P3 Biomed Instrument Inc.) the areas under the scan can be integrated for comparative purposes.

Two-dimensional IF-LDS PAGE: IF and LDS-PAGE were performed according to the procedures described by O'Farrell (24) as modified by Vlasuk et al. (32) and Perdew et al. (26), except a constant current setting of 15 mA was used rather than 18 mA. pH gradient was measured using a Bio-Rad surface electrode (Bio-Rad Laboratories) as described by O'Farrell (24).

Protein Blotting: Electrophoretically separated proteins were transferred from LDS-acrylamide gels to nitrocellulose sheets by the methods of Burnette (4) and Glass et al. (8). The transfers were performed at 4 °C, at constant voltage (24 V) for 30 min in a GENIE electroblotter unit (Idea Scientific Co., Oregon). Glycoproteins on nitrocellulose transfers were detected by the method of Clegg (6), except 3,3'-diaminobenzidine

(30 mg/100 mL) was used as the enzyme substrate.

RESULTS AND DISCUSSION

In initial studies investigating which grape proteins contributed to protein instability in juices and wines very poor resolution and detection of grape proteins were obtained unless phenolic scavengers and enzyme inhibitors were used during protein extraction.

Therefore, a technique was developed utilizing a combination of XAD-4/PVPP (18) and PMSF/DECA (34) in extraction buffer (pH 5.0) to minimize the interaction between proteins and phenols. In addition, LNP of grapes were prepared to minimize undesirable reactions occurring before protein extraction. The improvement in detection and resolution of protein fractions obtained from Riesling and Gewurztraminer grape extracts prepared in the presence of XAD-4/PVPP and enzyme inhibitors is illustrated in Fig. II.1. Forty-one fractions are readily detectable in Riesling and 35 fractions in Gewurztraminer using this technique (Fig. II.1).

In contrast, the isolation of juice and wine proteins, in the presence of XAD-4/PVPP did not improve the resolution and detection of proteins (Fig. II.2). This is attributed to the interaction between proteins and phenols, which is prevented by XAD-4/PVPP and inhibitors during grape extraction, but occurs during the juice and wine processing. The shorter destaining time (30 min) is

presented to illustrate the presence of the higher MW fractions ($>45,000$) which were not readily detectable with the 60 min destaining. In contrast, the 60 min destaining presents the better resolution for the lower MW fractions ($<45,000$).

For comparative purposes and to permit equivalent application of protein to gels, the concentration of protein was determined in the different extracts. The total soluble proteins were determined by a modified Bio-Rad (Bradford) dye-binding procedure reported to be the method of choice for the determination of beer proteins (11). Previous studies in our laboratory had demonstrated that this procedure to be more reliable than modified Kjeldahl (14) and modified Diemaier and Maier (2) procedures for the determination of total soluble proteins in grape juices and wines (9, 23), there being no interference from phenols at concentration as high as 400 mg/L (7). The reproducibility of the procedure is further demonstrated in Table II.1, which also demonstrates the effect of XAD-4/PVPP treatment on total soluble protein and total phenol in grape, juice and wine.

The silver staining technique was developed for detection of proteins at concentrations which previously could be revealed only by autoradiographic method (31). The protein fractions in Fig. II.2 have been detected by a modification of this technique (35). The same gel stained

with Coomassie Blue failed to detect any of the 35 fraction shown in Fig. II.2. Previous studies on grapes, juices and wines using Coomassie Blue stain had to concentrate and purify proteins to permit detection. The silver stain is 100 times (detection at 10 ng for bovine serum albumin) more sensitive than the conventional Coomassie Blue stain (31, 35). This, in conjunction with the application of gradient slab gel electrophoresis which concentrates proteins in bands during electrophoresis, accounts for the increased sensitivity of our procedures compared with previously reported procedures, and permits the direct application of unconcentrated grape juice or wine samples onto gels.

As shown in Figs. II.2 & II.3, the soluble proteins in juice and wine are basically those found in the grape, which is in agreement with previous reports (2, 19, 25). Thirty-five protein fractions were detected in Gewurztraminer grape with MW from 11,200 to 120,000. Juice and wine from these grapes contained only 24 protein fractions with MW from 11,200 to 65,000. Forty-one protein fractions (11,200-190,000) were detected in Riesling grape (Fig. II.1(A)); and 25 fractions (11,200-65,000) in juice and wine from these grapes. In processing grape to juice and wine, there has been a decrease in total soluble proteins (Table II.1) and in high MW fractions, and an increase in low MW protein fractions (Fig. II.2 & II.3).

These observations tend to agree with previous claims that protein hydrolysis occurs during fermentation (2, 5, 22). It is also possible that during fermentation a higher percentage of larger MW fractions are "denatured" and/or precipitated than the lower MW fractions. Yeast cells may also secrete proteins during fermentation (2).

A laser scanning densitometer was used to demonstrate relative concentrations of protein in different fractions in silver stained gels. Fig. II.3 demonstrates the utilization of this technique for following the effects of processing from grape to juice and wine on the individual protein fractions detected in Fig. II.2. Although the data are not presented here, relative concentrations of protein fractions present in the gels were determined by utilizing a software package (Model ER1P3 Biomed Instrument Inc.) to integrate the relative peak area.

Although there have been reports of grape juice and wine proteins possibly containing reactive reducing sugar moieties (14, 20), the isolation and separation of glycoproteins by electrophoresis and protein blotting by specific glycoprotein detection has not been reported. In this study which utilizes a technique developed for the detection of glycoproteins in virus and virus-infected cells (6), protein fractions were eluted from gels and immobilized on a nitrocellulose membrane by protein blotting. A lectin, concanavalin A, is used for detection

of glycoprotein on nitrocellulose transfer, and can specifically bind to glucose or mannose residues of glycoproteins (6, 28). With this technique, three protein fractions with MW of 12,600, 25,000 and 28,000 were identified as containing glycoprotein in Gewurztraminer wine (Fractions 2a, 4, and 5a, Fig. II.2). Only two fractions with MW of 25,000 and 28,000 were detected in Riesling wine.

Previous studies in this laboratory (10) and elsewhere (1, 15, 19, 20, 36) have reported the application of isoelectric focusing for separation of grape juice and wine proteins, and for the determination of their pI. In this study, we investigated the application of 2-dimensional electrophoretic techniques, which had been used for characterizing bacteria and microsomal proteins (24, 26), for determination of soluble wine proteins. This system, 2-dimensional IF-LDS PAGE, permits simultaneous determination of MW and pI. The application of this technique to the Gewurztraminer wine in Fig. II.2 is shown in Fig. II.4. Isoelectric focusing has further separated each of the wine protein fractions into several fractions on the basis of their pI (Fig. II.4). For instance, the fraction with MW of 28,000 has been separated in 5 major fractions with pI from 4.1-5.8. It can be seen that a high proportion of the wine proteins are of both low pI (4.1-5.8) and low MW (20,000-30,000).

The advantages and convenience of using these techniques were further demonstrated in subsequent studies for characterizing the unstable proteins in grape juices and wines, and investigating their removal by bentonite, heat, ultrafiltration and protease enzymes (12, 13, 33).

Fig. II.1 LDS-PAGE of extracts of grape proteins, in the (1) absence and (2) presence of XAD-4/PVPP and enzyme inhibitors during extraction of proteins, followed by silver staining. (A) White Riesling. (B) Gewurztraminer. The gels were run as described under Materials and Methods. The sample protein concentration was 3 ug (two wells were used for each sample). Molecular weight (K = 1,000 daltons) of standards are given on left-hand side of each gel.

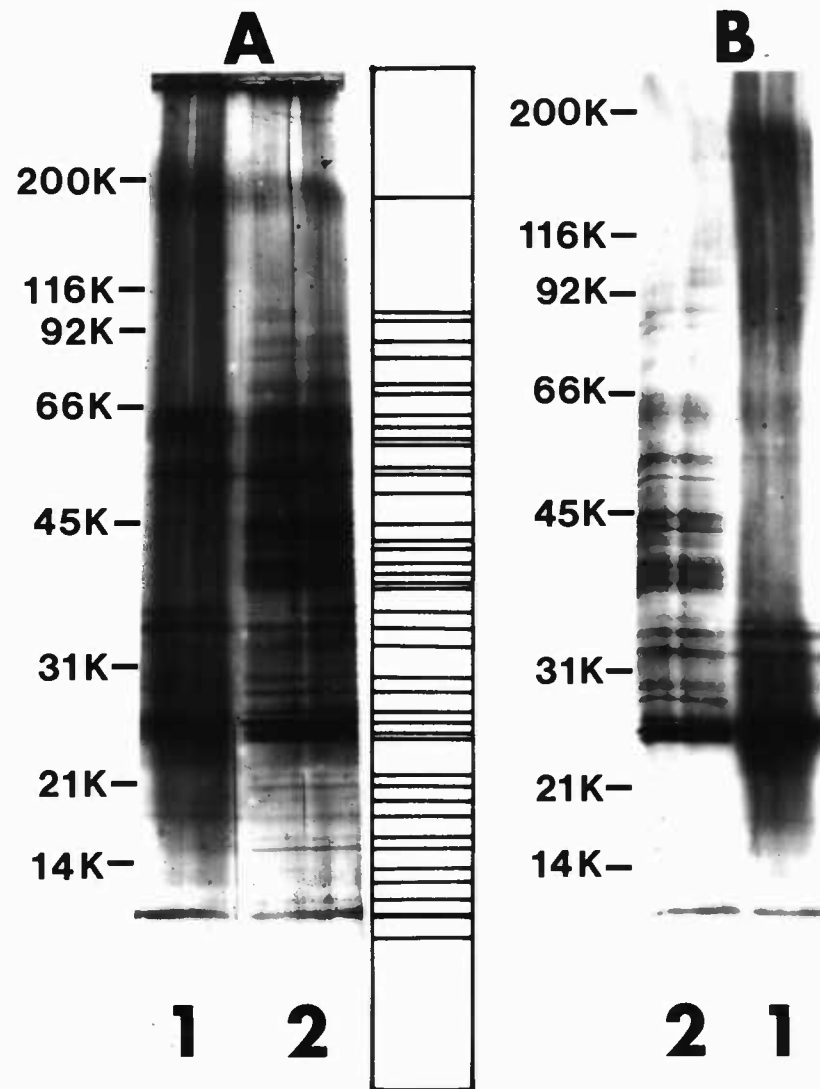


Fig. II.1

Fig. II.2 LDS-PAGE of Gewurztraminer grape extract (1,2), juice (3,4), and wine (5,6) followed by silver staining with destaining time of: (A) 30 min, (B) 60 min. Samples (1,3,5) were untreated. Samples (2,4,6) were treated with XAD-4/PVPP and enzyme inhibitors as described under Materials and Methods. The gels were run as described under Materials and Methods. The sample protein concentration was 3 ug. Molecular weight ($K = 1,000$ daltons) of standards are given on right-hand side of gels.

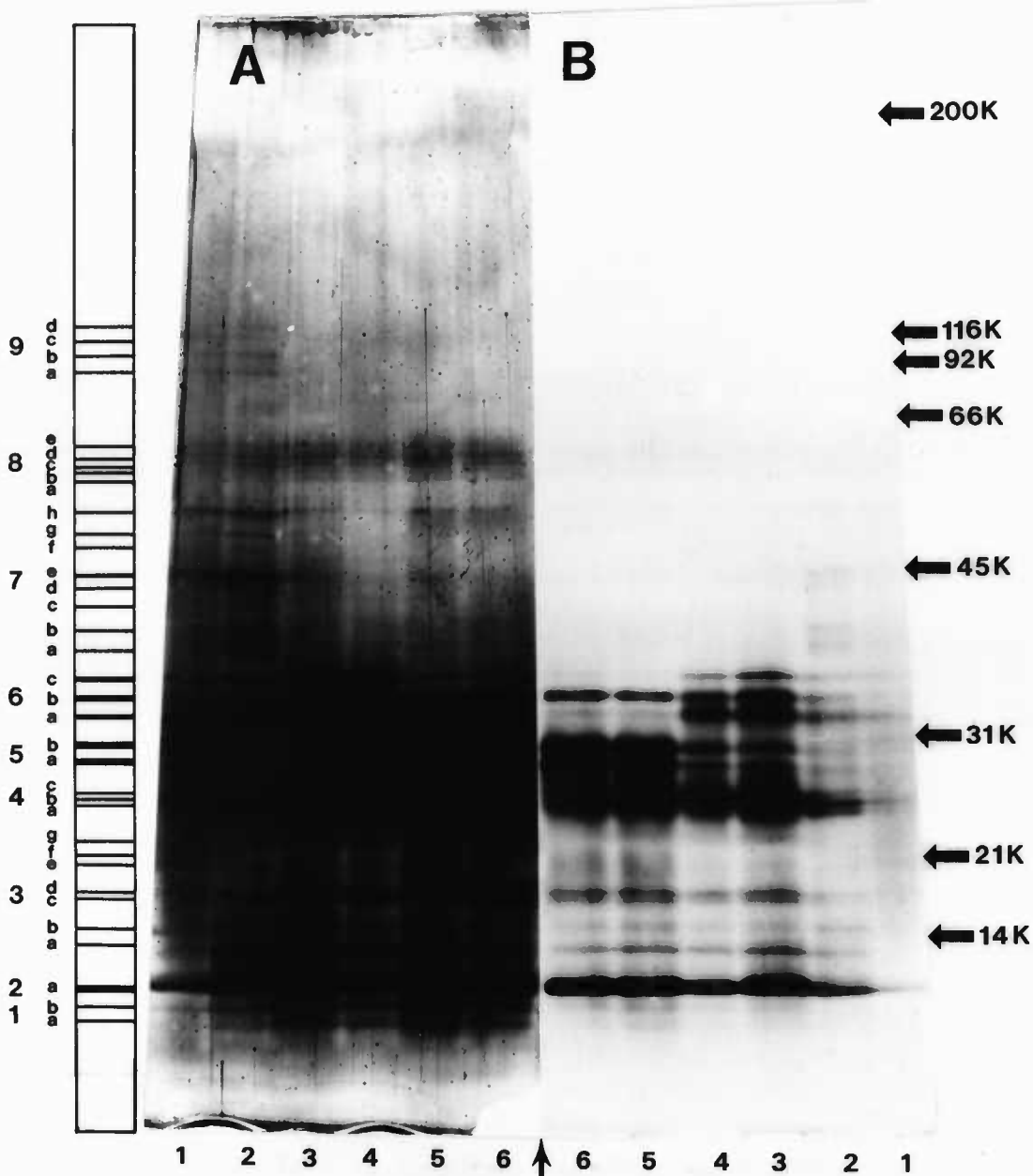


Fig. II.2

Table II.1. Concentration of soluble proteins and total phenols in Gewurztraminer grape, juice and wine: Effect of treatment with XAD-4/PVPP and enzyme inhibitors.

Sample	Protein (mg/L) ^a	Phenol (mg/L) ^a
Grape		
Untreated	101.2 \pm 1.8	98.4 \pm 4.2
Treated ^b	65.8 \pm 2.4	55.3 \pm 3.7
Juice		
Untreated	77.1 \pm 1.5	297.6 \pm 13.7
Treated ^b	58.2 \pm 0.3	153.4 \pm 2.1
Wine		
Untreated	41.3 \pm 0.2	291.0 \pm 6.2
Treated ^b	29.4 \pm 0.5	124.4 \pm 6.3

^aMean of triplicate determinations \pm standard deviation.

^bTreated with XAD-4/PVPP and enzyme inhibitors as described under Materials and Methods.

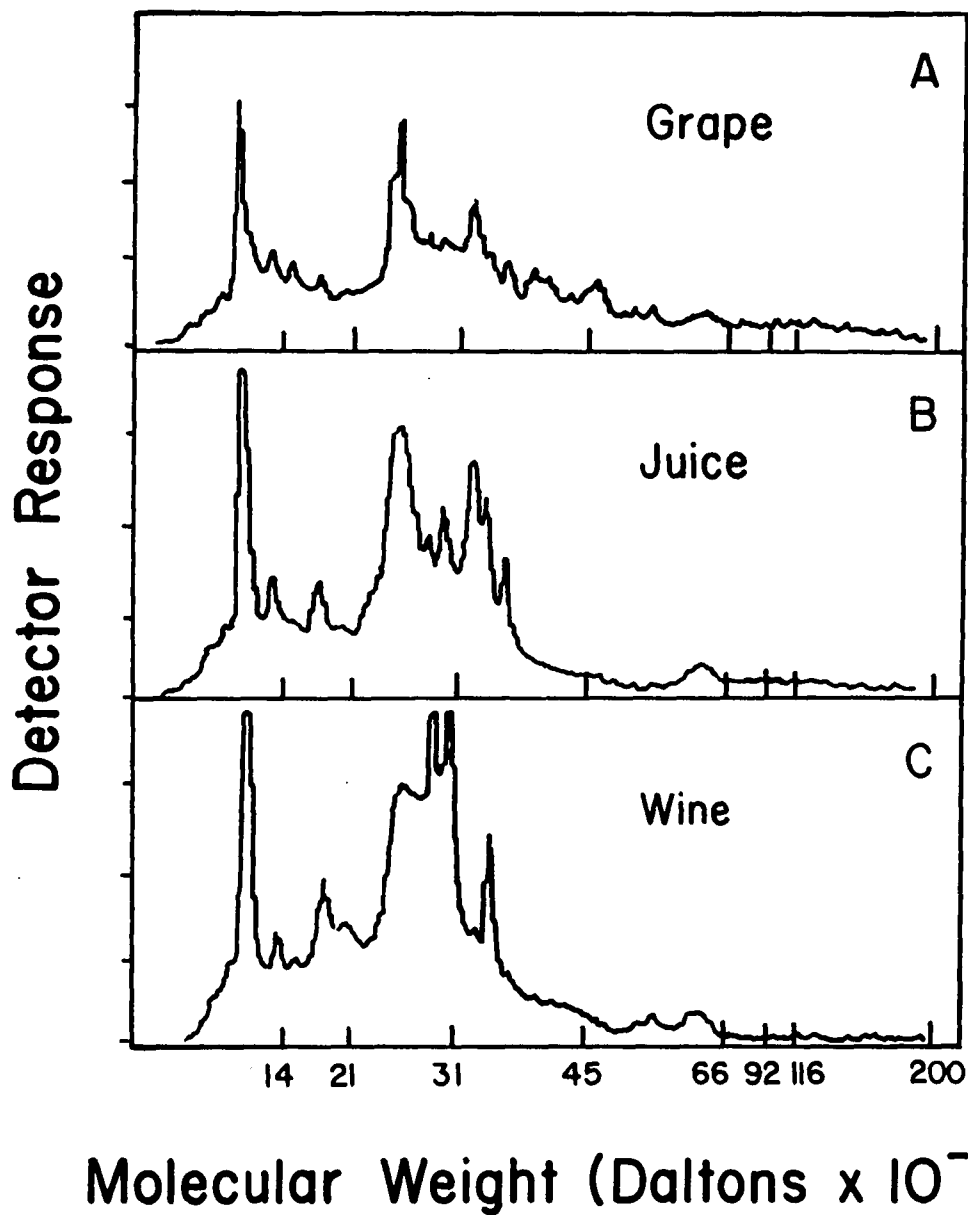


Fig. II.3 Densitometric scans of electrophoretic patterns of Gewurztraminer (A) grape, (B) juice, and (C) wine proteins in LDS-polyacrylamide gels as shown in Fig. II.2.

Fig. II.4 Two dimensional IF-LDS PAGE of Gewurztraminer wine proteins followed by silver staining. The gel was run as described under Materials and Methods. The sample protein concentration was 5 ug. Molecular weight ($K = 1,000$ daltons) of standards are given on left-hand side of gel. The pH gradient is labeled above the gel.

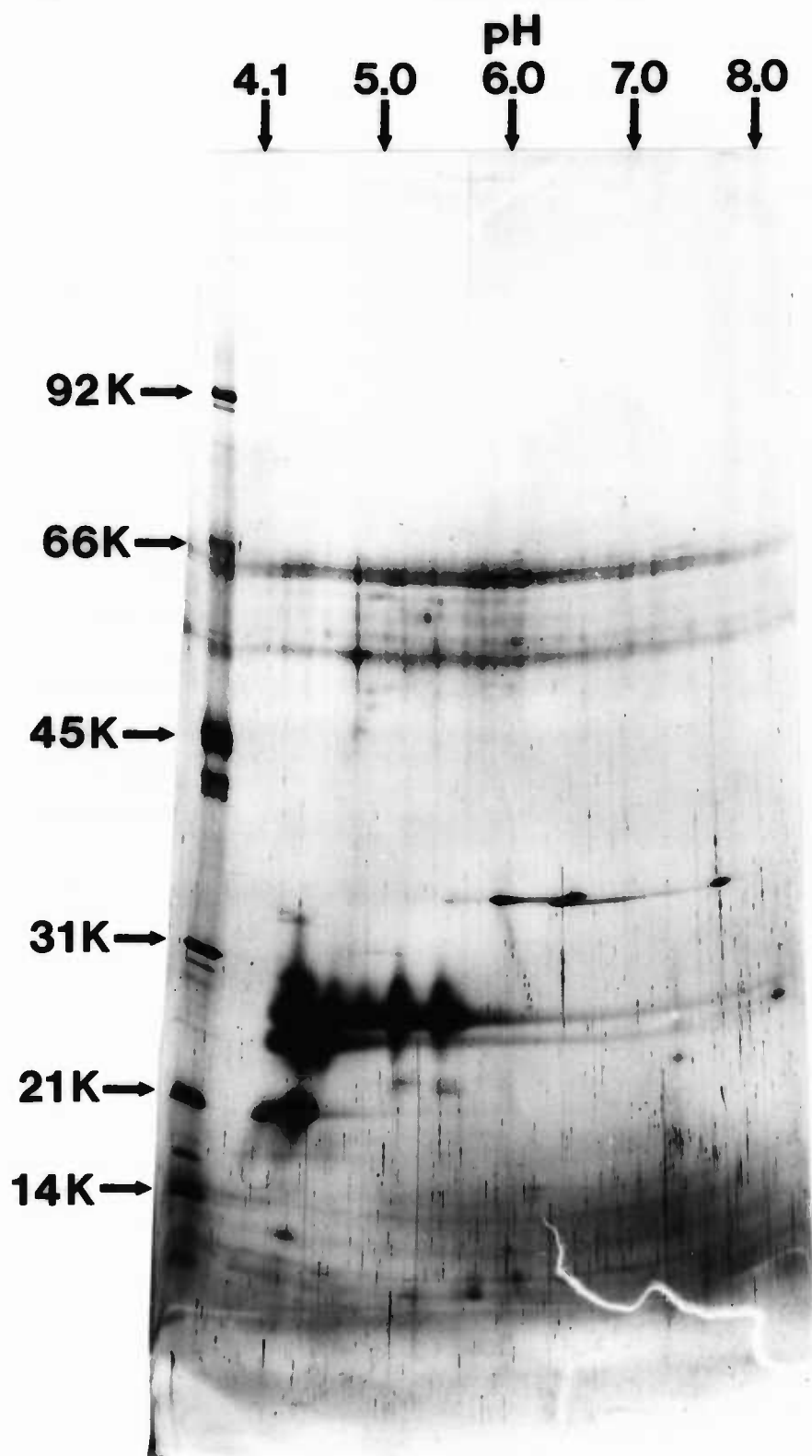


Fig. II.4

LITERATURE CITED

1. Anelli, G. The proteins of musts. Am. J. Enol. Vitic. 28:200-3 (1977).
2. Bayly, F.C. and H.W. Berg. Grape and wine proteins of white wine varieties. Am. J. Enol. Vitic. 18:18-32 (1967).
3. Broglie, R.M., Hunter, C.N., Delepelaire, P., Nieserman, R.A. Chua, N-H., and Clayton R.K. Isolation and characterization of the pigment-protein complexes of Rhodopseudomonas sphaeroides by lithium dodecyl sulfate/ polyacrylamide gel electrophoresis. Proc. Natl. Acad. Sci. 77:87-91 (1980).
4. Burnette, W.N. "Western Blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. 112:195-203 (1981).
5. Chazova, T.P. and V.I. Nilov. The proteins in grape juice and wine. Prik. Biokhim. Mikrobiol. 6:18-22 (1970). Abstract in: FSTA 4:8H1264 (1972).
6. Clegg, J.C.S. Glycoprotein detection on nitrocellulose transfers of electrophoretically separated protein mixtures using concanavalin A and peroxidase: application to arenavirus and flavivirus proteins. Anal. Biochem. 127:389-94 (1982).
7. Fombin, J.F. Ultrafiltration of grape juice: Effects on composition, quality and preservation. 84pp. M.S. thesis. Oregon State University (1983).
8. Glass F.W. II, R.C. Briggs, and L.S. Hnilica. Use of lectins for detection of electrophoretically separated glycoproteins transferred onto nitrocellulose sheets. Anal. Biochem. 115:219-24 (1981).
9. Heatherbell, D.A., P.R. Ngaba, B.T. Watson, and E.E. Dodd. Determination of protein and investigation of protein instability in Pacific Northwest wines. Presented at the 32th Annual Meeting of the American Society of Enology and Viticulture, San diego, California (June 1981).
10. Heatherbell, D., P. Ngaba, B. Fombin, B. Watson Jr., Z. Garcia, J. Flores, and J. Hsu. Recent developments in the application of ultrafiltration and protease enzymes

- to grape juice and wine processing. Heatherbell, D.A., P.B. Lombard, F.W. Bodyfelt, and S.F. Price (Eds.). Proceedings of the International Symposium on Cool Climate Viticulture and Enology, June 25-26, 1984, Eugene, Oregon. Oregon State University, pp418-45 (1985).
11. Hii, V. and W.C. Herwig. Determination of high molecular weight proteins in beer using Coomassie Blue. J. Am. Soc. Brew. Chem. 40:46-50 (1982).
 12. Hsu, J.C. and D.A. Heatherbell. Heat unstable proteins in wine: I. Characterization and removal by bentonite fining and heat treatment. Submitted to Am. J. Enol. Vitic. (1986).
 13. Hsu, J.C., D.A. Heatherbell, J.H. Flores, and B.T. Watson. Heat unstable proteins in grape juice and wine: II. Characterization and removal by ultrafiltration. Submitted to Am. J. Enol. Vitic. (1986).
 14. Koch, J. and E. Sajak. A review and some studies on protein. Am. J. Enol. Vitic. 10:114-23 (1959).
 15. Lee, T.H. Protein instability: Nature, characterization and removal by bentonite. Lee, T.H. Ed. Physical stability of wine. Proceeding of a seminar, October 10, 1985. Reynella. SA. Adelaide. SA. Australian Society of Viticulture and Oenology. Australian Wine Research Institute, pp23-39 (1986).
 16. Loomis, W.D. and J. Battaile. Plant phenolic compounds and the isolation of plant enzymes. Phytochem. 5:423-38 (1966).
 17. Loomis W.D. Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. In, Methods in Enzymology. 31:528-44. Ed. by Fleischer, S. and Parker, L. Academic Press, New York (1974).
 18. Loomis W.D., J.D. Lile, R.P. Sandstrom, and A.J. Burbot. Absorbent polystyrene as an aid in plant enzyme isolation. Phytochem. 18:1049-54 (1979).
 19. Luis, E.S. A study of proteins during grape maturation, juice preparation and wine processing. 266pp. Ph.D. thesis. University of New South Wales, Australia (1983).

20. Lyubarevich, T.A., R.K. Mindadze, N.M. Pavlenko, and E.N. Datunashvili. The proteins of wine grapes and their properties and stability in wine. *Vinodel. Vinograd. USSR* 4:58-9 (1975). Abstract in: *FSTA* 8:3H365 (1976).
21. Matheis, G. and J.R. Whitaker. Modification of proteins by polyphenol oxidase and peroxidase and their products. *J. Food Biochem.* 8:137-62 (1984).
22. Moretti, R.H. and H.W. Berg. Variability among wines to protein clouding. *Am. J. Enol. Vitic.* 16:69-78 (1965).
23. Ngaba, P.R. Investigation of methods for determination and prevention of protein instability in wines. 92pp. Ph.D. thesis. Oregon State University (1981).
24. O'Farrell, P.H. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007-21 (1975).
25. Ough, C.S. and G. Anelli. Zinfandel grape juice protein fractions and their amino acid makeup as affected by crop level. *Am. J. Enol. Vitic.* 30:8-10 (1979).
26. Perdew, G.H., H.W. Schaup, and D.P. Selivonchick. The use of a zwitterionic detergent in two-dimensional gel electrophoresis of trout liver microsomes. *Anal. Biochem.* 135:453-5 (1983).
27. Selivonchick, D.P., J.L. Williams, and H.W. Schaup. Alteration of liver microsomal proteins from rainbow trout (Salmo gairdneri) fed cyclopropenoid fatty acids. *Lipids.* 16:211-4 (1981).
28. Sharon, N. and H. Lis. Lectins: Cell-agglutinating and sugar-specific proteins. *Science.* 177:949-59 (1972).
29. Singleton, V.L. and J.A. Rossi. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* 16:144-58 (1965).
30. Sommers, T.C. and G. Ziemelis. Direct determination of wine proteins. *Am. J. Enol. Vitic.* 24:47-50 (1973).
31. Switzer, R.C. III, C.R. Merrie, and S. Shifrin. A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. *Anal. Biochem.* 98:231-7 (1979).

32. Vlasuk, G.P. and F.G. Walz Jr. Liver endoplasmic reticulum polypeptides resolved by two-dimensional gel electrophoresis. *Anal. Biochem.* 105:112-20 (1980).
33. Watson, B.T., D.A. Heatherbell, B.M. Yorgey, and J.C. Hsu. Application of protease enzymes for protein stabilizing wine. Submitted to *Am. J. Enol. Vitic.* (1986).
34. Wesche, P.A.E. Purification of strawberry polyphenol oxidase and its role in anthocyanin degradation. 171pp. Ph.D. thesis. Oregon State University (1984).
35. Wray, W., T. Boulikas, V.P. Wray, and R. Hancock. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* 118:197-203 (1981).
36. Yokutsuka, K., M. Yoshii, T. Aihara, and T. Kushida. Isolation and characterization of proteins from juices, musts and wines from Japanese grapes. *J. Ferment. Technol.* 55:510-5 (1977).

Heat Unstable Proteins in Wine: I. Characterization
and Removal by Bentonite Fining and Heat Treatment

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ABSTRACT

The effect of bentonite fining on the total proteins and the heat (80 °C, 6 h; 4 °C, 12 h) unstable proteins in Gewurztraminer, White Riesling, Sauvignon Blanc, and Sylvaner wines was investigated. Protein molecular weights (MW), isoelectric points (pI) and glycoproteins were determined by using LDS-polyacrylamide gel electrophoresis and 2-dimensional IF-LDS electrophoretic techniques with silver staining as well as protein blotting for glycoprotein detection. Relative concentrations of proteins in stained gels were determined by laser scanning densitometry. Bentonite fining tends to remove the higher pI (5.8-8.0) and intermediate MW (32,000-45,000) protein fractions first. However these represent only a small proportion of the soluble proteins. In general, it is necessary to remove the lower pI (4.1-5.8), lower MW (12,600 and 20,000-30,000) fractions, which contain glycoproteins and represent the major component of the proteins, to "protein stabilize" wines to heat testing. Protein fractions with MW of 60,000-65,000 and having a

wide range of pI (4.1-8.0) were highly resistant to removal by bentonite and remained in "protein stabilized wine". In addition, trace amounts of fractions with MW of 28,000 may remain in Gewurztraminer and Riesling wines; and of 25,000 in Sauvignon Blanc wines. Unstable proteins precipitated by heat tests were recovered and analyzed. Protein fractions with MW of greater than 14,000 were more heat sensitive than lower MW fractions. The heat precipitated proteins found in sediments were mainly of low MW (<30,000) and primarily glycoproteins. It is concluded that the protein fractions of lower MW (12,600 and 20,000-30,000), lower pI (4.1-5.8) and glycoproteins are the major and most important fractions contributing to protein instability in wines.

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INTRODUCTION

Although there have been numerous investigations on grape juice and wine proteins (9), the nature of the proteins responsible for wine turbidity remains unclear. Protein instability does not correlate well with the total protein content and there appears to be conflicting information in the literature as to which proteins (protein fractions) are responsible for haze and sediment formation. Koch and Sajak (10) using paper electrophoresis showed that grapes and wines contained two major protein fractions, both being decreased by heat treatment and addition of bentonite. Moretti and Berg (15) associated specific protein fractions with wine turbidity and concluded that only a part of the protein mixture was responsible for protein stability rather than total protein content. Bayly and Berg (3) further classified protein fractions according to their heat stability and concluded that removal of protein fractions by addition of bentonite did not occur in equal proportions, but removed the highly charged protein molecules first. Millies (14) using silica sol/gelatin fining and ultrafiltration to fractionate the wine proteins, claimed that the protein fractions with MW of less than 10,000 do not take part in turbidity formation, fractions between 10,000 and 30,000 are only partly involved and those with MW of greater than 30,000 are the

main unstable proteins. Mesrobian *et. al.* (13) indicated that the protein-clouding is mainly caused by the protein fraction with lower pI and lower MW. Recently, using gel electrophoresis and isoelectric focusing, we determined the MW and pI of heat stable and unstable proteins present in Oregon and Washington wines, and investigated their removal by bentonite fining, ultrafiltration and protease enzymes (8, 16). Protein fractions detected had a MW range of 16,000-90,000 daltons and pI from 4.5-8.0. With progressive bentonite fining, it was not until the lower MW (approximately 16,000-25,000 daltons) and higher pI (5.6-8.0) fractions were removed by bentonite fining that the wines became protein stabilized to heat testing. A more recent report by Lee (12) for Australian wines claimed that the major protein fractions of wine had MW of 40,000-200,000 daltons and pI of 4.8-5.7 and "these fractions must be largely removed from wine before stability ... is conferred".

In this study, we further investigated the nature of the unstable proteins and their removal by bentonite and heat treatment by applying improved sensitive techniques (9) for the detection and characterization of proteins and glycoproteins.

MATERIALS AND METHODS

Preparation of wines: Gewurztraminer, White Riesling, Sauvignon Blanc and Sylvaner grapes from OSU experimental vineyards were harvested and processed into wines by conventional procedures in the OSU experimental winery. Young wines (after 2 rackings) were sampled for this study.

Bentonite fining: For determination of the amount of bentonite required to stabilize wines to heat testing, each wine sample was fined with 5-80 g bentonite per hL wine and thoroughly mixed. Bentonite (sodium bentonite, Volclay) was added as a smooth aqueous 3% suspension. (one mL of bentonite solution when added to 100 mL of wine, corresponding to 30 g bentonite per hL or 2.5 lb bentonite per 1,000 gal of wine). The wines were held at room temperature for at least 48 h, and then filtered through 0.45 μ m Millipore membrane before analysis. Filtered wines were prepared for gel electrophoresis as previously described (9). Concentration of soluble protein and total phenol were determined as previously described (9).

Heat stability test: The heat stability of the wines was determined by the procedure recommended by Pocock and Rankine (17). Fifty mL of filtered wine was transferred into 65 mL bottles. Bottles were sealed with screw caps and

heated in a 80 °C water bath for 6 h, held at 4 °C for 12 h and allowed to warm to room temperature. Formation of turbidity was measured by visual observation under a strong beam of light, and by a Hunter Model D25P-2 Color Difference Meter (Hunter Associates Lab., Inc.) operated in the transmittance mode for the transmission haze reading; percent haze = $Y_{\text{(Arrangement I)}}/Y_{\text{(Arrangement III)}} \times 100$. Filtered distilled water was used as the blank giving a reading of 2 units.

Preparation of haze/sediment from heat stability test:

Fifty mL of unfined wine was heated at 80 °C in a water bath for 6 h, and then stored at 4 °C for 12 h. Protein haze/sediment was collected by centrifuging at 12,100 X g for 20 min at 4 °C in a Sorvall RC-5 centrifuge. The precipitate was collected and suspended in 2 mL of sample buffer for gel electrophoresis (9). The supernatant was filtered through 0.45 um Millipore filter membrane, and the filtrate was prepared for gel electrophoresis as previously described (9).

Gel electrophoresis: LDS-polyacrylamide gel electrophoresis (LDS-PAGE), two-dimensional isoelectric focusing-LDS PAGE (2-D IF-LDS PAGE), silver staining, laser scanning, protein blotting and glycoprotein detection were performed as previously described (9).

Carboxymethyl cellulose concentration of wine proteins:

In some instances for 2-dimensional IF-LDS PAGE, it was desirable to concentrate proteins. Proteins were concentrated using a modification of the procedure described by Calbiochem-Behring (1) using carboxymethyl cellulose (CMC, Aquacide II, Calbiochem., California). Twenty to thirty mL samples were placed in dialysis tubing and then packed in CMC for 2-3 h until up to 10 fold concentration was achieved.

RESULTS AND DISCUSSION

Bentonite fining: The effect of bentonite fining on the "protein stability" (as determined by heat test), soluble protein and total phenol of Gewurztraminer, Riesling, Sauvignon Blanc and Sylvaner wines was investigated (Fig. III.1). There was a good agreement between visible observations and Hunter haze readings following heat testing. Whereas heat induced haze formation progressively decreased with bentonite fining and protein reduction, bentonite addition had no effect on total phenol ($p > 0.05$). In these four wines, protein concentration of ca. 5 mg/L (mean = 5.4 ± 1.6 mg/L) coincided with wine stability (Fig. III.1). This occurred irrespective of the initial concentration of protein in the wines which ranged 19-44 mg/L (Fig. III.1). However, it should be noted that higher concentrations of proteins have been observed to be present in wine protein stabilized by bentonite fining both in our laboratory (commonly 10-20 ppm) and in the literature (12, 17). Relative large amounts of bentonite were required to remove the "proteins" which are most resistant to removal by bentonite (Fig. III.1). These fractions which contribute to persistent residual haze are present in small amounts, as low as 1-2 mg/L as previously reported (8). In addition, low concentration of phenolics may be contributing to persistent residual haze in juices and wines, either

independently or most likely in association with the proteins (7, 8, 18, 19).

To obtain further information about the removal of individual protein fractions from wines by bentonite, the protein fractions were separated and their MWs determined by using LDS-polyacrylamide gradient slab gel electrophoresis (LDS-PAGE). Although there were differences in the electrograms of these four wines, bentonite fining had a similiar effect on the removal of the protein fractions present. Protein fractions with MW of 12,600 and between 20,000-30,000 are the last fractions to be removed to "protein stabilize" wine to heat testing. For instance, the last fractions to be removed by bentonite fining in Gewurztraminer and Riesling wines were the 12,600 and 25,000 fractions; and in Sauvignon Blanc wine the 12,600 and 28,000 fractions.

A typical electrogram for Gewurztraminer wine is shown in Figure III.2 (wine A in Fig. III.1). Bentonite removed intermediate MW fractions (32,000-45,000) first, before removing the lower MW fractions (11,200-25,000). However, the fractions in the higher MW range of 60,000-65,000 were highly resistance to removal by bentonite. The same pattern of results was also obtained for Riesling and Sauvignon Blanc wines. The densitometric patterns of the stained gel are shown in Fig. III.3. These results indicate that the low MW fractions (12,600 and 25,000) are important to

protein instability, because their removal by bentonite fining coincides with protein stabilization of wines to heat testing (Figs. III.2(E) & III.3(E)). In contrast, protein fractions with MW of 60,000-65,000 remained in the bentonite fined protein stabilized wines (Figs. III.2(E) & III.3(E)). In addition trace amounts (limit of detection 10 ng (20)) of the 28,000 fraction was detectable in some instances.

Protein blotting combined with glycoprotein staining (9) was used for the detection of glycoproteins. With this technique, the three protein fractions with MW of 12,600, 25,000 and 28,000 in Fig. III.2 were identified as containing glycoproteins in Gewurztraminer wine. Only two fractions with MW of 25,000 and 28,000 were detected in Riesling wine. However, no glycoproteins were detected in the bentonite stabilized wines.

The effect of bentonite fining on wine proteins was further investigated by subjecting wines to two-dimensional IF-LDS gel electrophoresis (9). A typical result for a Gewurztraminer wine is shown in Fig. III.4 (wine A in Fig. III.1) which shows that the major protein fractions in Gewurztraminer wine are of low MW (20,000-30,000) and low pI (4.1-5.8). The absence of the major fraction with MW of 12,600 (Fig. III.2) in Fig. III.4 is not accounted for. The possibility exists that it may have been lost through dialysis tubing during concentration (wine samples applied

to gels in Fig. III.4 were concentrated as indicated in an attempt to detect low concentrations of some fractions, particularly in the bentonite fined sample). Isoelectric focusing has further separated each of the protein fractions in Fig. III.2(A) into several fractions on the basis of their pI (Fig. III.4(A)). For instance, the fraction with MW of 28,000 has been separated in 5 major fractions with pI from 4.1-5.8. When the same 2-dimensional technique was applied to bentonite stabilized wine (wine E, Fig. III.2), none of the fractions known to be important contributors to heat instability (8) could be detected (data not shown). However, with sufficient concentration (ca. 10 fold), trace amounts of these fractions could be demonstrated to remain in the stabilized wine (Fig. III.4(B)). In contrast, considerable amounts of the protein fractions with MW of 60,000-65,000 which were highly resistant to removal by bentonite and had a wide range of pI (4.1-8.0), remained in the bentonite fined wine (Fig. III.4(B)) and were readily detectable without concentration. In interpreting these results consideration must be given to the extreme sensitivity of the method; trace amounts of protein (10 ng) being detectable (20). The following interpretations are based upon analysis of "single strength" (unconcentrated) wine. In general, the application of this technique confirmed and extended our previous studies (8). Bentonite fining removed the

intermediate MW (32,000-45,000) (Fig. III.2) and higher pI (5.8-8.0) (Fig. III.4) fractions first. However, these fractions together only compose a small proportion of the soluble proteins present in these wines, and although they may contribute to instability, their removal did not stabilize wines. It was necessary to remove the lower pI (4.1-5.8), the lower MW (12,600 and 20,000-30,000) fractions and glycoproteins to "protein stabilize" wines. Specifically, for Gewurztraminer wine, the fractions with MW of 12,600 (containing glycoproteins) and MW of 25,000 (having pI of 4.1-4.8 and containing glycoproteins) are the last removed by bentonite to "protein stabilize" wine to heat testing (Figs. III.2, III.3, III.4). In contrast, the protein fractions with MW of 60,000-65,000 and trace amounts of the 28,000 fraction remained in wine which had been "protein stabilized" by bentonite fining.

Heat treatment: A standard heat test (80 °C, 6 h; 4 °C, 12 h) recommended by Pocock and Rankine (17) was used to evaluate the heat stability of wine proteins. In previous studies (6), we had demonstrated that for Oregon and Washington wines this one day test correlated well with the longer 5 day test (49 °C, 4 days; 5 °C, 1 day) proposed by Berg and Akiyoshi (4). To assist in the further characterization of the heat unstable proteins, proteins precipitated by the heat test (1 day test) were recovered

and analyzed. Heat treatment of wines precipitated ca. 50% (15-30 mg/L) of the proteins (Table III.1). Similar results were reported by Kock and Sajak (10) who claimed over 60% (20-45 mg/L) Voigt-N content remained in heat-treated (75 °C, 2 min) wine. In contrast, Pocock and Rankine (17) and later Lee (11) claimed that heat treatment of wine at 80 °C for 6 h appeared to completely coagulate the soluble protein present and remove all proteins from the Australian wines tested. A loss ($p < 0.05$) of up to 7.8% of total phenol was observed in heat-treated wine (Table III.1), and may be significant in haze formation during heat testing. The combination of phenolics with proteins in hazes and sediments from wines (5, 10, 18), juices (7, 11) and beer (2) has been reported frequently.

The MW of protein fractions, their relative concentrations, and the presence of glycoproteins in initial wine, heat-treated wine and in recovered sediments were determined (Figs. III.5 & III.6). Protein fractions with MW of greater than 14,000 were heat-sensitive and were removed by heat treatment (Fig. III.6(B)). In contrast, protein fractions with MW of lower than 14,000 including one major MW fraction (12,600) containing glycoproteins remained in the wine after heat testing (Fig. III.6(B)). In some wines trace amounts of fractions (60,000-65,000) also remained after heat testing. Protein "hydrolysis/dissociation" may have occurred during heat

treatment, there being an increase in the low MW fractions (<30,000) present in the sediment (Fig. III.6). It is also possible that a higher percentage of the larger MW fractions were "heat-sensitive/denatured" than the lower MW fractions during heat testing. The heat precipitated proteins found in sediments were mainly of low MW (<30,000) (Fig. III.6(C)) and primarily glycoproteins. These findings support the conclusions we obtained for characterizing the nature of the heat unstable proteins removed by bentonite fining.

It is concluded that the protein fractions of lower MW (12,600 and 20,000-30,000), lower pI (4.1-5.8) and glycoproteins are the major and most important fractions contributing to protein instability in wines.

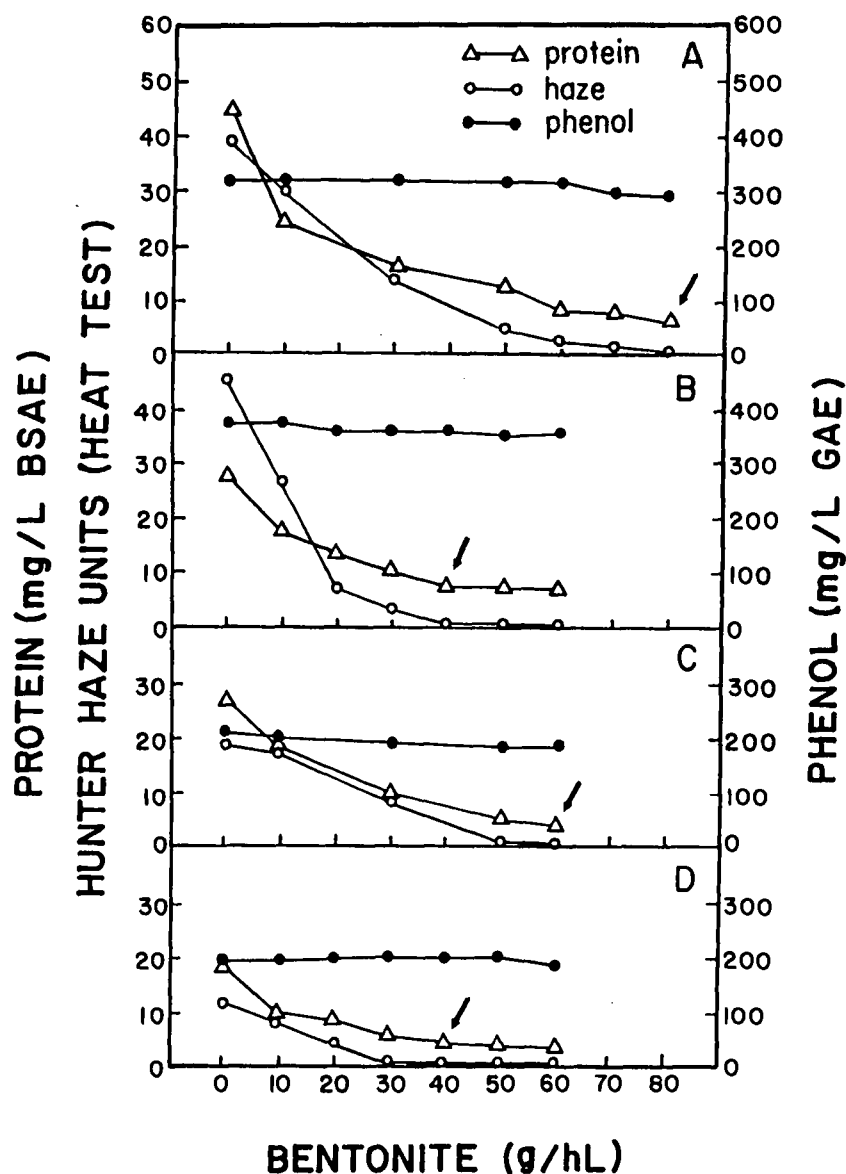


Fig. III.1 Effect of bentonite fining on protein stability¹ and concentration of soluble protein and phenol in wines.
 (A) Gewurztraminer, (pH 3.67, 12.2% ethanol).
 (B) White Riesling, (pH 3.15, 11.7% ethanol).
 (C) Sauvignon Blanc, (pH 3.20, 11.9% ethanol).
 (D) Sylvaner, (pH 3.20, 11.1% ethanol). BSAE = bovine serum albumin equivalent. GAE = gallic acid equivalent.

¹ Determined as Hunter haze formation or by visible observation following heat test. Arrow (↓) represents bentonite treatment required to protein stabilize wine as determined by visual assessment.

Fig. III.2 LDS-PAGE of Gewurztraminer wine proteins in wine fined with different levels of bentonite: (A) 0 g/hL, (B) 20 g/hL, (C) 50 g/hL, (D) 70 g/hL, (E) 80 g/hL (heat stable). One hundred uL of each sample was applied to gel. Molecular weight ($K = 1,000$ daltons) of standards are given on left-hand side of gel.

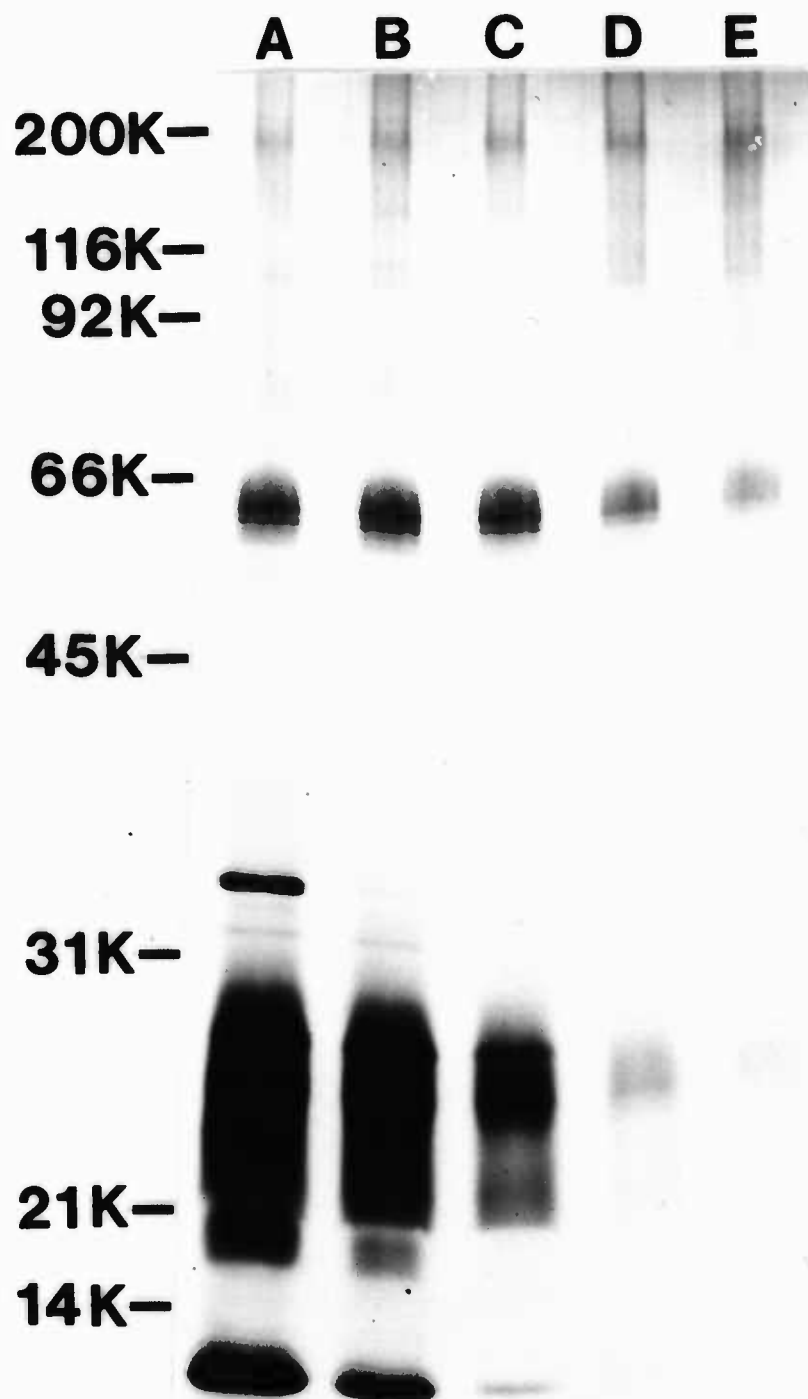


Fig. III.2

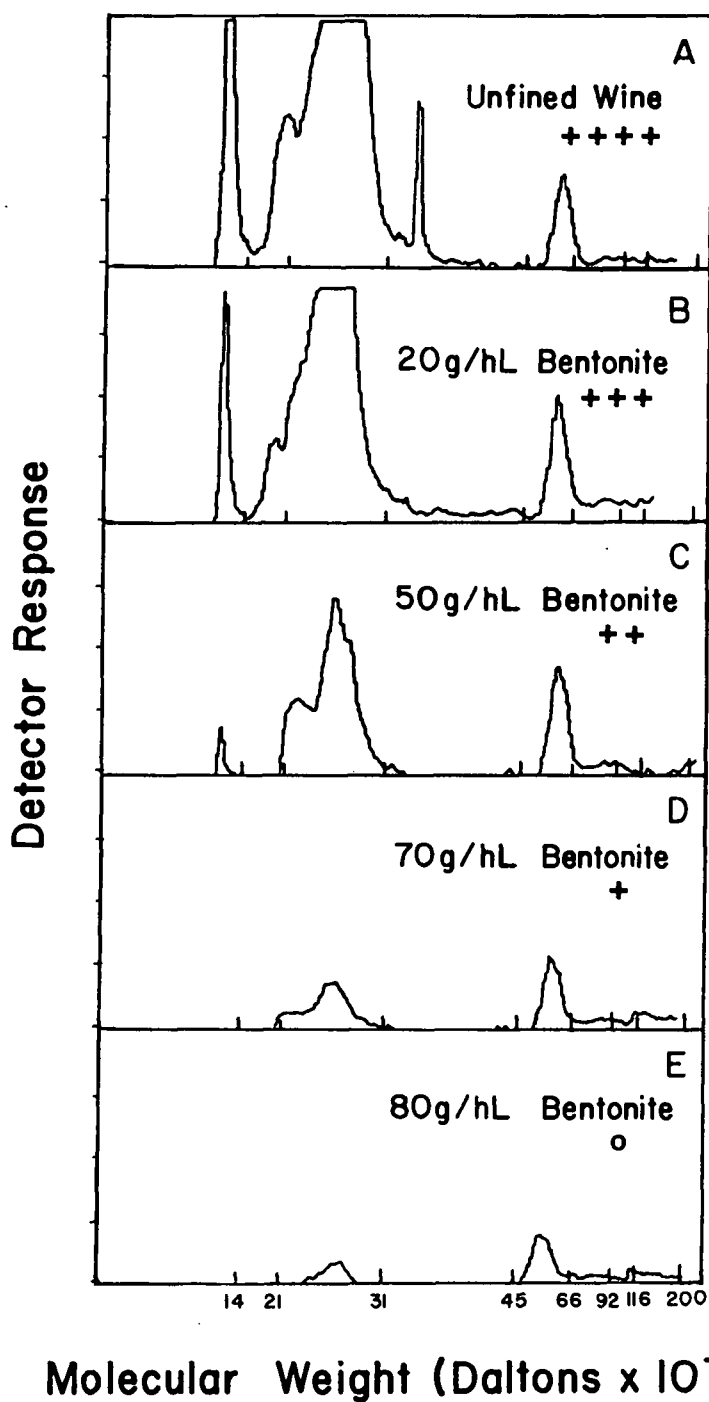


Fig. III.3 Densitometric scans of electrophoretic patterns of Gewurztraminer wine proteins in wine fined with different levels of bentonite (Fig. III.2): (A) 0 g/hL, (B) 20 g/hL, (C) 50 g/hL, (D) 70 g/hL, (E) 80 g/hL. Protein instability indicated by +.

Fig. III.4 Two dimensional IF-LDS PAGE of Gewurztraminer wine proteins followed by silver staining. (A) Unfined wine, concentrated 2 fold before electrophoresis. (B) Wine was fined with 80 g/hL bentonite and concentrated 10 fold before electrophoresis. Molecular weight ($K = 1,000$ daltons) of standards are given on left-hand side of each gel. The pH gradient is labeled above each gel.

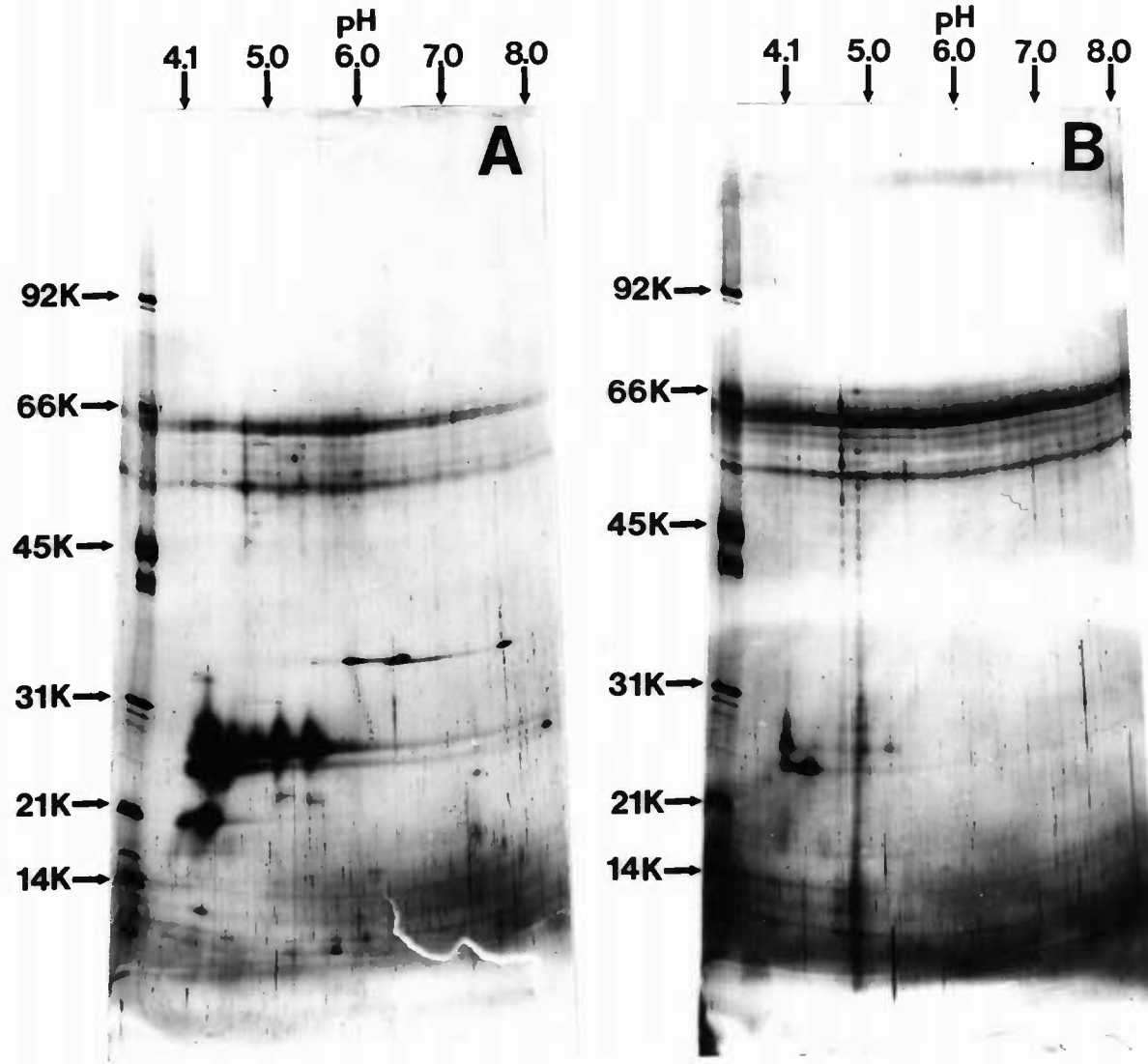


Fig. III.4

Table III.1 Effect of heat treatment^a on concentration of soluble protein and phenol in wines.

Sample	Protein (mg/L) ^b	Phenol (mg/L) ^b
Gewurztraminer		
Before Treatment	59.2 ± 0.2	302.1 ± 7.7
After Treatment	28.6 ± 1.5	282.9 ± 5.4
% Removal	51.7	6.4
Riesling		
Before Treatment	36.1 ± 0.7	371.3 ± 3.1
After Treatment	17.8 ± 0.8	342.3 ± 1.3
% Removal	50.7	7.8

^aHeat test for determining protein stability (6 h, 80 °C; 12 h, 4 °C).

^bMean of triplicate determinations ± standard deviation.

Fig. III.5 LDS-PAGE of Gewurztraminer wine proteins in wine subjected to heat test: (A) before heat treatment, (B) after heat treatment, (C) sediment. Molecular weight (K = 1,000 daltons) of standards are given on left-hand side of gel.

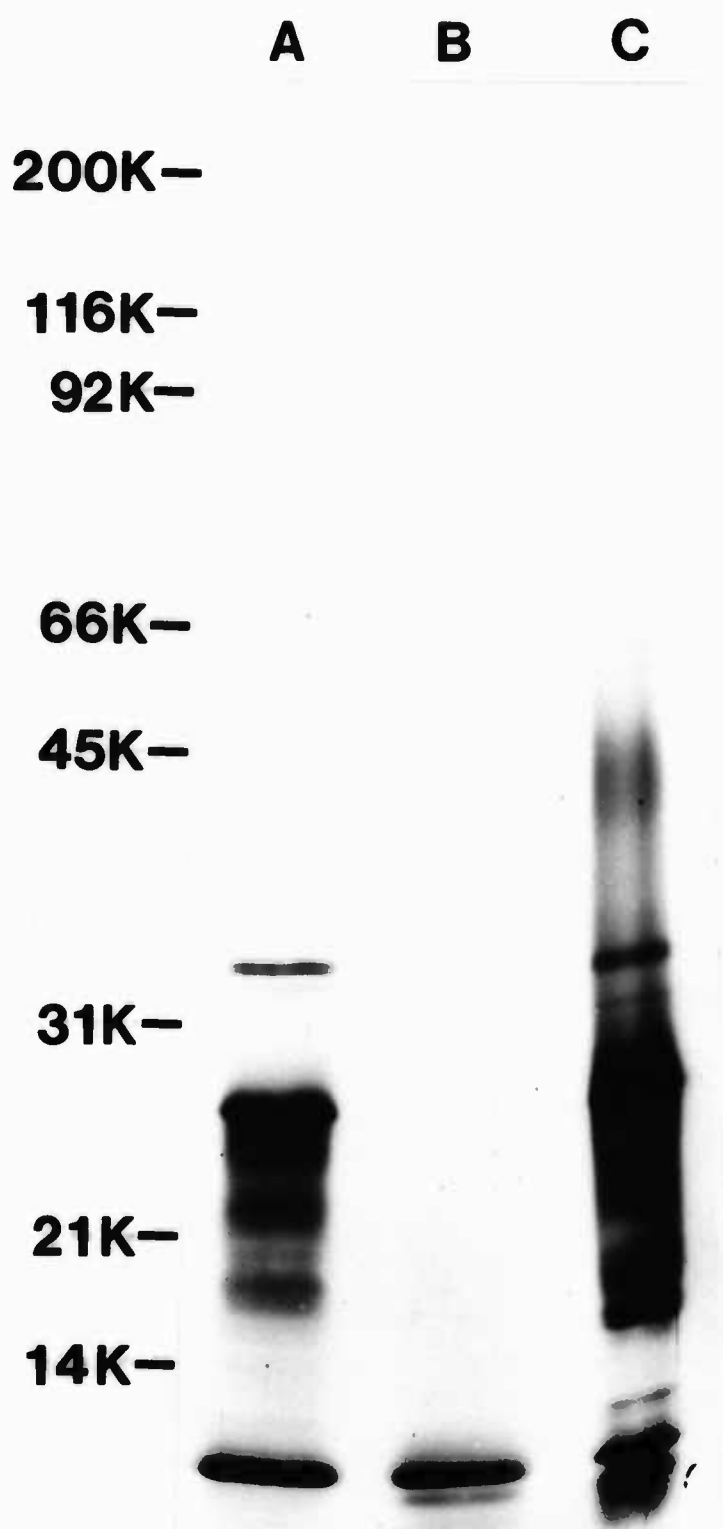


Fig. III.5

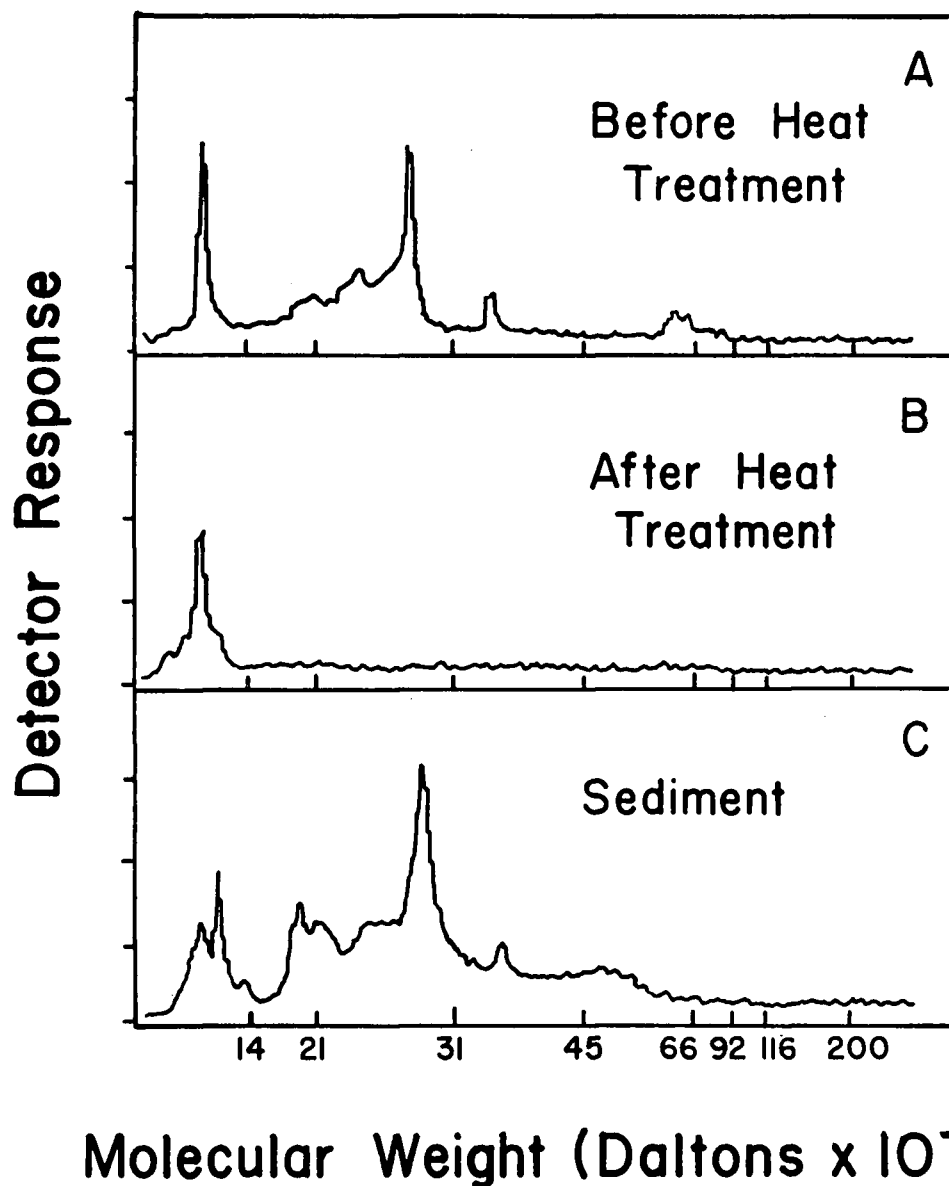


Fig. III.6 Densitometric scans of electrophoretic patterns of Gewurztraminer wine proteins in wine subjected to heat test (Fig. III.5): (A) before heat treatment, (B) after heat treatment, (C) sediment.

LITERATURE CITED

1. Anonymous. Aquacides. Biologics Technical Information, Calbiochem-Behring, Division of American Hoechst Corporation, La Jolla, CA 92037 (1985).
2. Asano, K., K. Shinagawa, and N. Hashimoto. Characterization of haze-forming proteins of beer and their roles in chill haze formation. J. Am. Soc. Brew. Chem. 40:147-54 (1982).
3. Bayly, F.C. and H.W. Berg. Grape and wine proteins of white wine varieties. Am. J. Enol. Vitic. 18:18-32 (1967).
4. Berg, H.W. and M. Akiyoshi. Determination of protein stability in wine. Am. J. Enol. Vitic. 12:107-10 (1961).
5. Gortges, S. Problems with protein stabilization in winemaking. Weinwirtschaft. 118:931-5 (1982). Abstract in: FSTA 15:8H1138 (1983).
6. Heatherbell, D.A., P.R. Ngaba, B.T. Watson, and E.E. Dodd. Determination of protein and investigation of protein instability in Pacific Northwest wines. Presented at the 32th Annual Meeting of the American Society for Enology and Viticulture, San Diego, California (June 1981).
7. Heatherbell, D.A. Fruit juice clarification and fining. Confructa. 28:192-7 (1984).
8. Heatherbell, D., P. Ngaba, B. Fombin, B. Watson Jr., Z. Garcia, J. Flores, and J. Hsu. Recent developments in the application of ultrafiltration and protease enzymes to grape juice and wine processing. Heatherbell, D.A., P.B. Lombard, F.W. Bodyfelt, and S.F. Price (Eds.). Proceedings of the International Symposium on Cool Climate Viticulture and Enology, June 25-26, 1984, Eugene, Oregon. Oregon State University. pp418-45 (1985).
9. Hsu, J.C. and D.A. Heatherbell. Isolation and characterization of soluble proteins in grapes, grape juices and wines. Submitted to Am. J. Enol. Vitic. (1986).
10. Koch, J. and E. Sajak. A review and some studies on protein. Am. J. Enol. Vitic. 10:114-23 (1959).

11. Lea, A.G.H. Tannin and colour in English cider apples. *Fluss. Obst.* 8:356-61,399-400 (1984).
12. Lee, T.H. Protein instability: Nature, characterization and removal by bentonite. Lee, T.H. (Eds.) *Physical stability of wine. Proceeding of a seminar, October 10, 1985. Reynella. SA. Adelaide. SA. Australian Society of Viticulture and Oenology. Australian Wine Research Institute, pp23-39 (1986).*
13. Mesrob, B., N. Gorinova, and D. Tzakov. Characterization of the electrical properties and of the molecular weights of the proteins of white wines. *Nahrung.* 27:727-33 (1983). Abstract in: *FSTA* 16:3H690 (1984).
14. Millies, K. Protein stabilization of wine using silica sol/gelatin fining. *Mittielungoblatt der GDCH-Fachgruppe. Lebensm. Gerich. Chemie.* 29:50-53 (1975). Abstract in: *FSTA* 7:10H1484 (1975).
15. Moretti, R.H. and H.W. Berg. Variability among wines to protein clouding. *Am. J. Enol. Vitic.* 16:69-78 (1965).
16. Ngaba, P.R. and D.A. Heatherbell. Protein instability in wine: I. Characterization of unstable proteins. II. The application of protease enzymes as bentonite substitutes in protein stabilization wine. Presented at the 32th Annual Meeting of the American Society for Enology and Viticulture, San Diego, California (June 1981).
17. Pocock, K.F. and B.C. Rankine. Heat test for detecting protein instability in wine. *Aust. Wine, Brew. & Spirit Rev.* 91:42-3 (1973).
18. Somers, T.C. The inevitability of phenolic instability. Lee, T.H. (Eds.) *Physical stability of wine. Proceeding of a seminar, October 10, 1985. Reynella. SA. Adelaide. SA. Australian Society of Viticulture and Oenology. Australian Wine Research Institute, pp81-90 (1986).*
19. Thomas, B. and R.B. Boulton. Studies of proteinaceous materials in white wines. Presented at the 35th Annual Meeting of the American Society for Enology and Viticulture, San Diego, California (June 1984).
20. Wray, W., T. Boulikas, V.P. Wray, and R. Hancock. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* 118:197-203 (1981).

Heat Unstable Proteins in Grape Juice and Wine:
II. Characterization and Removal by Ultrafiltration

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ABSTRACT

Riesling and Gewurztraminer wine and juice were ultrafiltered with Romicon & Millipore systems operated with membranes of "nominal molecular weight cut-off" (MWCO) of 10,000-100,000 daltons. A progressive increase in membrane retention of soluble protein was observed with decreasing MWCO, up to 99% of the protein in wine being retained with membranes of 10,000 dalton MWCO. In the order of 3-20 mg/L protein frequently remained in ultrafiltration (UF) wine permeates, this correlated with the periodic detection of heat instability and of a low bentonite requirement for "protein stability" as determined by sensitive heat testing. "Protein stability" could be obtained with MWCO of 10,000 and 30,000; however, when not obtained, reductions in the order of 80-95% in the bentonite requirement were achieved. The nature of unstable proteins and their removal by UF was further characterized by determining protein MW, pI and glycoprotein by LDS-polyacrylamide gel electrophoresis, 2-dimensional

IF-LDS gel electrophoresis, silver staining and protein blotting. Protein stabilization of wines by UF is similar to that by bentonite fining in that it is not until the lower MW (12,600-30,000), lower pI (4.1-5.8) fractions, and glycoproteins were removed that wines became protein stabilized to heat testing. Protein fractions in juices and wines behaved differently with UF. UF membranes were more effective at retaining wine proteins than juice proteins. Membranes were capable of retaining all glycoproteins in wines but not in juices. UF juices were more susceptible to heat induced haze formation than UF wines.

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INTRODUCTION

In a recent paper (14) we investigated the heat unstable proteins in wine; their characterization and removal by bentonite fining and heat treatment. The present paper further characterizes the unstable proteins in grape juice and wine by investigating their removal by ultrafiltration (UF) with membranes of different nominal molecular weight cut-off (MWCO), and evaluates the effectiveness of UF as a "bentonite substitute" for "protein stabilizing" grape juice and wine.

UF is emerging as a versatile membrane separation process which is increasingly finding application in the beverage industry (25). Its application as an alternative to conventional processes for clarifying and preserving fruit juices was demonstrated as early as 1977 by Heatherbell et. al. (9) for apple juice. More recently, there has been increased interest in the application of UF for processing a wide range of fruit juices (18-20, 26, 27) including grape, pear, and berry juices in our own laboratory (3, 4, 11, 12, 15). Industrial scale operations for juice processing are in operation in several countries (1, 8, 18, 19, 23, 25). The possibility of using membrane ultrafiltration for clarifying and protein stabilizing grape juice and wine has been recognized (1, 2, 5, 17, 22, 28). Apart from a recent report by Gnekow et. al. (6)

claiming protein stabilization of wine with an industrial scale operation with California wines, commercial development of UF by the wine industry does not appear as advanced as in the juice industry. However, it is known that commercial scale units are in operation in several countries including France, Italy, Australia, and Germany as well as in the U.S..

Recently we reported on the effect of UF membrane MWCO on the retention of proteins and protein stability in grape juices and wines (12). In the present study, we further investigate the effect of MWCO and the effectiveness of UF as a "bentonite substitute". Furthermore, UF is combined with bentonite fining to further characterize the nature of unstable proteins in wine.

MATERIALS AND METHODS

Preparation of grape juices and wines: White Riesling and Gewurztraminer grapes were processed into juices and wines by conventional procedures in the OSU experimental winery. Settled press juices (overnight settling at 18 °C) and young wines (after 2 rackings) were used for this study. Settled juice was filtered through a 200 u mesh filter bag (Filter Specialists, Inc., Michigan) before ultrafiltration.

Ultrafiltration of grape juices and wines: The UF units used in pilot plant trials in this study were: Romicon UF-Lab 5 Ultrafiltration system (Romicon Inc., Massachusetts) with PM-10, PM-50 and PM-100 cartridges (membrane nominal molecular weight cut-off 10,000, 50,000 and 100,000 daltons respectively, membrane area 0.46 m^2), and a Millipore Pellicon cassette system (Millipore Co., Massachusetts) with PTGC-10 and PTTK-30 cassettes (membrane nominal molecular weight cut-off 10,000 and 30,000 daltons respectively, membrane area 0.46 m^2). Commercially processed wine which had been ultrafiltered with a Millipore commercial PUF (process ultrafiltration) system with spiral-wound cartridges (polysulfone membrane with nominal molecular weight cut-off 10,000 daltons) was also evaluated. All units were operated in a batch mode, under

standardized operating conditions as previously determined (6, 12) and as specified under tables and figures.

Familiarity with the following terms is necessary to interpret the results discussed in the tables and figures:

$$\begin{aligned} 1) \quad \text{Volume Concentration Ratio (VCR)} &= V_I/V_R \\ &= V_I/(V_I - V_P) \end{aligned}$$

Where V_I = initial (unfiltered) volume

V_R = retentate volume

V_P = permeate volume

$$\begin{aligned} 2) \quad \Delta P_T &= \text{average transmembrane pressure} \\ &= (P_{\text{inlet}} + P_{\text{outlet}})/2 \end{aligned}$$

Where P = pressure

$$3) \quad \% \text{ Retention by membrane} = \sigma \times 100$$

$$\begin{aligned} \text{Where } \sigma &= \text{membrane rejection coefficient} \\ &= 1 - (C_P/C_R) \end{aligned}$$

In pilot plant trials with the Romicon unit, 20 L lots, and with the Millipore unit, 4 L lots, of juice or wine were ultrafiltered to the VCR specified. In the commercial trial, 5,500 gals of wine was ultrafiltered at 10-13 °C, to VCR = 92. Initial (unfiltered) juice or wine and total UF permeates and retentates (total collected volumes) were sampled for analysis.

Bentonite fining and heat stability testing: Wines were bentonite fined (sodium bentonite, Volclay) and their heat stability tested as previously described (14).

Determination of soluble proteins: Total protein determination, electrophoresis of proteins including lithium dodecyl sulfate (LDS)-polyacrylamide gel electrophoresis (PAGE), two-dimensional isoelectric focusing (IF)-LDS PAGE, silver staining, laser scanning and protein blotting for glycoprotein detection were performed as previously described (13). A 10-15 % gradient slab gel was used for LDS-PAGE.

RESULTS AND DISCUSSION

Ultrafiltration of wine: The main objective of this study was to investigate the effectiveness of UF for the removal of unstable proteins from wine and juice, and to determine its effectiveness as a bentonite substitute. In a previous study (12) using a smaller scale Romicon HFXS and Millipore cassette units with different nominal membrane molecular weight cut-off (MWCO) from 10,000-100,000 daltons, we demonstrated that the potential advantage of increasing flux with higher MWCO coincided with decreasing retention of proteins by the membranes. In this study Riesling and Gewurztraminer wines were ultrafiltered using Romicon and Millipore systems with MWCO from 10,000-50,000. Table IV.1 illustrates decreasing retention of protein with increasing MWCO. However, table IV.2 illustrates that the 30,000 MWCO membrane has performed as effectively as the 10,000 for this particular wine, both retaining 99% of the protein (96% retention was reported for the 30,000 MWCO membrane in our earlier study (12)). This demonstrates the importance of recognizing that the MWCO reported for these membranes are "nominal" rather than absolute figures. Up to 99% of the protein was retained in wine with membranes of 10,000 MWCO (Table IV.2). Even at the lowest MWCO, it was not possible to completely remove all wine proteins by UF, in the order of 3-20 mg/L protein

remaining in the UF wine permeate (Tables IV.1, IV.2 & Fig. IV.1). The % retention of wine (& juice) proteins reported for the Romicon unit (Tables IV.1 & IV.3) were not as high as we had observed previously (12), for instance in that study up to 92 % of the wine proteins were retained with 50,000 MWCO. No attempt is made to make comparisons between UF equipment in this report. Different wines were used for each study and some variation in the % retention of proteins has been observed between different wines (or juices) filtered with the same UF system and MWCO.

The effect of MWCO on the protein stability of these wines as determined by heat test, is illustrated in Fig. IV.1. Decreasing MWCO has resulted in increasing "protein stability". Protein stability has been achieved with the Gewurztraminer wine filtered with the Millipore 10,000 MWCO membrane. Trace instability remains for the same wine filtered with the Millipore 30,000 MWCO membrane, and a Riesling wine filtered with the Romicon 10,000 MWCO membrane (0-5 g/hL bentonite required to protein stabilize to heat testing). These results are typical of what we have found previously for several wines (12). In general, although "protein stability" can be obtained with MWCO of 10,000 and even 30,000, in most instances small amounts of heat unstable protein are passing through the membranes. Although stabilization is not always obtained, reductions of in the order of 80-95% in the "bentonite demand" are

achieved. Similar results were obtained for wine processed on a commercial scale (Fig. IV.1(C)), in which a reduction of 95% in "bentonite demand" was achieved by using membranes of 10,000 MWCO.

To further understand the behavior of proteins during UF of wine, LDS-polyacrylamide gel electrophoresis, laser scanning, 2-dimensional IF-LDS PAGE, and protein blotting for glycoprotein detection were used to characterize individual protein fractions present in wines referred to in Tables IV.1 & IV.2. The effect of membrane MWCO on individual protein fractions is demonstrated in Figs. IV.2-IV.5. Although there is a progressive reduction in total proteins present with decreasing MWCO, the membranes tested did not remove all protein fractions. With 10,000 MWCO (Figs. IV.2(B) & IV.3(C)) and 30,000 MWCO (Figs. IV.4(B) & IV.5(C)), protein fractions with MW of 12,600, 20,000-30,000 and trace amounts of 60,000-65,000 fractions still remained in UF wine permeate, which required 5 g/hL of bentonite to stabilize to heat testing (Fig. IV.1). Protein stabilization of UF wine by the addition of 5 g/hL bentonite, coincided with the removal of the 12,600 and 28,000, but not of the 60,000-65,000 fractions (Fig. IV.4(D)). This supports our previous study in demonstrating that it is necessary to remove these low MW fractions (20,000-30,000) which are known to have pI 4.1-5.8 and to contain glycoproteins, to achieve protein stabilization to

heat testing (14).

Two-dimensional IF-LDS gel electrophoresis was used for further characterization of proteins remaining in UF stabilized wine (Fig. IV.4(B)). Although trace amounts of proteins were still detected by LDS-PAGE in UF stabilized wine (Fig. IV.4(B)), the application of this technique failed to detect any fractions, except the 60,000-65,000 fractions, even in samples which had been concentrated 10 fold.

The observation, that even membranes of MWCO 10,000 do not retain all proteins, indicates that earlier studies using UF or dialysis as analytical tools for protein concentration may have lost protein fractions that are important to wine (& juice) instability. This was further confirmed by analyzing wine (& juice) which had been dialyzed with 6,000-8,000 MWCO membrane tubing (Spectrum Medical Industries, Inc., Los Angeles), and then followed by LDS-PAGE. The results showed that the 12,600 MW fraction was completely lost with dialysis. Furthermore, this accounts for the loss of the 12,600 fraction in our recent study (14), where wine proteins had been concentrated using dialysis tubing before 2-dimensional IF-LDS PAGE.

Ultrafiltration of juice: Increasing membrane MWCO from 10,000 to 100,000 daltons resulted in a progressive reduction in membrane retention of Riesling juice proteins,

up to 90% of protein being retained by UF with 10,000 MWCO membrane (Table IV.3). Even with 10,000 MWCO it was not possible to remove all juice proteins by UF. The concentration of proteins remaining in juice permeates (Table IV.3) is greater than in the wine permeates (Table IV.1), the wine being fermented from this juice. This is further demonstrated by comparing the electrophoresis of juice permeates (Figs. IV.6 & IV.7) and wine permeates (Figs. IV.2 & IV.3). It can be seen that a larger percentage of fractions with MW of 20,000-40,000 remained in the juice (Fig. IV.7) than in the wine (Fig. IV.3) even with 10,000 MWCO. Interestingly, a higher percentage of the protein fraction with MW of 12,600 passed through the 10,000 MWCO membrane than the 50,000 and 100,000 MWCO membranes (Figs. IV.6 & IV.7). This is in contrast to the finding for wine made from this juice (Figs. IV.2 & IV.3), and further indicates that proteins of juices and wines may behave differently with UF. In addition, glycoprotein was detected in the protein fraction with MW of 25,000 in the initial juice and UF juice permeates with MWCO >10,000 (Fig. IV.7). An increasing quantity of glycoprotein was detected in permeates with increasing MWCO from 50,000 to 100,000. In contrast, no glycoprotein was detected in UF wine permeates.

Attempts to apply the heat test for protein stability to juices, produced unreliable results that could not be

used as an index of removal of unstable proteins. For instance, heat induced haze formation was greater in UF juice than in UF wine. Furthermore, haze formation in UF juice filtered with 10,000 MWCO was greater than in the initial juice. This is suggested that UF may be removing colloid stabilizing materials such as pectins and phenolics, which may modify susceptibility to heat induced haze formation. In addition, juices processed in this study are being subjected to quality and stability testing in long-term storage studies at 2 °C and 20 °C, the results to be published elsewhere.

CONCLUSIONS

UF is an effective technology for removing soluble protein in grape juice and wine. However, even with membrane MWCO as low as 10,000 daltons, small amounts of protein which can contribute to heat instability remains in UF permeates. Protein stabilization by UF is similar to that by bentonite in that it is necessary to remove the fractions of lower MW (12,600-30,000), lower pI (4.1-5.8) and known to contain glycoprotein, to finally "protein stabilize" wines. These trace amount of the heat unstable "protein" that is most resistant to removal by either bentonite or UF may be modified by conjugation with phenolics or carbohydrates (7, 12, 14, 16, 24). In addition, polymerized phenolics themselves may form haze (10) and may be contributing to residual heat induced haze that is difficult to remove by bentonite (14), heat or UF treatment. However, it is likely they do so as complexes with small amounts of proteins; the difference between a wine being protein unstable and stable to heat tests often coinciding with the removal of as little as 1-2 mg/L protein by bentonite fining (12, 14) or UF.

To place these conclusions in perspective it is necessary to comment on the use of heat tests by the wine industry and by research institutes as an index of protein instability in wines. The assumption is made that the tests

correlate with stability under longer-term commercial storage conditions. Using bentonite fining as a standard, past experience has shown this assumption to be generally true (16). The heat test used in this study (80 °C, 6 h; 4 °C, 12 h) was recommended by Pocock and Rankine (21) for Australian wines. In our preliminary studies with Pacific Northwest wines (12), the test was found to correlate well with the longer test recommended by Moretti and Berg for Californian wines (16), while generally being less sensitive than the Bentotest (Fritz Merkel) widely used in Germany. All these tests are very sensitive, haze formation being detected by observation with a strong light such as a microscope lamp. The difficult to remove trace amounts of residual haze described in this study is not readily detectable in normal daylight.

Therefore, the possibility that the wines produced by UF processing may very well be "protein stable" to normal commercial conditions, must be considered.

Table IV.1 Effect of membrane nominal molecular weight cut-off on the concentration of soluble protein in ultrafiltered Riesling wine.

Sample	Protein (mg/L) ^a			
	10,000 ^b	10,000 ^b	50,000 ^b	50,000 ^b
Initial wine	30.1 ± 2.3	30.5 ± 0.8	33.8 ± 0.7	27.6 ± 0.1
Permeate	11.4 ± 0.7	10.6 ± 0.4	15.1 ± 0.6	16.5 ± 0.6
Retentate	108.3 ± 3.5	104.8 ± 4.0	93.5 ± 1.6	90.5 ± 0.4
% Retention ($\sigma \times 100$)	90.1	89.9	83.9	81.8

^aMean of triplicate determinations ± standard deviation.

^bMembrane nominal molecular weight cut-off (daltons), Romicon HF-Lab 5 system, operated with PM-10 and PM-50 cartridges at $\Delta P_T = 1.23 \text{ Kg/cm}^2$, 20 °C, to VCR = 6.5; duplicate processing trials.

Table IV.2 Effect of membrane nominal molecular weight cut-off on the concentration of soluble protein in ultrafiltered Gewurztraminer wine.

Sample	Protein (mg/L) ^a			
	10,000 ^b	10,000 ^b	30,000 ^b	30,000 ^b
Initial wine	61.5 ± 1.6	60.8 ± 1.0	54.5 ± 1.6	55.0 ± 1.6
Permeate	9.0 ± 0.1	9.9 ± 0.2	9.5 ± 0.6	8.9 ± 0.2
Retentate	1120.0 ± 14.1	1193.0 ± 15.6	900.6 ± 14.6	1007.5 ± 7.2
% Retention ($\sigma \times 100$)	99.3	99.2	98.9	99.1

^aMean of triplicate determinations ± standard deviation.

^bMembrane nominal molecular weight cut-off (daltons), Millipore Pellicon cassette system, operated with PTGC-10 and PTTK-30 cassettes at $\Delta P_T = 0.88 \text{ Kg/cm}^2$, 20 °C, to VCR = 13; duplicate processing trials.

Table IV.3 Effect of membrane nominal molecular weight cut-off on the concentration of soluble protein in ultrafiltered Riesling juice.

Sample	Protein (mg/L) ^a			
	10,000 ^b	10,000 ^b	50,000 ^b	100,000 ^b
Initial juice	39.9 ± 0.1	40.2 ± 0.2	49.0 ± 1.6	41.7 ± 0.1
Permeate	14.8 ± 0.2	15.9 ± 1.3	26.1 ± 0.5	32.3 ± 1.4
Retentate	125.4 ± 1.7	172.8 ± 4.4	145.7 ± 3.7	114.0 ± 1.7
% Retention ($\sigma \times 100$)	88.2	90.8	82.1	71.7

^aMean of triplicate determinations ± standard deviation.

^bMembrane nominal molecular weight cut-off (daltons), Romicon HF-Lab 5 system, operated with PM-10, PM-50 and PM-100 cartridges at $\Delta P_T = 1.23$ Kg/cm², 20 °C, to VCR = 6.5.

Fig. IV.1 Effect of membrane nominal molecular weight cut-off on concentration of protein and protein stability in (A) Riesling wine (pH 3.15, 11.7% ethanol) filtered with Romicon UF-Lab 5 system*, (B) Gewurztraminer wine (pH 3.67, 12.2% ethanol) filtered with Millipore Pellicon cassette system*, and (C) Gewurztraminer wine (pH 3.19, 12.1% ethanol) filtered with Millipore commercial PUF system.

*Mean of duplicate processing trials
(Brackets indicate range).

¹Determined by modified Bio-Rad (Bradford) dye-binding procedure.

²"Protein haze" induced by heat test (80 °C, 6 h; 4 °C, 12 h) and measured by Hunter meter (model D25P-2).

³Bentonite required to protein stabilize wine to heat test.

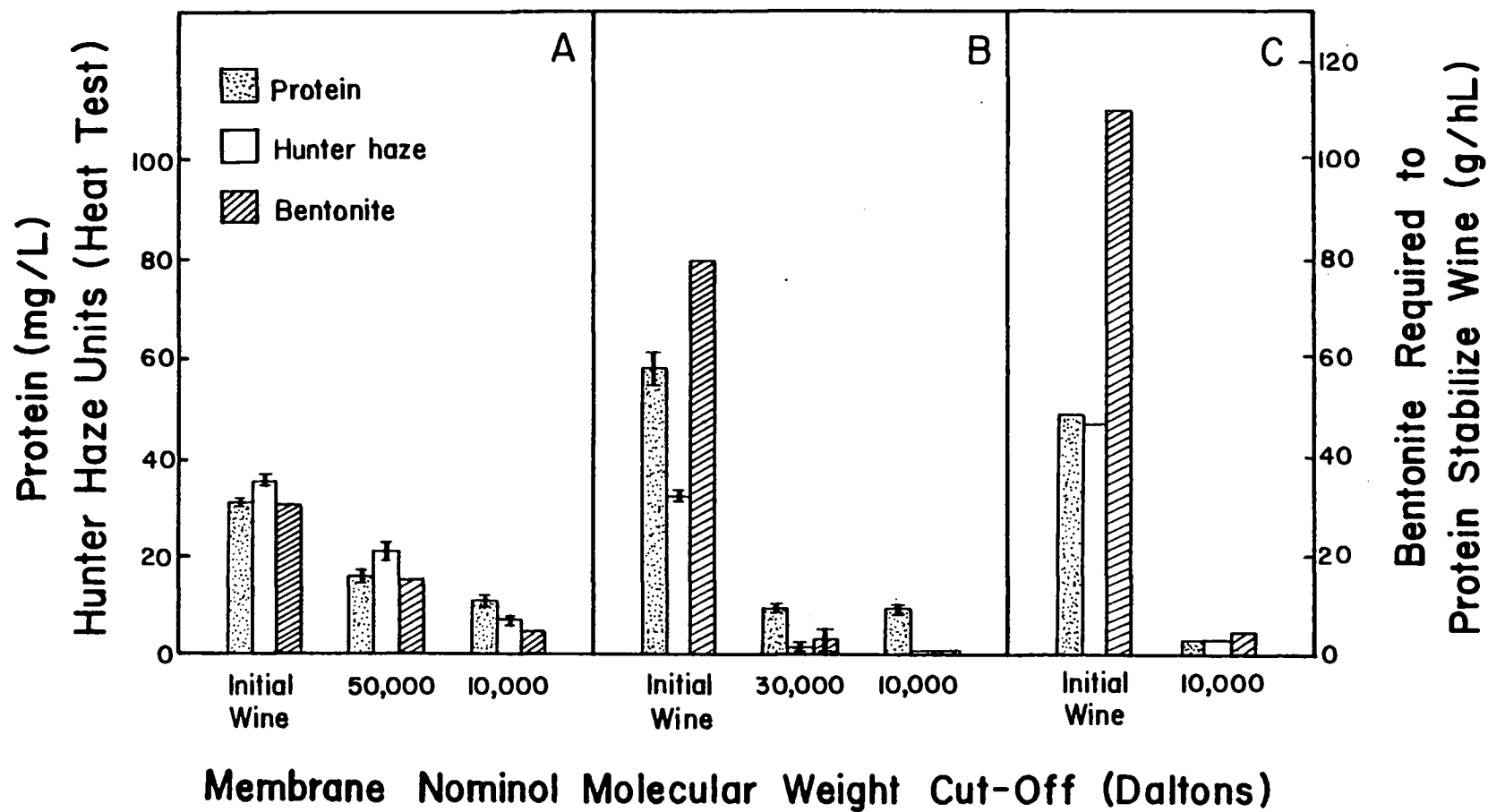


Fig. IV.1

Fig. IV.2 LDS-PAGE of Riesling wine proteins filtered with different membrane nominal molecular weight cut-off: (A) initial wine, (B) permeate of 10,000 MWCO, (C) permeate of 50,000 MWCO, (D) retentate of 10,000 MWCO, (E) retentate of 50,000 MWCO. One hundred μ L of each sample was applied to gel. Molecular weight ($K = 1,000$ daltons) of standards are given on left-hand side of gel.

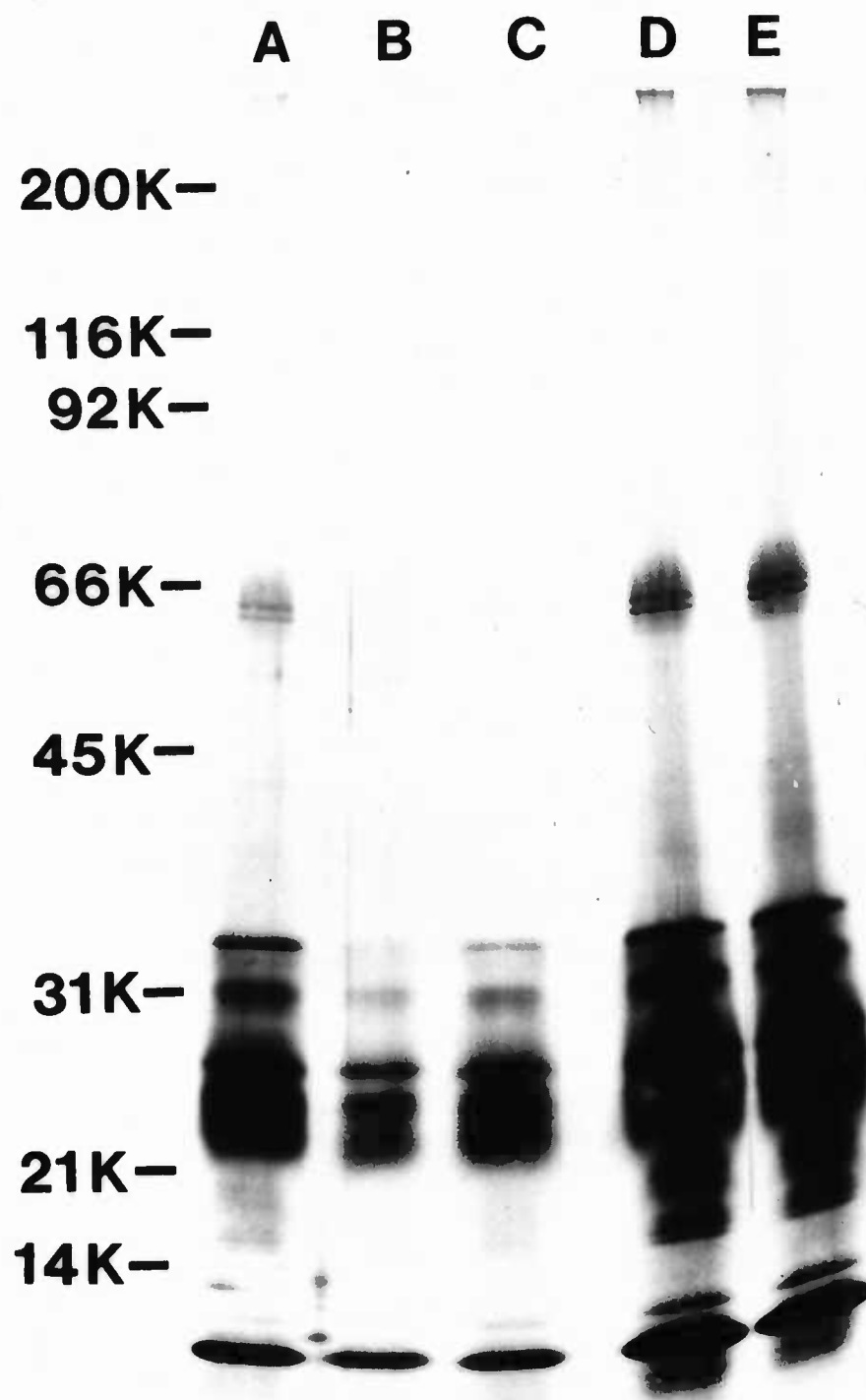


Fig. IV.2

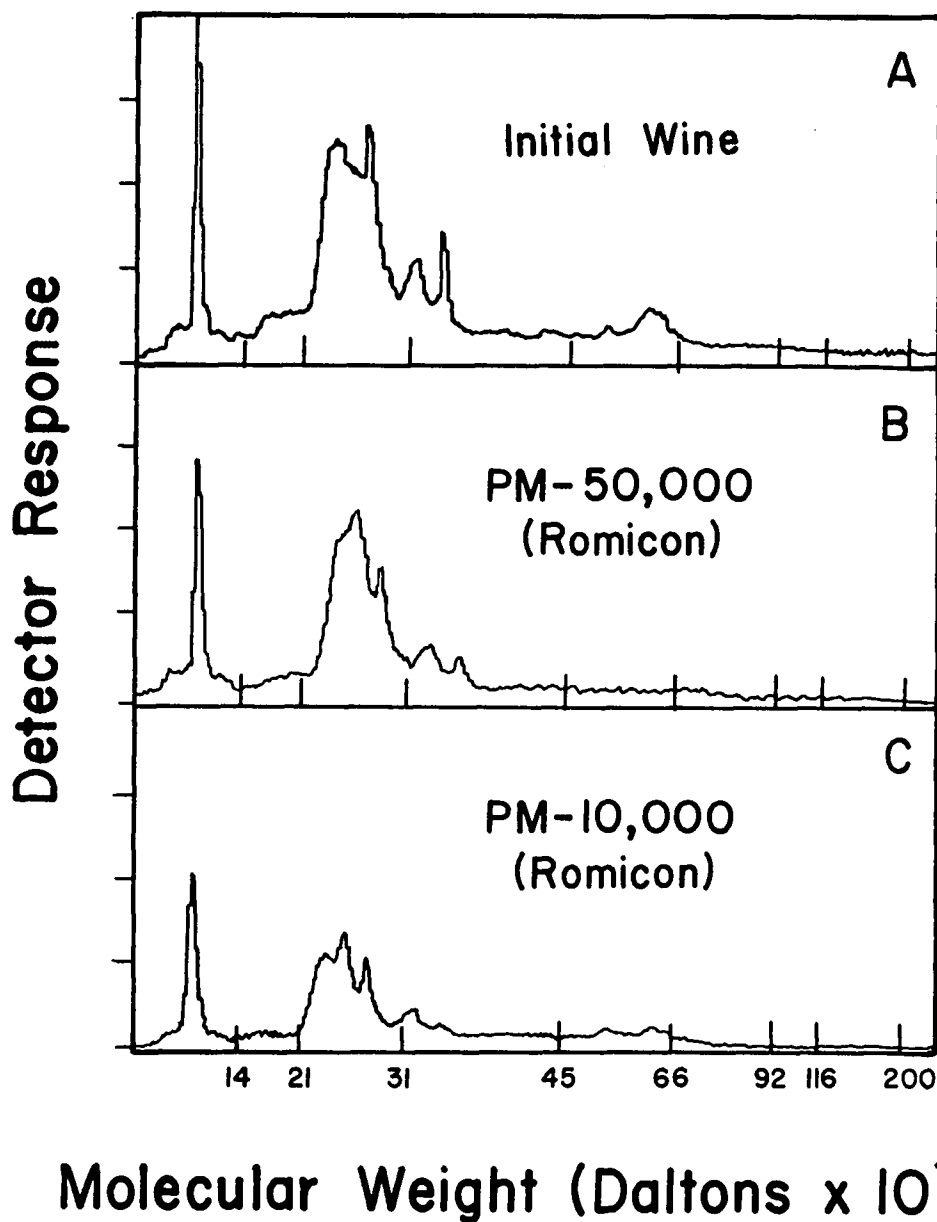


Fig. IV.3 Densitometric scans of electrophoretic patterns of Riesling wine proteins filtered with different membrane nominal molecular weight cut-off (Fig. IV.2): (A) initial wine, (B) permeate of 50,000 MWCO, (C) permeate of 10,000 MWCO.

Fig. IV.4 LDS-PAGE of Gewurztraminer wine proteins filtered with different membrane nominal molecular weight cut-off: (A) initial wine, (B) permeate of 10,000 MWCO, (C) permeate of 30,000 MWCO, (D) sample C fined with 5 g/hL bentonite. One hundred uL of each sample was applied to gel. Molecular weight ($K = 1,000$ daltons) of standards are given on left-hand side of gel.

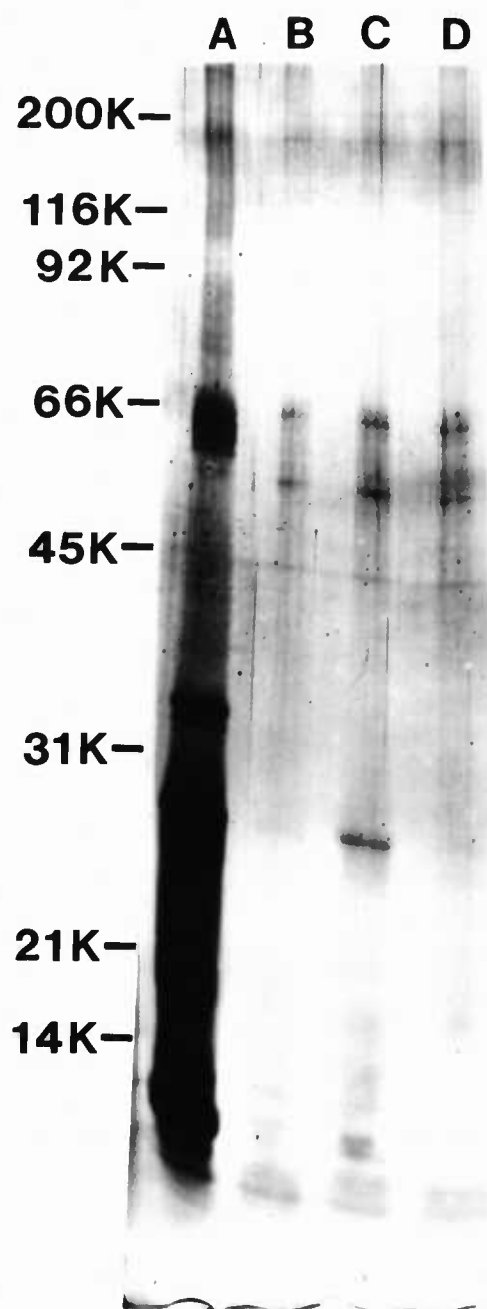


Fig. IV.4

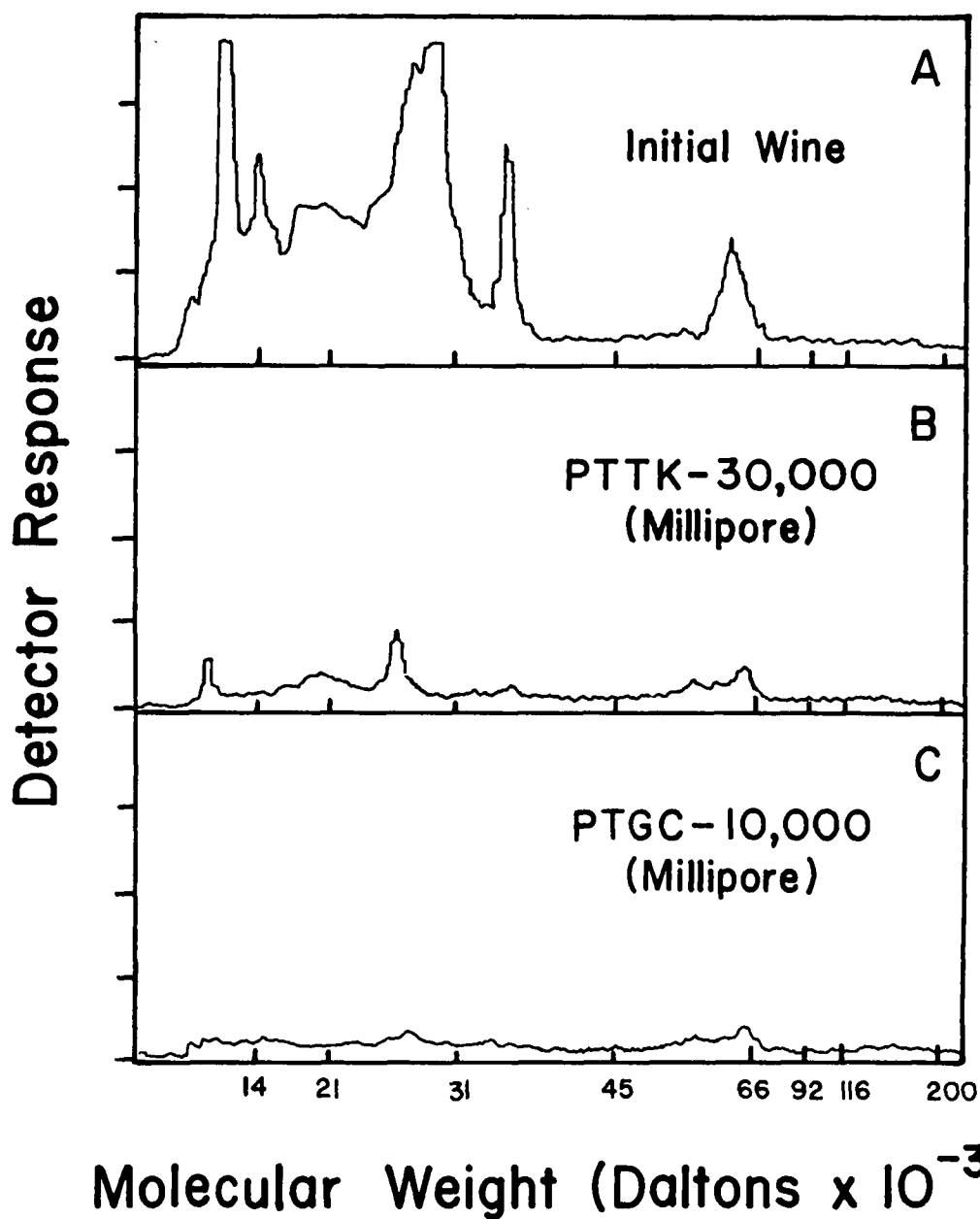


Fig. IV.5 Densitometric scans of electrophoretic patterns of Gewurztraminer wine proteins filtered with different membrane nominal molecular weight cut-off (Fig. IV.4): (A) initial wine, (B) permeate of 30,000 MWCO, (C) permeate of 10,000 MWCO.

Fig. IV.6 LDS-PAGE of Riesling juice proteins filtered with different membrane nominal molecular weight cut-off: (A) initial juice (20.4 °Brix, pH 3.13), (B) permeate of 10,000 MWCO, (C) permeate of 50,000 MWCO, (D) permeate of 100,000 MWCO, (E) retentate of 10,000 MWCO, (F) retentate of 50,000 MWCO, (G) retentate of 100,000 MWCO. One hundred μ L of each sample was applied to gel. Molecular weight ($K = 1,000$ daltons) of standards are given on left-hand side of gel.

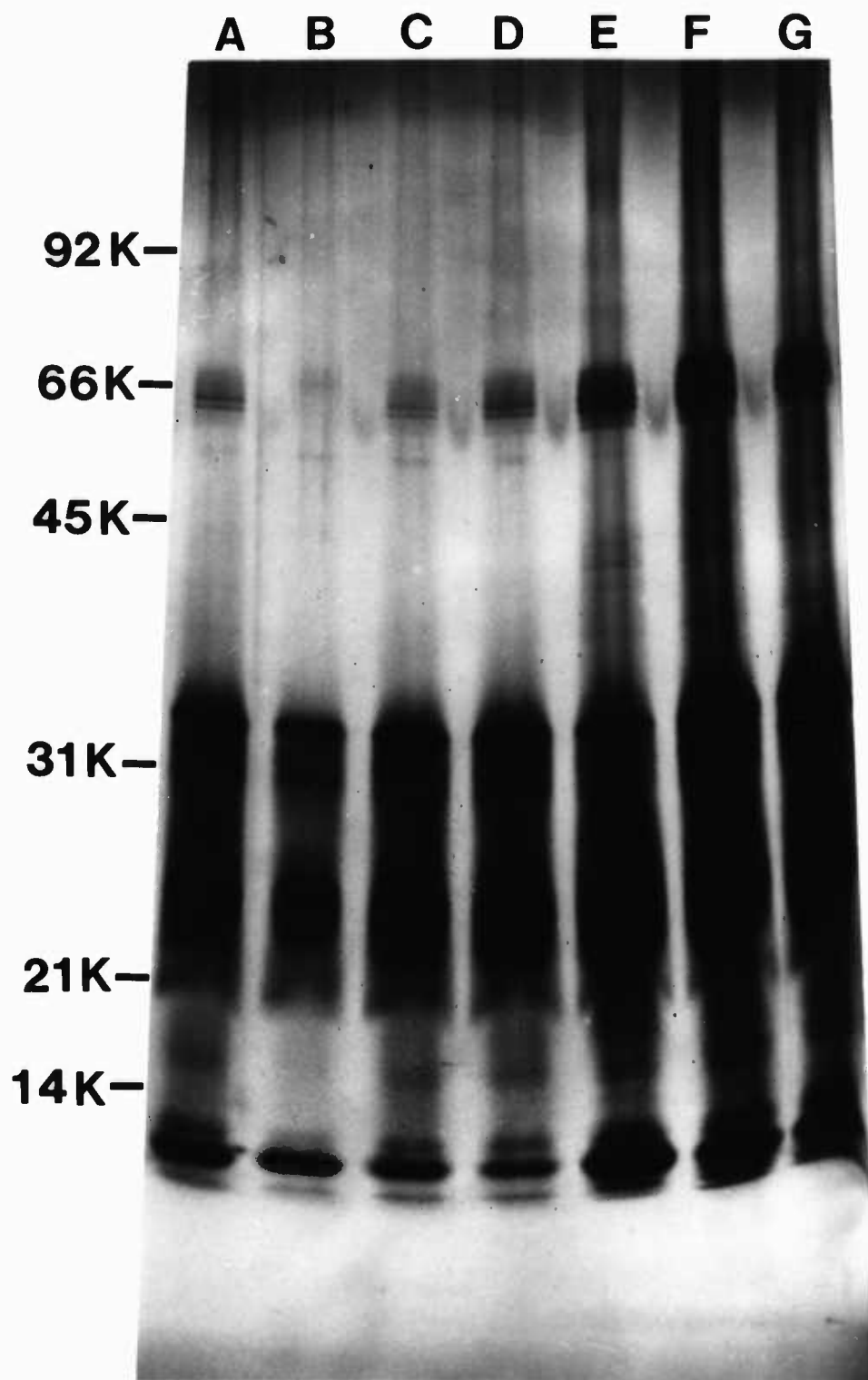
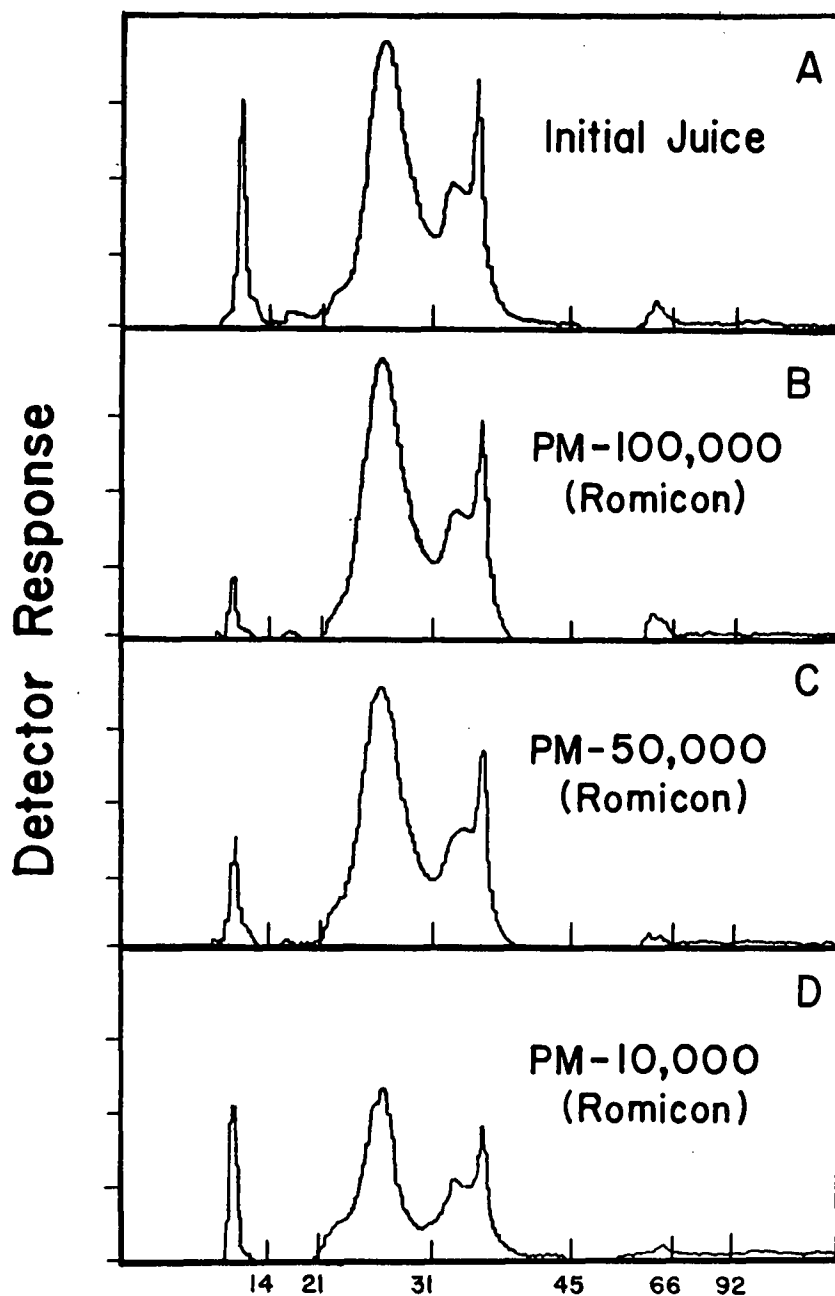


Fig. IV.6



Molecular Weight (Daltons x 10⁻³)

Fig. IV.7 Densitometric scans of electrophoretic patterns of Riesling juice proteins with different membrane nominal molecular weight cut-off (Fig. IV.6): (A) initial juice, (B) permeate of 100,000 MWCO, (C) permeate of 50,000 MWCO, (D) permeate of 10,000 MWCO.

LITERATURE CITED

1. Anonymous. Ultrafiltration et microfiltration tangentielle en oenologie. Compte rendu des Journees Techniques Organisees par I'ITV les 23-24 janvier (22 papers). Institut Technique de la Vigne et du Vin. 21, rue Francois 1^{er}-75008 Paris (1985).
2. Drioli, E., G. Orlando, S. D'Ambra, and A. Amati. Membrane processing in must and wine treatment. ACS Symposium Series 154, Synthetic Membranes. 2:17-26 (1981).
3. Fombin, J.F. Ultrafiltration of grape juice: Effects on composition, quality and preservation. 84pp. M.S. thesis, Oregon State University (1983).
4. Fombin, J.F., D.A. Heatherbell, and D.L. Griffith. Ultrafiltration of grape juice: Effects on composition, quality and preservation. Presented at the 34th Annual Meeting of the American Society for Enology and Viticulture, Reno, Nevada (June 1983).
5. Gaillard, M. Ultrafiltration et microfiltration en flux tangentiel. Les possibilites d'utilisation des processus membranaires en oenologie. Vignes et Vin. 329:26-33 (1984).
6. Gnekow, B., Meir, P.M., Mezynski, D., and Mook, M. Protein stabilization of Sauvignon Blanc by ultrafiltration. Presented at the 34th Annual Meeting of the American Society for Enology and Viticulture, Reno, Nevada (June 1983).
7. Gortges, S. Problems with protein stabilization in winemaking. Weinwirtschaft. 118:931-5 (1982). Abstract in: FSTA 15:8H1138 (1983).
8. Hackert, R. and R.J. Swientek. Ultrafiltration system clarifies 25,000 gal/day of apple juice. Food Processing. 47:80-1 (1986).
9. Heatherbell, D.A., J.L. Short, and P. Struebi. Apple juice clarification by ultrafiltration. Confructa. 22:157-69 (1977).
10. Heatherbell, D.A. Fruit juice clarification and fining. Confructa. 28:192-7 (1984).
11. Heatherbell, D.A., J.L. Short, B.R. Breslau, and B.A. Milnes. The appilcation of ultrafiltration to fruit

- juice processing. Abstract in: International Membrane Technology Conference, The University of New South Wales, Sydney, Australia. pp125 (November 1983). Pub. School of Chem. Engineering & Ind. Chem., University of NSW, P.O. Box 1, Kensington, Australia 2033.
12. Heatherbell, D., P. Ngaba, B. Fombin, B. Watson Jr., Z. Garcia, J. Flores, and J. Hsu. Recent developments in the application of ultrafiltration and protease enzymes to grape juice and wine processing. Heatherbell, D.A., P.B. Lombard, F.W. Bodyfelt, and S.F. Price (Eds). Proceedings of the International Symposium on Cool Climate Viticulture and Enology, 25-26 June, 1984, Eugene, Oregon. Oregon State University, pp418-45 (1985).
 13. Hsu, J.C. and D.A. Heatherbell. Isolation and characterization of soluble proteins in grape, grape juice and wine. Submitted to Am. J. Enol. Vitic. (1986).
 14. Hsu, J.C. and D.A. Heatherbell. Unstable proteins in wine: I. Characterization and removal by bentonite fining and heat treatment. Submitted to Am. J. Enol. Vitic (1986).
 15. Kirk, D.E., M.W. Montgomery, and M.G. Kortekaas. Clarification of pear juice by hollow fiber ultrafiltration. J. Food Sci. 48:1663-6 (1983).
 16. Koch, J. and E. Sajak. A review and some studies on protein. Am. J. Enol. Vitic. 10:114-23 (1959).
 17. Miller, G.C., J.M. Amon, R.L. Gibson, and R.F. Simpson. Loss of wine aroma attributable to protein stabilization with bentonite or ultrafiltration. The Australian Grapegrower & Winemaker. pp46-50 (April 1985).
 18. Moslang, H. Ultrafiltration in the fruit juice industry. Confructa. 28:219-24 (1984).
 19. Nagel, C.W. and U. Schobinger. Untersuchungen uber die bildung von trubungen in apfel- und birnensaftkonzentraten nach vorheriger ultrafiltration. Confructa. 29:16-21 (1985).
 20. Paulson, D.J., R.L. Wilson, and D.D. Spatz. Reverse osmosis and ultrafiltration applied to the processing of fruit juices. Am. Chem. Soc. Symp. Ser. 281:325-44 (1985).

21. Pocock, K.F. and B.C. Rankine. Heat test for detecting protein instability in wine. *Aust. Wine, Brew. & Spirit Rev.* 91:42-3 (1973).
22. Poirier, D., F. Maris, M. Bennasar, J. Gillot, D.a Garcera, and B. Tarodo De La Fuente. Clarification et stabilisation des vins par ultrafiltration tangentielle sur membranes minerales. *Ind. Aliment. Agric.* 101:481-90 (1984).
23. Rosch, E. Experience with ultrafiltration in the 1984 season. *Confructa.* 29:27-31 (1985).
24. Somers, T.C. The inevitability of phenolic instability. Lee, T.H. Ed. *Physical stability of wine. Proceeding of a seminar, October 10, 1985. Reynella. SA. Adelaide. SA. Australian Society of Viticulture and Oenology. Australian Wine Research Institute, pp81-90 (1986).*
25. Swientek, R.J. Ultrafiltration's expanding role in food & beverage processing. *Food Processing.* 47:71-74,76,78,80,82,83. (1986).
26. Wilson, E.L. and D.J.W. Burns. Kiwifruit juice processing using heat treatment techniques and ultrafiltration. *J. Food Sci.* 48:1101-5 (1983).
27. Wucherpfennig, K. and S. Neubert. Applicabilities of ultrafiltration for protein stabilization and of reverse osmosis for concentration of grape juice. *Fluess. Obst.* 44:46-9,52-6 (1977).
28. Wucherpfennig, K. Possibilities for the application of membranes for stabilizing wines (ultrafiltration, reverse osmosis, electrodialysis). *Ann. Technol. Agric.* 27:319-31 (1978).

Bibliography

- Almog, R. and Berns, D.S. 1981. A sensitive assay for proteins and billiproteins. *Anal. Biochem.* 114:336-341.
- Amerine, M.A., Berg, H.W., Kunkee, R.E., Ough, C.S., Singleton, V.L., and Webb, A.D. 1980. *The technology of wine making*. 4th ed. Westport, CT: The AVI Publishing Company, Inc..
- Amerine, M.A. and Roessler, E.B. 1976. *Wine*. p.23. Freeman, San Francisco, California.
- Anderson, R.A. and Sowers, J.A. 1968. Optimum conditions for bonding of plant phenols to insoluble polyvinylpolypyrrolidone. *Phytochem.* 7:1973-1988.
- Anelli, G. 1977. The proteins of musts. *Am. J. Enol. Vitic.* 28:200-203.
- Anonymous. 1985a. Ultrafiltration et microfiltration tangentielle en oenologie. *Compte rendu des Journees Techniques Organisees par I'ITV les 23-24 janvier (22 papers)*. Institut Technique de la Vigne et du Vin. 21, rue Francois 1^{er}-75008 Paris.
- Anonymous. 1985b. *Aquacides*. Biologics Technical Information, Calbiochem-Behring, Division of American Hoechst Corporation, La Jolla, CA 92037.
- Asano, K., Shinagawa, K., and Hashimoto, N. 1982. Characterization of haze-forming proteins of beer and their roles in chill haze formation. *J. Am. Soc. Brew. Chem.* 40:147-154.
- Badran, A.M. and Jones, D.E. 1965. Polyethylene glycols-tannins interaction in extracting enzymes. *Nature*. 206:622-624.
- Bayly, F.C. and Berg, H.W. 1967. Grape and wine proteins of white wine varietals. *Am. J. Enol. Vitic.* 18:18-32.
- Bearden, J.C. 1978. Quantitation of submicrogram quantities of protein by an improved protein-dye binding assay. *Biochim. Biophys. Acta.* 583:525-529.
- Benjamin, N.D. and Montgomery, M.W. 1973. Polyphenol oxidase of Royal Ann cherries: Purification and characterization. *J. Food Sci.* 38:799-806.

- Berg, H.W. and Akiyoshi, M. 1961. Determination of protein stability in wine. *Am. J. Enol. Vitic.* 12:107-110.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal. Biochem.* 72:248-254.
- Broglie, R.M., Hunter, C.N., Delepelaire, P., Nieserman, R.A. Chua, N-H., and Clayton R.K. 1980. Isolation and characterization of the pigment-protein complexes of Rhodospseudomonas sphaeroides by lithium dodecyl sulfate/ polyacrylamide gel electrophoresis. *Proc. Natl. Acad. Sci.* 77:87-91.
- Burnette, W.N. 1981. "Western Blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal. Biochem.* 112:195-203.
- Chazova, T.P. and Nilov, V.I. 1970. The proteins in grape juice and wine. *Prik. Biokhim. Mikrobiol.* 6:18-22. Abstract in: *FSTA* 4:8H1264, 1972.
- Clegg, J.C.S. 1982. Glycoprotein detection on nitrocellulose transfers of electrophoretically separated protein mixtures using concanavalin A and peroxidase: application to arenavirus and flavivirus proteins. *Anal. Biochem.* 127:389-394.
- Cordonnier, R. 1966. Etude des proteines et des substances azotees. *Bull. O.I.V.* 430:1474-1489.
- Drioli, E., Orlando, G., D'Ambra, S., and Amati, A. 1981. Membrane processing in must and wine treatment. *ACS Symposium Series* 154, Synthetic Membranes. 2:17-26.
- Ebermann, R., Barna, J., and Prillinger, F. Jr. 1972. Differentiation of white wines by separation of characteristic proteins on polyacrylamide gel. *Mitt. Rebe, Wein, Obstbau Fruch.* 22:414-416. Abstract in: *FSTA* 5:6H928, 1973.
- Ferenczi, S. 1966. Proteines et substances azotees. *Bull. O.I.V.* 39:1313-1336.
- Fields, M.A. and Tyson, H. 1973. Total phenolic content and peroxidase isozymes in Linum usitatissimum. *Phytochem.* 12:2133-2143.

- Fombin, J.F., Heatherbell, D.A., and Griffith., D.L. 1983. Ultrafiltration of grape juice: Effects on composition, quality and preservation. Presented at the 34th Annual Meeting of the American Society for Enology and Viticulture, Reno, Nevada (June 1983).
- Fombin, J.F. 1983. Ultrafiltration of grape juice: Effects on composition, quality and preservation. Master Thesis. Oregon State University, Corvallis, Oregon.
- Gaillard, M. 1984. Ultrafiltration et microfiltration en flux tangentiel. Les possibilités d'utilisation des processus membranaires en oenologie. Vignes et Vin. 329:26-33.
- Glass F.W. II, Briggs, R.C., and Hnilica L.S. 1981. Use of lectins for detection of electrophoretically separated glycoproteins transferred onto nitrocellulose sheets. Anal. Biochem. 115:219-224.
- Gnekow, B., Meir, P.M., Mezynski, D., and Mook, M. 1983. Protein stabilization of Sauvignon Blanc by ultrafiltration. Presented at the American Society of Enologists annual meeting, Reno, Nevada, June 22-24, 1983..
- Gorinstein, S., Goldblum, A., Kitov, S., Deutsch, J., Loinger, C., Cohen, S., Tabakman, H., Stiller, A., and Zykerman A. 1984. The relationship between metals, polyphenols, nitrogenous substances and treatment of red and white wines. Am. J. Enol. Vitic. 35:9-15.
- Gornall, A.G., Bardawill, C.J., and David, M.M. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177:751-766.
- Gortges, S. 1982. Problems with protein stabilization in winemaking. Weinwirtschaft. 118:931-935. Abstract in: FSTA 15:8H1138, 1983.
- Gray, J.C. 1978. Adsorption of polyphenols by polyvinylpyrrolidone and polystyrene resins. Phytochem. 17:495-497.
- Greenberg, S. and Shipe, W.F. 1979. Comparison of the abilities of trichloroacetic, picric sulfosalicylic and tungstic acids to precipitate protein hydrolysates and proteins. J. Food Sci. 44:735-737.

- Hackert, R. and Swientek, R.J. 1986. Ultrafiltration system clarifies 25,000 gal/day of apple juice. Food Processing. 47:80-81.
- Heatherbell, D.A., Short, J.L., and Struebi, P. Apple juice clarification by ultrafiltration. Confructa. 22:157-69 (1977).
- Heatherbell, D.A., Ngaba, P.R., Watson, B.T., and Dodd, E.E. 1981. Determination of protein and investigation of protein instability in Pacific Northwest wines. Presented at the American Society of Enologists annual meeting, San diego, California, June 24-27, 1981.
- Heatherbell, D.A., Short, J.L., Breslau, B.R., and Milnes, B.A. 1983. The appilcation of ultrafiltration to fruit juice processing. Abstract in: International Membrane Technolog Conference, The University of New South Wales, Sydney, Australia. ppl25 (November 1983). Pub. School of Chem. Engineering & Ind. Chem., University of NSW, P.O. Box 1, Kensington, Australia 2033.
- Heatherbell, D.A. 1984. Fruit juice clarification and fining. Confructa. 28:192-197.
- Heatherbell, D., Ngaba, P., Fombin, B., Watson, B. Jr., Garcia, Z., Flores, J., and Hsu, J. 1985. Recent developments in the application of ultrafiltration and protease enzymes to grape juice and wine processing. Ed. by Heatherbell, D.A., Lombard, P.B., Bodyfelt, F.W., and Price, S.F. Proceedings of the International Symposium on Cool Climate Viticulture and Enology, 25-26 June, 1984, Eugene, Oregeon. Oregon State University, 1985:418-445.
- Henning, K. 1970. Bilans de l' azote dans les mouts et les vins nouveaux en fermentation. Bull. O.I.V. 16:82-86.
- Hii, V. and Herwig, W.C. 1982. Determination of high molecular weight proteins in beer using Coomassie Blue. J. Am. Soc. Brew. Chem. 40:46-50.
- Hsu, J.C. and Heatherbell, D.A. 1986. Isolation and characterization of soluble proteins in grapes, grape juices and wines. Submitted to Am. J. Enol. Vitic.
- Hsu, J.C. and Heatherbell, D.A. 1986. Heat unstable proteins in wine: I. Characterization and removal by bentonite fining and heat treatment. Submitted to Am. J. Enol. Vitic.

- Hsu, J.C., Heatherbell, D.A., Flores, J.H., and Watson, B.T. 1986. Heat unstable proteins in grape juice and wine: II Characterization and removal by ultrafiltration (UF). Submitted to Am. J. Enol. Vitic.
- Jakob, L. 1975. The use of bentonite in treatment of must and wine. In: Proceedings of 4th International Enological Symposium, Valencia, Spain. Ed. by Lemperle, E. and Frank, J. International Association of Modern Winery Technology.
- Jones, J.D., Hulme, A.C., and Woollorton, S.C. 1965. The use of polyvinylpyrrolidone in the isolation of enzymes from apple fruits. Phytochem. 4:659-676.
- Joslyn, M.A. 1953. The theoretical aspects of clarification of wine by gelatin fining. Presented at the 4th Annual Meeting of the American Society of Enology and Viticulture, 1953:39-68.
- Kirk, D.E., Montgomer, M.W., and Kortekaas, M.G. 1983. Clarification of pear juice by hollow fiber ultrafiltration. J. Food Sci. 48:1663-1666.
- Koch, J. and Geiss, E. 1955. Der Einfluss verschiedener kellertechnischer Massnahmen auf den Aufbau der Weissweine. II. Die kurzzeiterhitzung der Weine. Z. Lebensm. Untersuch. Forsch. 100:15-24.
- Koch, J. and Sajak, E. 1959. A review and some studies on protein. Am. J. Enol. Vitic. 10:114-123.
- Kuridze, M.A. and Mindadze, R.K. 1979. Control of protein level in wine. Vinodel. Vinograd. USSR. 5:57-59. Abstract in: FSTA 12:6H0978, 1980.
- Lam, T.H. and Shaw, M. 1970. Removal of phenolics from plant extracts by grinding with anion exchange resin. Biochem. Biophys. Res. Com. 39:965-968.
- Layne, E. 1957. Spectrophotometric and turbidimetric methods for measuring proteins. In: Methods in Enzymology. 3:447-454. Ed. by Colowick S.P. and Kaplan N.O., Academic Press, New York.
- Lea, A.G.H. 1984. Tannin and colour in English cider apples. Fluss. Obst. 8:356-361, 399-400.

- Lee, T.H. 1986. Protein instability: Nature, characterization and removal by bentonite. Lee, T.H. Ed. Physical stability of wine. Proceeding of a seminar, October 10, 1985. Reynella. SA. Adelaide. SA. Australian Society of Viticulture and Oenology. Australian Wine Research Institute, 1986:23-39.
- Loomis, W.D. and Battaile, J. 1966. Plant phenolic compounds and the isolation of plant enzymes. *Phytochem.* 5:423-438.
- Loomis W.D. 1969. Removal of phenolic compounds during the isolation of plant enzymes. In, *Methods in Enzymology*. 13:555-563. Ed. by Lowenstein, J.M., Academic Press, New York.
- Loomis W.D. 1974. Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. In, *Methods in Enzymology*. 31:528-544. Ed. by Fleischer, S. and Parker, L. Academic Press, New York.
- Loomis W.D., Lile, J.D., Sandstrom, R.P., and Burbot, A.J. 1979. Absorbent polystyrene as an aid in plant enzyme isolation. *Phytochem.* 18:1049-1054.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Luis, E.S. 1983. A study of proteins during grape maturation, juice preparation and wine processing. Ph.D. Thesis. University of New South Wales, Australia.
- Lyubarevich, T.A., Mindadze, R.K., Pavlenko, N.M., and Datunashvili, E.N. 1975. The proteins of wine grapes and their properties and stability in wine. *Vinodel. Vinograd. USSR* 4:58-59. Abstract in: *FSTA* 8:3H365, 1976.
- Matheis, G. and Whitaker, J.R. 1984. Modification of proteins by polyphenol oxidase and peroxidase and their products. *J. Food Biochem.* 8:137-162.
- Mesrob, B., Gorinova, N., and Tzakov, D. 1983. Characterization of the electrical properties and of the molecular weights of the proteins of white wines. *Nahrung*. 27:727-733. Abstract in: *FSTA* 16:3H690, 1984.

- Miller, G.C., Amon, J.M., Gibson, R.L., and Simpson, R.F. 1985. Loss of wine aroma attributable to protein stabilization with bentonite or ultrafiltration. *The Australian Grapegrower & Winemaker*. April. p46-50.
- Millies, K. 1975. Protein stabilization of wine using silica sol/gelatin fining. *Mittielungobblatt der GDCH-Fachgruppe. Lebensm. Gerich. Chemie*. 29:50-53. Abstract in: FSTA 7:10H1484.
- Molnar, I. 1975. Study of the soluble proteins of grape and wine by disc-electrophoresis, isoelectric focusing and isotachophoresis. *Zeszyty Problemowe Postepow Nauk Rolniczych*. 167:109-114. Abstract in: FSTA 8:8H1435, 1976.
- Moretti, R.H. and Berg, H.W. 1965. Variability among wines to protein clouding. *Am. J. Enol. Vitic*. 16:69-78.
- Moslang, H. 1984. Ultrafiltration in the fruit juice industry. *Confructa*. 28:219-224.
- Nagel, C.W. and Schobinger, U. 1985. Untersuchungen uber die bildung von trubungen in apfel- und birnensaftkonzentraten nach vorheriger ultrafiltration. *Confructa*. 29:16-21.
- Ngaba, P.R. 1981. Investigation of methods for determination and prevention of protein instability in wines. Ph.D. Thesis. Oregon State University, Corvallis, Oregon.
- Nilov, V.I., Datunashvili, G.M., Zudova, G.M., Ezhov, V.N., and Anancheikova, G.M. 1975. Effect of treatment with bentonite clays on the polysaccharide complex in wines. *Sadov. Vinograd. Vinodel. Moldav*. 30:25-27. Abstract in: FSTA 8:1H16, 1976.
- O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem*. 250:4007-4021.
- Olsson, L., Renne, N., and Samuelson, O. 1976. Chromatography of aromatic compounds on cross-linked polyvinylpyrrolidone and on anion exchange resins. *J. Chromatog*. 123:355-365.
- Ough, C.S. and Anelli, G. 1979. Zinfandel grape juice protein fractions and their amino acid makeup as affected by crop level. *Am. J. Enol. Vitic*. 30:8-10.

- Paulson, D.J., Wilson, R.L., and Spatz, D.D. 1984. Crossflow membrane technology and its applications. Food Tech. 38:77-87,111.
- Paulson, D.J., Wilson, R.L., and Spatz, D.D. 1985. Reverse osmosis and ultrafiltration applied to the processing of fruit juices. Am. Chem. Soc. Symp. Ser. 281:325-344.
- Pavlenko, N.M. 1976. Isolation of grape proteins and their properties. Prik. Biokhim. Mikrobiol. 5:464-468. Abstract in: FSTA 8:7H1262.
- Perdew, G.H., Schaup, H.W., and Selivonchick, D.P. 1983. The use of a zwitterionic detergent in two-dimensional gel electrophoresis of trout liver microsomes. 135:453-455.
- Pocock, K.F. and Rankine, B.C. 1973. Heat test for detecting protein instability in wine. Aust. Wine, Brew. & Spirit Rev. 91:42-43.
- Poirier, D., Maris, F., Bennasar, M., Gillot, J., Garcera, D.a, and Tarodo De La Fuente, B. 1984. Clarification et stabilisation des vins par ultrafiltration tangentielle sur membranes minerales. Ind. Aliment. Agric. 101:481-490.
- Rankine, B.C. 1962. Bentonite and wine fining. Aust. Wine Brew. Spirit Rev. 81:18-20.
- Rankine, B.C. and Emerson, W.W. 1963. Wine clarification and protein removal by bentonite. J. Sci. Food. Agric. 14:685-689.
- Rankine, B.C. and Pocock, K.F. 1971. A new method for detecting protein instability in white wines. Aust. Wine, Brew. & Spirit Rew. 89:10.
- Rhodes, M.J.C. 1977. The extraction and purification of enzymes from plant tissues. In: Regulation of Enzyme Synthesis and Activity in Higher Plants. Ed. by H. Smith. Academic Press, N.Y. 245-269.
- Ribereau-Gayon, J. and Peynaud, E. 1961. Traite d'Oenologie. Beranger, Pairs. 2:770.
- Ribereau-Gayon, J., Peynaud, E., Ribereau-Gayon, P., and Sudraud, P. 1976. Sciences et Techniques du Vin. tome 3. Vinifications. Transformations du Vin., p591-594. Dunod, Pairs, Francis.

- Robinson, T. 1979. The determination of proteins in plant extracts that contain polyphenols. *Plant Sci. Letters*. 15:211-216.
- Rosch, E. 1985. Experience with ultrafiltration in the 1984 season. *Confructa*. 29:27-31.
- Sanderson, G.W. 1964. Extraction of soluble catechol oxidase from tea shoot tips. *Biochem. Biophys. Acta*. 92:24P-25P.
- Sedmark, J.J. and Grossberg, S.E. 1977. A rapid, sensitive and versatile assay for protein using Coomassie Brilliant G-250. *Anal. Biochem*. 79:544-552.
- Selivonchick, D.P., Williams, J.L., and Schaup, H.W. 1981. Alteration of liver microsomal proteins from rainbow trout (Salmo gairdneri) fed cyclopropanoid fatty acids. *Lipids*. 16:211-214.
- Sharon, N. and Lis, H. 1972. Lectins: Cell-agglutinating and sugar-specific proteins. *Science*. 177:949-959.
- Singleton, V.L. and Rossi, J.A. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic*. 16:144-158.
- Singleton, V.L. 1967. Fining-phenolic relationships. *Wines Vines*. 48:23-26.
- Sommers, T.C. and Ziemelis, G. 1973. Direct determination of wine proteins. *Am. J. Enol. Vitic*. 24:47-50.
- Somers, T.C. 1986. The inevitability of phenolic instability. Lee, T.H. Ed. *Physical stability of wine. Proceeding of a seminar, October 10, 1985. Reynella. SA. Adelaide. SA. Australian Society of Viticulture and Oenology. Australian Wine Research Institute, 1986:81-90.*
- Swientek, R.J. 1986. Ultrafiltration's expanding role in food & beverage processing. *Food Processing*. 47:71-74, 76, 78, 80, 82-83.
- Switzer, R.C. III, Merrie, C.R., and Shifrin, S. 1979. A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. *Anal. Biochem*. 98:231-237.

- Taran, N.G., Takking, L.R., Borko, M.M., and Rozhneva, L.V. 1975. Defecation of wine using natural and modified earth sorbents. *Isv. Vys. Ucheb. Zaved. Pish. Tekhnol.* 5:71-74. Abstract in: FSTA 8:8H1380, 1976.
- Thomas, B. and Boulton, R.B. 1984. Studies of proteinaceous materials in white wines. Presented at the American Society of Enologists annual meeting, San diego, California, June 21-23, 1984.
- Troost, G. 1961. *Die Technologie des Weines*. Uimer, Stuttgart, pp283.
- Tyson, P.J., Luis, E.S., Day, R.W., Walker, B., and Lee, T.H. 1981. Estimation of soluble protein in must and wine by high-performance liquid chromatography. *Am. J. Enol. Vitic.* 32:241-243.
- Usseglio-Tomasset, L. and Stefano, R. di. 1977. Observations on nitrogenous constituents in colloids of musts and wines, and colloids formed by wine yeasts. *Riv. Viticolt. Enol.* 30:452-469. Abstract in: FSTA 11:1H56, 1979.
- Usseglio-Tomasset, L. 1978. Colloidal phenomena in musts and wines. *Ann. Technol. Agric.* 27:261-274. Abstract in: FSTA 11:8H1324, 1979.
- Van K.H. and Hale, S.M. 1977. Assay for protein by dye binding. *Anal. Biochem.* 81:485-487.
- Villettaz J-C, Steiner, D., and Trogus, H. 1984. The use of a beta glucanase as an enzyme in wine clarification and filtration. *Am. J. Enol. Vitic.* 35:253-256.
- Vlasuk, G.P. and Walz, F.G., Jr. 1980. Liver endoplasmic reticulum polypeptides resolved by two-dimensional gel electrophoresis. *Anal. Biochem.* 105:112-120.
- Walker, J.R.L. and Hulme, A.C. 1965. The inhibition of the phenolase from apple peel by polyvinylpyrrolidione. *Phytochem.* 4:677-685.
- Watson, B.T., Heatherbell, D.A., Yorgey, B.M., and Hsu, J.C. 1986. Application of protease enzymes for protein stabilizing wine. Submitted to *Am. J. Enol. Vitic.*
- Weiss, H., Rousett, A., Maillard, M., and Bonnet, R. 1960. L'evolution qualitative et semi-quantitative des acides amines au cours de la fermentation des mouts de raisin provenant de cepage d'alsace. *Compt. Rend.* 250:1322-1324.

- Wesche, P.A.E. 1984. Purification of strawberry polyphenol oxidase and its role in anthocyanin degradation. Ph.D. Thesis. Oregon State University, Corvallis, Oregon.
- Wilson, E.L. and Burns, D.J.W. 1983. Kiwifruit juice processing using heat treatment techniques and ultrafiltration. J. Food Sci. 48:1101-1105.
- Wray, W., Boulikas, T., Wray, V.P., and Hancock, R. 1981. Silver staining of proteins in polyacrylamide gels. Anal. Biochem. 118:197-203.
- Wucherpfennig, K. and Neubert, S. 1977. Applicabilities of ultrafiltration for protein stabilization and of reverse osmosis for concentration of grape juice. Fluess. Obst. 44:46-49, 52-56.
- Wucherpfennig, K. 1978. Possibilities for the application of membranes for stabilizing wines (ultrafiltration, reverse osmosis, electrodialysis). Ann. Technol. Agric. 27:319-331.
- Wucherpfennig, K. and Dietrich, H. 1983. Determination of the colloid content of wines. Lebensmitteltechnik. 15:246, 248, 250, 252-253. Abstract in: FSTA 16:10H1905, 1984.
- Wucherpfennig, K., Dietrich, H., and Schmitt, H. 1984. Effect of colloidal substances originating from yeast on wine filterability. Z. Lebensm. Unters. Forsch. 179:119-124. Abstract in: FSTA 17:12H28, 1985.
- Yokotsuka, K., Yoshii, M., Aihara, T., and Kushida, T. 1977. Isolation and characterization of proteins from juices, musts and wines from Japanese grapes. J. Ferment. Technol. 55:510-515.