The effects of post-fermentation and post-bottling heat treatment on Cabernet Sauvignon (*V. vinifera* L.) glycosides and quantification of glycosidase activities in selected strains of *Brettanomyces bruxellensis and Oenococcus oeni.*

by

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ABSTRACT

Thermal processing has been used as a means of modifying the sensory aspects of wine. Cabernet Sauvignon wines were heated prior to dejuicing (3ºC per day from 25ºC to 42ºC) or after bottling (42°C for 21 days) to determine the effects on total glycosides and glycosidic fractions. Total and phenol-free glycosidic concentrations in the wine and skins were quantified by analysis of glycosyl-glucose. Pre-dejuicing thermal vinification resulted in higher total glycosides (12%), phenol-free glycosides (18%), total hydroxycinnamates (16%), large polymeric pigments (LPP) (208%) small polymeric pigments (SPP) (41%), and lower monomeric pigments (42%) in wines. Skins had lower total glycosides (-16%), and no significant difference in phenol-free glycosides. Post-bottling heat treatment resulted in lower total (-15%) and phenol-free (-16%) glycosides, increased hue (25%), a 62% increase in LPP and a 29% decrease in monmeric pigments.

A second study investigated the potential of enological spoilage microorganisms to affect wine aroma, flavor, and color. The activities of β-glucosidase were determined in model systems for fourteen strains of *Brettanomyces bruxellensis* yeast and nine strains of lactic acid bacteria (*Oenococcus oeni*). All *Brettanomyces* strains and seven

Oenococcus strains exhibited enzymatic activity. *B. bruxellensis* β-glucosidase activity was primarily intracellular; *O. oeni* showed some extracellular activity. Yeasts and bacteria showing activity greater than 1000 nmole mL^{-1} g^{-1} for *Brettanomyces*, or 100 nmole mL^{-1} g^{-1} for *Oenococcus*, were evaluated for their effect on Viognier grape glycosides. Neither was active on native grape glycosides.

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INTRODUCTION

Potential wine quality is dependent, in part, on the pool of glycosylated secondary metabolites present in the grape. These metabolites are largely present in the form of potential volatile compounds glycosidically bound to mono- or disaccharide conjugates. In this form, the conjugated aglycone is rendered non-volatile, and has no effect on wine aroma and flavor. Enzymatic or acid hydrolysis can be utilized to free these bound components, releasing the aglycones.

Thermal vinification, or the heat treatment of wines, holds unexplored potential to increase free aglycones through heat-induced glycoside hydrolysis. Heat treatment of musts and wines has been shown to cause change in wine aroma by speeding oxidation, esterification, polymerization, and precipitation. Heating must can also affect hydrolysis of grape glycosides catalyzed by endogenous acids. This hydrolysis has been shown to accelerate with increased temperature.

Microorganisms present in wine, such as bacteria and yeasts, have been shown in some cases to increase the number of free aglycones present in wine. *Brettanomyces bruxellensis* are spoilage yeasts historically associated with off-flavors in wine; lactic acid bacteria (LAB), particularly *Oenococcus oeni*, are often used in winemaking to induce secondary or Malolactic fermentation. Little research, however, has been done on the hydrolytic effects of *B. bruxellensis* or LAB enzymes on grape glycosides.

This research is being conducted to determine the hydrolytic effects of thermal vinification and the enzymatic products of *Brettanomyces bruxellensis* and *Oenococcus oeni* on grape glycosides.

CHAPTER I: REVIEW OF THE LITERATURE

A. Glycosides

Wine aroma and flavor is determined, to some degree, by the glycosidic compounds which exist in small part as free aglycones and largely as bound glycoconjugates (Abbott *et al*., 1993, Williams *et al*., 1996). Glycosides are primarily found in grape juice rather than the skin or pulp fractions (Wilson *et al*., 1986.) Bound glycosides exist mainly as monoglucosides or disaccharides, with sugar moieties occurring as β-D-glucose, 6-O-α-L-rhamnopyranosyl-β-D-glucopyranose, 6-O-α-L-arabinofuranosyl-β-D-glucopyranose and 6-O-α-L-apiofuranosyl-β-D-glucopyranose (Strauss *et al*, 1986; Salles, 1989; Voirin *et al*., 1990). In Riesling grapes, the aglycone portion frequently is a terpenol, most notably linalool, nerol, geraniol, and in come cases linalool oxides, terpene diols and triols. Other aglycones include aliphatic or cyclic alcohols such as hexanol, 2-phenylethanol, benzylalcohol, C13 norisoprenoids, phenol acids and some volatile phenols, such as vanillan (Strauss *et al*., 1986, Guanata *et al*., 1988, Williams *et al.*, 1982, Salles, 1989.) Glycosidic complexes are formed during grape maturation. It is theorized that glycotransferases catalyze the transfer of carbohydrates from sugar-carrying nucleotides to aglycones (Williams *et al*., 1982). These complexes must be hydrolyzed to produce aglycones, a group of complex chemical compounds which may directly affect wine aroma, flavor, color and structure. Liberation of aglycones may occur enzymatically through β-glucosidases, or via heat or acid hydrolysis (Francis *et al*., 1992, 1996, Guanata *et al*, 1985, Williams *et al*., 1982). Glycoside hydrolysis results in equimolar concentrations of aglycones and D-glucose, also known as glycosyl-glucose or G-G (Williams *et al*, 1996). Determination of glycosyl-glucose

concentration can therefore be extrapolated to provide an estimate of the pool of aglycones present (Williams *et al*., 1996). Therefore, tracking concentrations of total glycosides and their phenol-free and red-free fractions allows determination of potential hydrolysis and release of grape-derived volatiles.

β-Glucosidase

Enzymatic Hydrolysis: Enzymatic hydrolysis of diglucoside glycosides generally occurs as a two-step process. Terminal must first be cleaved from the D-glucose by a hydrolase groups (α-L-arabinofuranosidase, α-L-rhamnosidase, or β-apiosidase) before the desired aglycone can be removed by the β-glucosidase (Guanata *et al*., 1982.) β-glucosidases found in the grape promote hydrolysis during fruit maturation, but these enzymes show low activity and cannot liberate the entire pool of aromatic precursors (Gueguen *et al*., 1997). Exogenous enzymes have also been used to split the β-glucosidic bonds between monoterpenes and sugars (Bayonove *et al.,* 1984, Aryan *et al*, 1987, Cordonnier *et al*, 1986, Gunata *et al*, 1990.) Williams *et al* (1982) indicated that enzymes from grape, sweet almond, and molds and yeasts were capable of catalyzing the release of aglycones from the glycosidic complex but did not modify the sensory characteristics of the finished wine. In contrast, Shiraz was found to have enhanced aroma and flavor characteristics after enzyme hydrolysis (Abbott *et al*., 1991.) Enzymes of plant origin, however, are fairly inactive at pH 3-4, are inhibited by glucose concentrations over 1%, and exhibit specificity in respect to aglycone cleavage (Bayonove et al., 1984; Aryan et al, 1987.)

Yeasts: β-glucosidases of yeast origin, particularly those produced by *Candida molischiana* and *C. wickeramii*, evince a low sensitivity to glucose and are active on a rather non-specific range of aglycones (Gunata et al., 1990.) Rosi et al (1994) found a

strain of *Debaryomyces hansenii* capable of producing an exocellular β-glucosidase with activity uninhibited by high ethanol and glucose concentrations and whose activity was largely unaffected by acidic pH and low environmental temperatures. Laboratory strains of *S. cerevisiae* have been found to possess β-glucosidase encoding genes, and do exhibit some hydrolytic activity, but non-Saccharomyces yeasts evince higher hydrolytic activity (Sanchez-Torres e*t al.,* 1998, Guanata *et al*., 1994.) *Hanseniaspora uvarum, Kloeckera apiculata, Metschnikowia pulcherrima,* and *Hansenula anaomala*, all isolated from native fermentations, have been shown to produce β-glucosidase *in vitro* (Reed and Nagodawithana, 1991; Rosi *et al.,* 1994.) The *Candida molischiana* β-glucosidase gene had been successfully produced in recombinant DNA (Sanchez-Torres e*t al.,* 1998.) *Brettanomyces bruxellensis* has been found to produce such volatile phenols as 4-ethyl guaiacol and 4-ethyl phenol (Fleet, 1992).

Enzyme Activity Inhibition: Typical conditions found in wine may severely inhibit the production and activity of enzymatic hydrolases. Low pH, lack of oxygen, and glucose and alcohol concentrations all serve as inhibitors (Guanata *et al*., 1984). Delcroix *et al*. (1994) monitered three strains of Saccharomyces cerevisiae for β-glucosidase concentration in Muscat juice and found that enzyme activity dropped 95% at wine pH.

Acidic conditions can inhibit activity through denaturation (Delcroix *et al*., 1994). *Candida wickerhamii* exhibits optimal hydrolytic enzyme activity at pH 4.5 (Leclerc *et al*., 1987). The optimum pH of *Debaryomyces hansenii* was found to be 3.2 (Rosi *et al.,* 1997). Wine typically exhibits a pH of less than 3.5 (Zoecklein *et al*., 1995).

Vasserot *et al.* (1989) demonstrated that concentrations of glucose lower than 0.5% (w/v) inhibits β-glucosidase production in *Hanseniaspora vineae*. *Debaryomyces*

hansenii evinced optimum enzyme production at glucose concentrations of 2-8% and was inhibited at concentrations above 9% (Rosi *et al*., 1997). Aryan *et al*. (1987), Delcroix *et al.* (1993) and Dubourdieu *et al*. (1988) showed that some indigenous grape and enological yeast enzymes seem more resistant to glucose inhibition. Indeed, *Candida wickerhamii* retains almost half of its enzymatic activity at 500mM glucose, levels normally found in grape juice (Guanata *et al*., 1994)

Eleven to 15% ethanol levels normally found in wines can effect loss of enzymatic activity. Grape and almond β-glucosidases have been shown to lose up to 60% of their enzymatic activity at such ethanol levels (Aryan *et al.,* 1987; Gunata *et al.,* 1984). *Candida entomphilia* β-glucosidases were stimulated by ethanol up to a concentration of 3.5%; higher concentrations effected inhibition, possibly because of protein denaturation (Guegen *et al*., 1995). In contrast, β-glucosidases produced by such species as *Dekkera intermedia* (Blondin *et al.,* 1983), *Candida molischiana* (Gonde *et al*., 1985), and *Hanseniaspora vineae* (Vasserot *et al.,* 1989), as well as other fungal and yeast enzymes, was found to remain unaffected by ethanol concentrations in table wines (Aryan *et al*., 1987; Delcroix *et al*., 1994; Leclerc *et al*., 1987.)

Lactic Acid Bacteria (LAB): Lactic acid bacteria have been shown to hydrolyze maltose and sucrose in bread by utilizing α-glucosidase and β-fructosidase (Antuna et al., 1993.) *Lactobacillus brevis* isolated from Belgian lambic beer produces an isolateable α-glycosidase during secondary fermentation (De Cort *et al*., 1994.) *Lactobacillus plantarum*-type strains have been found to hydrolyze oleurprotein in brined Spanish olives via β-glucosidase production, producing an aglycone and simple compounds such as β-3,4-dihydroxyphenylethanol (Ciafardini et al., 1994). These enzymes, however,

were found to be significantly inhibited by as little as 2% glucose concentrations (Ciafardini et al., 1994.)

Studies have indicated an increase in glucose concentration following malolactic fermentation, in which LAB plays an essential role (Costello *et al*., 1985; Davis *et al*., 1986.) While this increase could be due to glycoside hydrolysis, it could also result from latent hydrolytic enzymes present in the wine before malolactic fermentation (MLF) (Lafon-Lafourcade, 1983; Davis *et al*., 1986)

Acid Hydrolysis: Acid hydrolysis of grape glycosides occurs when protonated reagents break the glycosyl bond between D-glucose and the aglycone, producing one molecule of water. Acids naturally present in wine can cause such cleavage, but at normal wine pH (3.2-3.8) this reaction proceeds very slowly, if at all (Sefton *et al.*, 1998). Further, such hydrolysis can cause undesirable changes in the aglycone aroma (Gunata 1984.) Enzyme hydrolysis cleaves the glycosidic bond cleanly, leaving the aglycone unaltered. In contrast, acid hydrolysis seems to occur only with glycosides of activated (allylic) alcohols, and results from cleavage of the ether, rather than the glucose-aglycone glycosidic bond (Williams *et al.*, 1982). The carbocation produced can then set off a chain of reactions resulting in a variety of products (Sefton et al., 1996, 1998.) One acid-hydrolyzed reactant can subsequently yield a variety of volatiles potentially capable of affecting wine aroma, flavor, and color. Sefton et al. (1998) found that acid-hydrolyzed products of Merlot and Cabernet Sauvignon grape glycosides contributed desirable berry and plum aromas to the finished wine, while products of enzyme hydrolysis produced little aromatic effect. Acid hydrolysis products have also been shown to contribute varietal characteristics such as lime and honey to Chardonnay (Francis *et al.,* 1992.)

Acid hydrolysis can cause undesirable alterations of aroma (Gunata *et al.,* 1994), and can hydrolyze a smaller, but more sensorally important, pool of glycoconjugates than can enzymes (Sefton *et al.,* 1996, 1998).

B. Lactic Acid Bacteria (LAB)

Characterization/Classification: Lactic acid bacteria (LAB) constitute a wide range of organisms which are Gram positive, non-sporeforming, and non-motile (Pardo *et al*., 1992.) LAB occur naturally on the grape skin in the form of filamentous fungi and can be found on cellar equipment (Zoecklein *et al*., 1995). LAB are fairly fastidious, and have high nutritional requirements (Fugelsang, 1997; Jackson, 1994.) Pre-formed nutrients, such as B vitamins, purine and pyrimidine bases, and amino acids must be present in the environment, as well has Mn^{+}, which serves as a necessary antioxidant (Jackson, 1994). LAB are microaerophilic and grow best under low oxygen conditions (Fugelsang, 1997, Zoecklein *et al.*, 1995.)

Malolactic Fermentation (MLF): Malolactic fermentation generally alters the acidity and sensory characteristics of wine, producing a smoother and more palatable product (Henick-Kling *et al*., 1993, 1994., Lafon-Lafourcade 1983., Kunkee, 1974). LAB are important in winemaking as inducers of malolactic fermentation, in which malic acid present in the wine is converted into L-lactic acid and CO_2 (Pardo *et al*., 1992).

The most common lactic acid bacteria found in fresh must that are capable of performing uninoculated fermentation include *Oenococcus oeni*, *Pediococcus*, and several *Lactobacillli*, including *L. plantarum, L. higardii, L. brevis*., and *L. confusus* (Davis *et al*., 1986; Pardo *et al*., 1992). Malolactic fermentability of a wine is determined in part by the strain of *Saccharomyces cerevisiae* used for primary fermentation (Lafon-

Lafourcade *et al*., 1983). Some yeast strains produce necessary bacterial growth factors, such as amino acids, peptides and macromolecules; others directly inhibit LAB growth by producing SO_2 and fatty acids (Caridi *et al*, 1997.) Cryotolerant strains of *S. cerevisiae* can be utilized specifically to inhibit LAB; when used as starters in must of low acidity, these bacteria increase α-phenylethanol and succinic acid production, both of which slow growth (Caridi *et al*., 1997).

C. *Brettanomyces bruxellensis*

Brettanomyces species cost the wine industry several million dollars in wine loss each year (Fugelsang, 1997.) *Brettanomyces* spp. are known to be responsible for off-odors in wine, alternately described as similar to ammonia, band-aid, burnt beans, barnyard, and mouse droppings (Hock 1990; Chatonnet *et al*., 1992., Licker *et al.,* 1999). It is also possible, however, that *Brettanomyces* spp. may positively influence wine complexity and accelerate the aging process.

Brettanomyces bruxellensis has been found to produce volatile phenols such as 4-ethylguaiacol and 4-ethylphenol (Dubois and Dekimpe, 1982; Heresztyn, 1986a, b; Chatonnet et al., 1988, 1992, 1995)and the medium –chain octanioc (C8), dodecanoic (C12) (Rozes *et al*., 1992), isobutyric, isovaleric, and 2-methylbutric acids (Fugelsang, 1997). The low detection thresholds and distinctive aromas make ethylphenols important potential contributors to wine aroma (Dubois and Brulé, 1970; Dubois *et al*., 1971; Singleton and Noble, 1976; Schreier *et al*., 1980; Etiévant, 1981; Ducruet *et al*., 1983; Chatonnet *et al*., 1988, 1990; Etiévant *et al*., 1989). *B. Bruxellensis'* influence on the production of such volatiles, which may cause positive sensory changes in wine, has not

been fully explored; however, studies have indicated that several *Brettanomyces* strains display β-glucosidase activity (Blondin et al., 1983, McMahon et al., 1999.)

D. Thermal Vinification

Heat treatment of musts and wines has been shown to affect positive change in wine color, aroma and flavor to varying degrees (Coffelt *et al*, 1965, Rankine, 1973, Lowe *et al*, 1976.) Amerine *et al* (1980, also Lowe et al 1976) determined that brief exposure to high temperatures (73°C for 30 minutes) renders the greatest benefits concurrent with the smallest increase of off-flavor in the finished wine. Prolonged color extraction by heat can result in high tannin content, rendering the finished wine overly astringent (Amerine *et al*, 1980). Further, heat treatment performed on must during prefermentation and maceration provides greater color extraction than equivalent treatments of finished wine (Lowe *et al*, 1976).

Pre- and post-fermentation heat application has been reported to accelerate aging (Singleton, 1962; Singleton *et al*, 1964). Post-fermentation thermal processing improves sensory scores of Chardonnay and Semillion (Francis *et al*, 1994.) Zoecklein *et al* (1997) reported that low-temperature, moderate duration heat treatments (40°C for 20 days) applied under anaerobic conditions after fermentation increased glycoside hydrolysis.

Increases in color, flavor and aroma in heat-treated wines may be the result of increased hardiness of indigenous microorganisms. (For the purposes of this study, aroma is defined as the volatile compounds detected by the olfactory bulb during inhalation, and flavor as the volatile compounds warmed in the mouth during tasting and detected by the olfactory bulb during exhalation.) Guzzo *et al* (1994) indicated that

Oenococcus oeni, the lactic acid bacteria most commonly responsible for uninoculated malolactic fermentation in wine, induced the production of stress metabolites. The LAB was subsequently able to tolerate higher levels of ethanol and acid, and function longer in a wine environment (Guzzo *et al*, 1994.) In addition, the heat of thermal vinification treatments can serve to speed existing enzymatic hydrolysis of aroma and flavor precursors, and may act as a hydrolytic force in itself (Zoecklein *et al*. 1997).

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The Effects of Post-Fermentation and Post-Bottling Heat Treatment on Cabernet Sauvignon (*V. vinifera* L.) Glycosides

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ABSTRACT

Thermal processing has been used as a means of modifying the sensory aspects of wine. Cabernet Sauvignon wines were heated prior to dejuicing (3ºC per day from 25ºC to 42ºC) or after bottling (42°C for 21 days) to determine the effects on total glycosides and glycosidic fractions. Total and phenol-free glycosidic concentrations in the wine and skins were quantified by analysis of glycosyl-glucose. Pre-dejuicing thermal vinification resulted in higher total glycosides (12%), phenol-free glycosides (18%), total hydroxycinnamates (16%), large polymeric pigments (LPP) (208%) small polymeric pigments (SPP) (41%), and lower monomeric pigments (42%) in wines. Skins had lower total glycosides (-16%), and no significant difference in phenol-free glycosides. Post-bottling heat treatment resulted in lower total (-15%) and phenol-free (-16%) glycosides, increased hue (25%), a 62% increase in LPP and a 29% decrease in monmeric pigments.

INTRODUCTION

Grape-derived aroma and flavor compounds are present as free volatiles or non-volatile sugar-bound precursors, including glycosides (Abbott *et al.,* 1993, Williams *et al.,* 1996). Glycosides exist mainly as monoglucosides or diglucosides, with sugar moieties occurring as β-D-glucose, 6-O-α-L-rhamnopyranosyl-β-D-glucopyranose, 6-O-

α-L-arabinofuranosyl-β-D-glucopyranose and 6-O-α-L-apiofuranosyl-β-D-glucopyranose (Strauss *et al.*, 1986; Salles, 1989; Voirin *et al.,* 1990). Glycoside hydrolysis liberates aglycones, a complex group of chemical compounds that include aliphatic residues, monoterpenes, sesquiterpenes, norisoprenoids, and shikimic acid metabolites. (Abbott *et al.,* 1993; Sefton *et al.,* 1993; Sefton *et al.,* 1994; Winterhalter *et al.,* 1990). Members of this group may affect wine aroma, flavor, color and structure (Hardie *et al*., 1996).

The products of glycoside hydrolysis have been shown to influence wine varietal aroma and flavor (Francis *et al.,* 1992). Liberation of aglycones may occur enzymatically through β-glucosidases, or via heat-induced acid hydrolysis (Francis *et al.,* 1992, 1996; Guanata *et al.*, 1985; Williams *et al.,* 1982). Wine aging has the potential to induce acid hydrolysis of glycosides, breaking some of the glycosyl bonds between D-glucose and aglycones (Zoecklein *et al.,* 1997). Enzymatic hydrolysis can cleave glycosidic linkages, releasing the aglycone unaltered, while acid hydrolysis can act on a smaller pool of glycoconjugates and can result in structural rearrangement of the aglycone (Sefton *et al.,* 1996).

During wine production, pomace (grape skins, seeds and stems) is frequently removed at or before dryness. Studies indicate, however, that grape glycosides are found primarily in the skin and pulp cells (Gomez *et al*., 1994). Wine-pomace contact for up to 50 days post-dryness resulted in increased anthocyanin glycoside extraction (Scudamore-Smith *et al.,* 1990), which may suggest potential for extraction of glycosides containing other aglycones.

Heat treatment of musts and wines has been shown to cause change in wine aroma and flavor (Coffelt *et al.*, 1965, Rankine, 1973, Lowe *et al.*, 1976.) by speeding oxidation, esterification, polymerization, and precipitation (Singleton *et al.,* 1962). Early work in post-bottling heat treatment showed color changes in red wines heated anaerobically for 20 days (Singleton *et al*, 1964). Wines heated post bottling evinced small increases in glucose, perhaps as a result of hydrolysis of glycosidic bonds (Williams *et al*., 1995). Zoecklein *et al*. (1997) reported a 33% decrease in total glycosyl-glucose (TGG) concentrations after heating bottled White Riesling.

Heating must can accelerate hydrolysis of grape glycosides catalyzed by endogenous acids (Francis *et al.,* 1994). Thermal vinification can affect red wine color through the destruction of grape cell membranes, the hydrolytic release of anthocyanin aglycones (Gerbaux, 1999), and the polymerization of phenolic compounds (Rankine, 1973). For example, heating red Burgundy wines for two days at 40ºC post cold maceration resulted in a 22% increase of color intensity, 15% increase in anthocyanins, and 16% increase in polyphenols (Gerbaux *et al.,* 1999).

Gomez *et al.* (1994) and Francis *et al.* (1999) suggested that the transfer of glycosides from skins, and their subsequent hydrolysis, might enhance the flavor of wine. Heating grapes increases the permeability of epidermal cells, allowing anthocyanins to move into the juice (Coffelt and Berg, 1965, Rankine 1973). Gil-Munoz *et al.* (1999) found that grape temperature strongly affected the rate of extraction during the first days of alcoholic fermentation. Gerbaux (1999) found that both heating wine to 40°C for two days at the end of vatting and increasing in temperatures linearly (3ºC per day up to 42ºC) during the vatting period resulted in increased color, tannins, and overall sensory quality.

The potential for extraction of glycosidically bound compounds by extended post-fermentation contact, especially during heat-treatment, has not been fully explored.

This study was performed to determine the effect of post-fermentation wine-pomace contact in the presence of heat, and post-bottling thermal treatment on Cabernet Sauvignon glycosides.

MATERIALS AND METHODS

Pre-dejuicing heat treatment: Cabernet Sauvignon grapes (54 kg), grown in northern Virginia, were harvested at 21.5º Brix, divided into 8 equal replicates, hand-destemmed, and crushed. Berry breakage following crush was approximately 70%. Musts were treated with 200mg/L dimethyl dicarbonate (DMDC) (Velcorin™, Bayer Corp., Pittsburg, PA) and held at 7ºC for 48 hours of cold soak before inoculation with 120g/L *Saccharomyces cerevisiae* (Enoferm Bordeaux Red, Lallemond, Montreal, Canada) hydrated according to the manufacturer's instructions.

One-hundred and sixteen grams of sucrose were added to each treatment replicate to produce final alcohol levels one ºBrix higher than control replications (22.4°Brix and 21.5°Brix, respectively) as preliminary studies indicated a loss of alcohol (%v/v) in treatment replicates. Initial fermentable nitrogen was 160 mg/L, so diammonium phosphate was added at a rate of 120 mg/L.

Fermentation was conducted at ambient temperature (23-24°C) in 7L polycarbonate containers. Temperatures were recorded every 15 minutes on a data logger (5100 Logger, Electronic Controls Design, Inc., Milwaukie, OR), and temperatures in the cap and liquid varied less than 0.5°C at any time. Caps were punched down every eight hours to ensure liquid-pomace contact.

At dryness (<0.2% reducing sugar), a gas flow system was established to continuously blanket the wine with filtered CO_2 (3 : mol filter, Pall Gelman Sciences, Ann Arbor, MI). Treatments were assigned as follows: heat treatment (7 days heating; 3ºC increase per day, beginning at 23ºC and ending at 42ºC) and ambient (7 days at 23ºC). The four treatment replications were placed in a custom-designed, circulating-water-heating tank. Thermal vinification commenced at 24ºC and was increased by increments of 2-3ºC per day for seven days, to 42ºC, and held for one day. After the treatment, replicates were placed in a basket press and free-run wine was collected for 20 minutes. Must and skin samples were taken at crush, post-cold soak, dryness, after heat treatment, and at dejuicing. Samples were frozen in individual sample tubes at –20°C for subsequent analysis.

Berry weight was determined by triplicate measure of 50-berry samples. Juice pH was measured with an Accumet® Model 20 pH/conductivity meter (Fisher Scientific, Atlanta, GA), and °Brix determined by refractometer (American Optical 10430 Hand Refractometer, Scientific Instruments, Warner Lambert, Keene, NH). Fermentable nitrogen was measured as described by Gump *et al.* (2001). Titrateable acidity was determined as described by Zoecklein *et al.* (1995). Lactic, malic and tartaric acids were determined via HPLC (Hewlett Packard model 1100, Palo Alto, CA) using a Bio-Rad Fast Acid column (Bio-Rad Laboratories, Hercules, CA).

For each replicate, three grams of skins were scraped clean of pulp and homogenized in 30 mL of 50% (v/v) ethanol in a Waring blender for 30 seconds on high speed. The homogenate was agitated for an hour using a Thermolyne RotoMix

(Barnstead/Thermolyne, Dubuque, IA), then clarified by centrifugation at 34540 g for 30 minutes prior to analysis. Juice samples were clarified in the same manner.

Total (TGG), and phenol-free glycosides (PFGG) were estimated as described by Iland *et al.,* (1996) and Zoecklein *et al.* (2000), respectively. The phenol-free glycoside (PFGG) assay measures the concentration of glycosides without phenolic functional groups ionizable at pH 10 (Williams *et al.,* 1996; Zoecklein *et al.,* 2000). PFGG includes those compounds responsible for potential changes in aroma and flavor. Total phenols (A_{280nm} – 4), hydroxycinnamates (A_{320nm} – 1.4), and anthocyanins (20 x A_{520nm}) were estimated spectrophotometrically (Genesys 5, Spectronic Instruments Inc., Rochester, NY) as described by Somers and Evans. Hue (A_{520nm}/A_{420nm}) and intensity (A_{520nm} + A_{420nm}) were determined spectrophotometrically as described by Zoecklein *et al.* (1995). Monomeric, small, and large polymeric pigments were determined spectrophotometrically as described by Adams and Harbertson (1999).

Volatile compounds were analyzed by solid phase microextraction (SPME) using a Carbowax fiber and gas chromatography/mass spectroscopy (GC/MS) and a model 5972 Mass Selective Detector (Hewlett Packard, Palo Alto, CA) on a 30m x 0.25 mm DB-Wax column (J&W, Folsom, CA).

Post-bottling heat treatment: One 3.75 L lot of wine, produced as described above, was used for a second study. Total sulfur dioxide concentration was adjusted to 25 mg/L, and wines were sparged with nitrogen to help displace molecular oxygen. Three treatment wine replicates were decanted anaerobically into three standard 750 mL screw-cap glass bottles, flushed with CO_2, capped and stored 20 days at 45ºC. Two 750 mL control samples were treated in the same manner and held at 10ºC.

TGG and PFGG concentrations, total phenols, hydroxycinnamates, anthocyanins, hue, intensity, monomeric, small and large polymeric pigments and volatile compounds were determined as described above.

Data analysis: Data were statistically analyzed using the students' t-test and JMP (SAS Institute, Cary, NC) at a significance of $P \leq 0.05$.

RESULTS AND DISCUSSION

Pre-treatment glycoside extraction: Mean total glycoside (TGG) concentration increased 194% in juice after two days of 7°C cold soak (Fig. 1). McMahon *et al.* (1999) reported a 103% increase after three days at 10°C. The degree of fruit maturity and berry breakage may have influenced glycosidic extraction. Phenol-free glycoside (PFGG) concentrations in the juice rose 200% from crush to cold soak, resulting in maximum concentrations (Fig.1) and consistent with previous studies (McMahon *et al*., 1999). Increased glycoside concentrations may have resulted from the hydrolysis of complex macromolecular precursors, as suggested by Williams *et al.* (1996) and/or extraction from fruit components.

Skin TGG and PFGG concentrations also rose through pre-fermentation maceration (Fig. 2). In Cabernet Sauvignon, glycosidically-bound aroma and flavor compounds have been found in highest concentrations in the skins (Gomez *et al.*, 1994) located in primarily in vacuoles of internal skin cell layers (Castino *et al.,* 1994; Di Stefano *et al.,* 1995). Large cellules of pulp also contain some aroma and flavor precursors (Di Stefano *et al.,* 1995). The increased skin glycoside concentrations in this study may be the result of the activity of enzymes such as arabinosidase or rhamnosidase, which are capable of degrading macromolecules (Williams *et al*., 1996) detectable with

the GG assay. The adsorption of diffuse vacuole contents is also possible; anthocyanins are adsorbed on to grape solids in this manner (Somers and Evans, 1979). Skin TGG may have increased due to pectin hydrolysis occurring in the acidic media, resulting in macromolecule degradation.

TGG concentrations in wines continued to rise through fermentation (Fig. 1), while skin concentrations dropped (Fig. 2). This converse relationship may signify extraction by increased alcohol levels and the increased temperature occurring during fermentation. Prior work found maximum total glycoside concentration in fermenting Shiraz wines occurred at approximately 11 g/L reducing sugar (Williams *et al*., 1996), and Cabernet Sauvignon at 7 g/L (McMahon *et al.,* 1999). In this study, TGG concentrations rose throughout fermentation; prior work found Shiraz TGG remained steady after reaching maximum concentration (Williams *et al*., 1996). Extraction rates may vary due varietal differences, degree of fruit maturity and berry breakage. Wine PFGG concentrations at dryness (Figs. 1,2) were lower than at the completion of cold soak, consistent with previous studies (Williams *et al*., 1996; McMahon *et al*., 1999) Decreased PFGG concentrations may result from glycoside hydrolysis.

At crush and after cold soak, PFGG comprised 50% of TGG concentration, but had dropped to 18% by the end of fermentation (Fig. 1). McMahon *et al*. (1999) similarly found PFGG to be 50% of TGG concentration after cold soak and 23% after fermentation. Increased extraction of phenolic compounds from skins, described above, may have caused the decrease in PFGG percentage after fermentation.

Pre-dejuicing heat treatment: Heat treatment was begun at dryness. TGG concentrations in treatment and control wines decreased overall (15% and 5%,

respectively) from dryness until cessation of heat treatment (Fig. 3). Decreases in TGG concentrations may have resulted from a combination of factors including adsorption, precipitation, or hydrolytic activity. Lebert (1984) reported adsorption by lees, and variations among yeast strains have been recorded (Lubbers *et al.,* 1994). Bourzeix *et al.* (1970) and Somers and Evans (1979) demonstrated that anthocyanins may be absorbed by yeast lees. Color compounds have shown to precipitate following heat treatment (Somers and Evans, 1979) and other glycosides may behave similarly. In addition, glycoside hydrolysis may have resulted from endogenous enzyme activity. Treatment temperatures of 42°C were nearing the optimum β-glucosidase activity in *Saccharomyces cerevisiae* of 50°C (Delcroix *et al*., 1994); however, wine contains many components which may inhibit glycosidase activity. After pressing, treatment and control wines showed increased TGG concentrations (4% and 6%, respectively), possibly caused by physical release of glycosides due to cell tissue rupture in skins and pulp.

While treatment wine TGG's were higher than ambient control wines, treatment skin TGG's were lower than control skins (Figs. 3, 4). Total glycoside concentrations in the skin were 4% lower than control ambient-aged skins when treatment commenced, but dropped to 16% lower than control by the end of heat treatment (Fig. 4). Increased must temperature results in increased extraction of grape constituents into wine (Gil-Munoz *et al.,* 1999; Somers and Evans, 1977; Rankine *et al.,* 1973; Coffelt *et al.,* 1965; Gil-Munoz *et al.*, 1999). Higher glycoside concentrations in treatment wines may have resulted from increased extraction as a result of contributions from the skins, as indicated by the decrease in total skin glycosides.

Treatment and control wines had significantly different PFGG concentrations at dryness, so heat treatment commenced with treatment replicates 16% higher (Fig. 5). Following heat treatment, the PFGG concentration for treatment replicates remained 16% higher than control, indicating that little change in PFGG concentration occurred during treatment. PFGG concentrations in treatment and control skins were not significantly different at any time (Figure 6). Static PFGG concentrations in both wines and skins may suggest that no extraction occurred as a result of heat treatment. Percentage of PFGG to TGG was similar for control and treatment wines, rising from 18 to 20% during ambient aging and 19 to 21% in treated wines.

Red wine color is due primarily to extraction of skin anthocyanins (Ribereau-Gayon, 1959), which can exist as glycosides. In Syrah and Pinot Noir, anthocyanins are responsible for 70-80% of the measurable TGG (Iland *et al*., 1996). In this study, heat treatment lowered A_{520nm} readings in wines (-45%) more than ambient aging (-34%) (Table 1). Hue increased 20% in treatment wines compared to 11% in control wines. Young red wine color hue ($A_{420\ nm}/A_{520\ nm}$) is influenced by the equilibrium between color and colorless anthocyanin forms (Berg and Akiyoski, 1956; Liao *et al.,* 1992); the shift in absorbance maxima from 520 nm to 420 nm may be a result of enhanced polymerization with non-colored phenols (Nagel and Wulf, 1979; Somers and Verette, 1988). Such polymerization may result in long-term color stability (Scudamore-Smith *et al*., 1990) and may alter sensory characteristics (Auw *et al.*, 1996). Treatment increased total hydroxycinnamates (16%), but affected no change in intensity or total phenols (Table 1). Ambient aging of control wines diminished estimated phenols (-4%), elevated hue (11%) and caused no change in intensity or total hydroxycinnamates (Table 1).

Treatment wines had 208% more large polymeric pigments (LPP), 41% more small polymeric pigments (SPP), and 42% less monomeric pigments than did control wines, suggesting both increased extraction of anthocyanins and enhanced polymerization due to heat treatment. The increased incidence of both long and short polymeric pigments indicates the polymerization of four or more anthocyanins (Adams *et al.*, 2001). Coupled with the decrease in A_{520}, this polymerization may indicate changes in phenol structure similar to those found in aged wines (Nagel and Wulf, 1979, Scudamore-Smith *et al*., 1990). Anthocyanin polymerization causes wine to change from purple to tawny as the wine ages (Somers, 1971); visual inspection revealed that treatment wines were noticeably darker in color.

Volatile analysis indicated reductions in volatile esters in wine following treatment (Table 2). Of the compounds surveyed, there were no changes in concentrations of aroma and flavor compounds of glycoside origin.

Post-bottling heat treatment: Total and phenol-free glycoside concentrations of heat-treated wines were 15% and 16% lower, respectively, than in control wines (Fig. 7). Zoecklein *et al*. (1997) found Riesling wines held anaerobically for 20 days at 45°C averaged a 32.9% decrease in TGG concentrations, suggesting glycosidic hydrolysis. PFGG accounted for 52% of TGG concentration in both control and treatment wines, indicating no exclusive decrease in PFGG concentrations. This would suggest release of both phenolic and phenol-free aglycones occurs equally.

Hue increased by 25%, intensity decreased by 4%, and no significant differences were seen in total anthocyanins, total phenols, or total hydroxycinnamates between control and treatment wines (data not shown). These results are contrary to data reported

by Somers and Evans (1979), who noted a 75% increase in hue, 50% decrease in intensity and 30% decrease in both anthocyanins and total phenols following heat treatment. Adams *et al*. (2001) found that LPP's accounted for 37% of color in Syrah wine. In this study, LPP's were 62% higher in treatment wines, and no significant difference was seen in SPP's (Fig. 8). This suggests that the rate of monomer polymerization into small polymeric pigments is equivalent to that of small polymeric pigments into larger polymeric pigments. Monomeric pigments were lower in treated wine (29%, data not shown).

Volatile analysis indicated greater concentrations of several compounds possibly released through glycoside hydrolysis (Table 3). Vitispirane, a compound identified in acid hydrosylates from Cabernet Sauvignon and Merlot (Francis *et al*., 1999) was undetectable in the control wine but present in treatment wines. β-damascenone was also identified as a hydrosylate product (Sefton *et al*., 1998; Francis *et al*., 1999); in this study, treatment wines displayed 73% higher concentration of β-damascenone, which been found to contribute floral (Baumes *et al*., 1986) or "canned apple" (Kotseridis and Baumes, 2000) aromas to wine. Significant differences were not observed in the volatile ester concentrations (data not shown). Esters exist in equilibrium (Ough and Ramey, 1980), and any heat-induced volatile hydrolysis may have been balanced by esterification.

CONCLUSIONS

This study explored the impact of heat treatment of wine pre-dejuicing and post bottling on glycosides and glycoside fractions. Since glycosides are, in part, important

aroma and flavor precursors, this data may lead to an enhanced understanding of the effect of processing parameters on potential wine quality.

The pre-dejuicing heat treatment has the potential to both extract and hydrolyze glycosides; the post-bottling treatment can only affect hydrolysis. While the pre-dejuicing heat treatment showed evidence of glycoside extraction, release of aroma or flavor aglycones may have been offset of volatilization. Since the post-bottling heat treatment occurred in a closed system, hydrolysis products were retained. Cabernet Sauvignon heated post-bottling suggested the release of glycosidic aroma and flavor compounds and increased pigment polymerization. Since this research was performed under near-anaerobic conditions, further research might explore the effect of post-bottling heat treatment on grape-derived secondary metabolites in wine under strict anaerobic conditions. Exploration of the nature of released volatiles, and the sensory thresholds of each within a wine matrix, would be useful for enhanced understanding.

Table 1: Cabernet Sauvignon wine spectral analysis (AU) before and after pre-dejuicing heat treatment or ambient aging period.

		Treatment Wine		Control wine	
		Pre	**Post**	**Pre**	**Post**
Intensity	$A_{420\ nm} + A_{520\ nm}$	2.07 a	2.20 a	2.25 c	1.92 c
Hue	$A_{420\ nm} / A_{520\ nm}$	0.51 a	0.63 b	0.49 c	0.55 d
Total Phenols	$A_{280\ nm}$	1.02 a	2.70 a	1.10 c	2.96 d
Total Anthoxyanins	$20 * A_{520\ nm}$	167.42 a	115.65 b	168.60 c	125.85 d
Total Hydroxycinnamates	$A_{320\ nm} - 1.4$	1.95 a	2.31 b	1.98 c	1.92 c

Different letters within rows of each column indicate significance of t-test of treatment means at $P < 0.05$. $N = 4$.

Table 2: Percent difference in concentration of target compounds in treated wine following pre-dejuicing heat treatment

Compound	% Difference
Ethylacetate	- 58%
Ethylhexanoate	- 42%
Hexylacetate	- 48%
Ethyloctanoate	- 19%
Ethyldecanoate	- 23%
Ethyl-9-decenoate	- 30%
Diethylsuccinate	52%

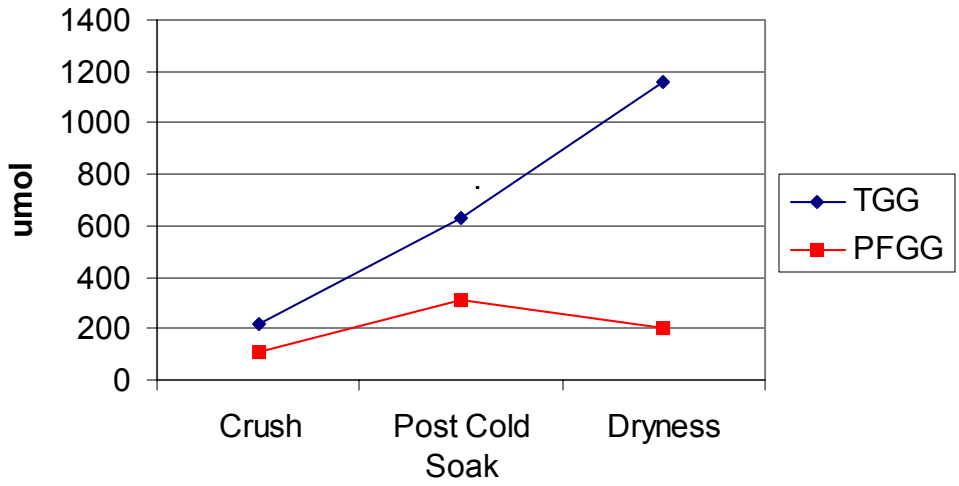
Values are the results of duplicate replication; n=4.

Table 3: Percent difference in concentrations of target compounds in control and treatment wine following post-bottling heat treatment

Compound	% Difference
Fufural	*
Vitispirane	*
Beta-Damascenone	73
BHT-Aldehyde	-18
Methionol	19
Gamma-Butyrolactone	67
Trimethyldihydronaphthalene	467

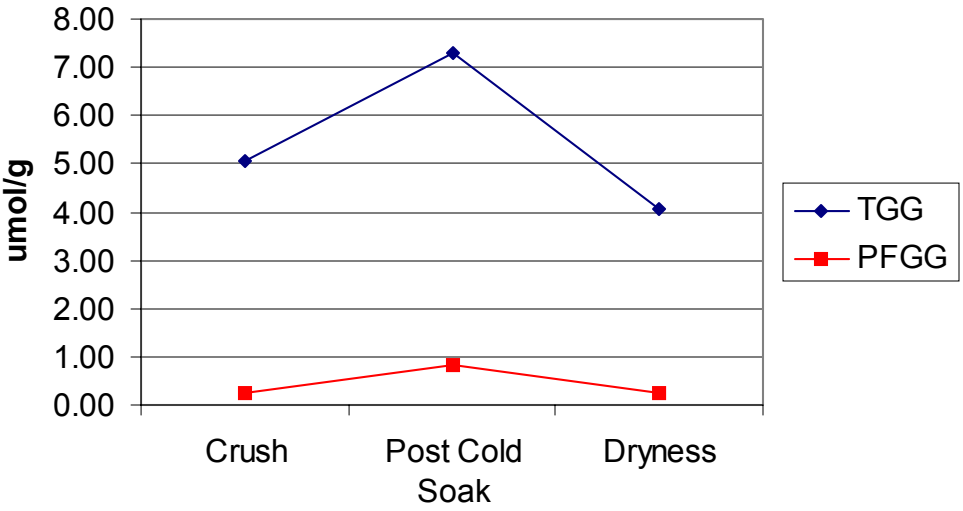
* Indicates compounds below detection levels in control wines, but present in significant numbers in treatment wines.
Data is the result of duplicate replications; n=4.

Figure 1:Total and Phenol-Free Glycosides (umol) of Cabernet Sauvignon Wine During Processing



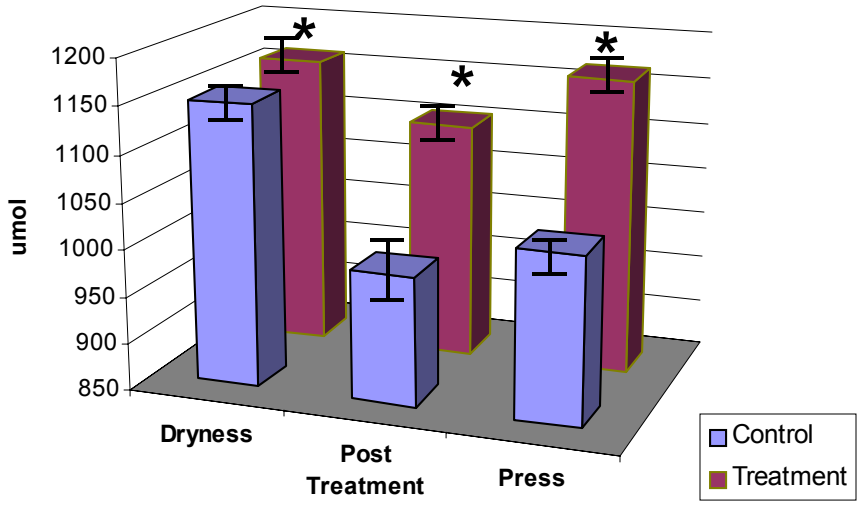
Values represent the mean of duplicate replications. n=8.

Figure 2: Total and Phenol-Free Glycosides (umol/g) of Cabernet Sauvignon Skins During Processing



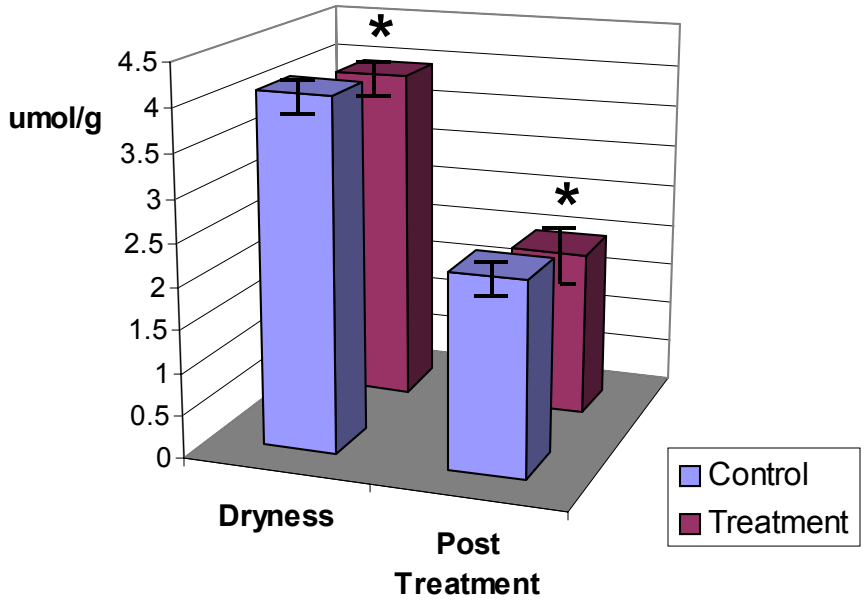
Values represent the mean of duplicate replications. n=8.

Figure 3: Total Glycosides (umol) of Cabernet Sauvignon Wine Before and After Pre-Dejuicing Heat Treatment and at Press



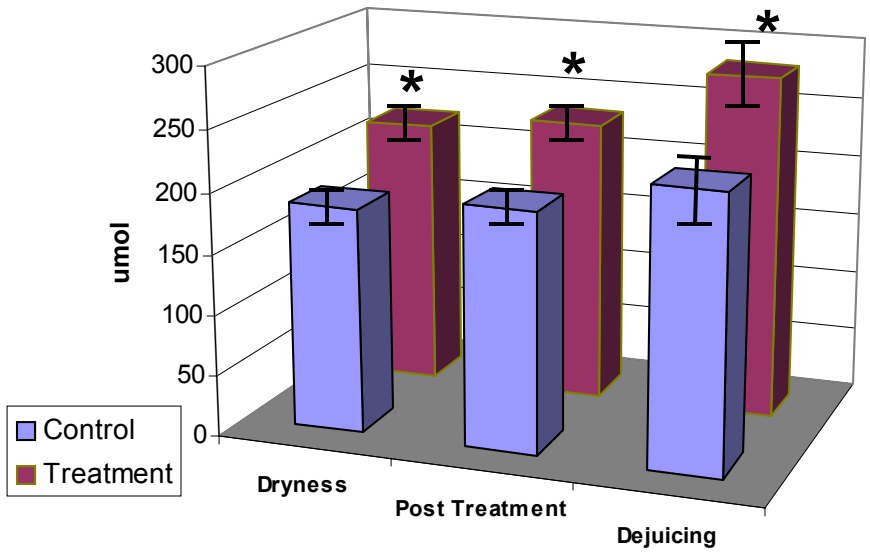
* Indicates significance at P < 0.05. Values are the results of duplicate replications; n=4.

Figure 4: Total Glycosides (umol/g) of Cabernet Sauvignon Skins Before and After Heat Treatment



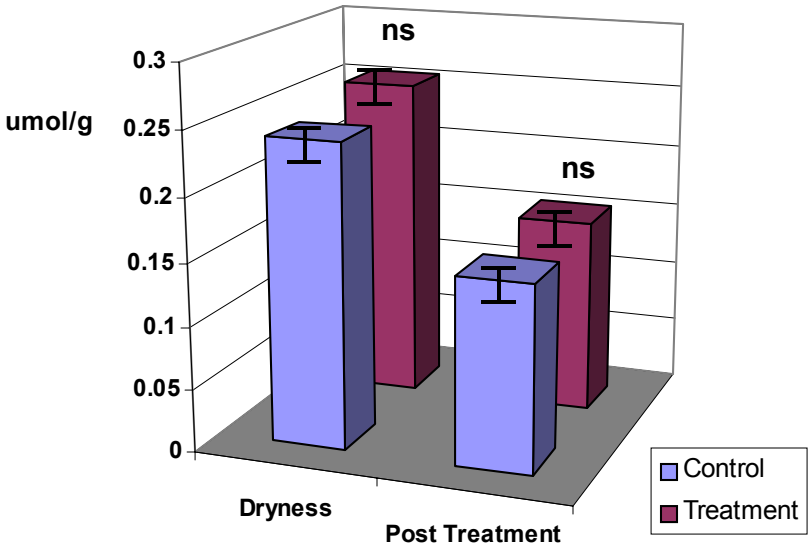
*Indicates significance at P <0.05. Values are the result of duplicate replications; n=4.

Figure 5: Phenol-Free Glycosides (umol) in Cabernet Sauvignon Wine Before and After Pre-Dejuicing Heat Treatment and at Dejuicing



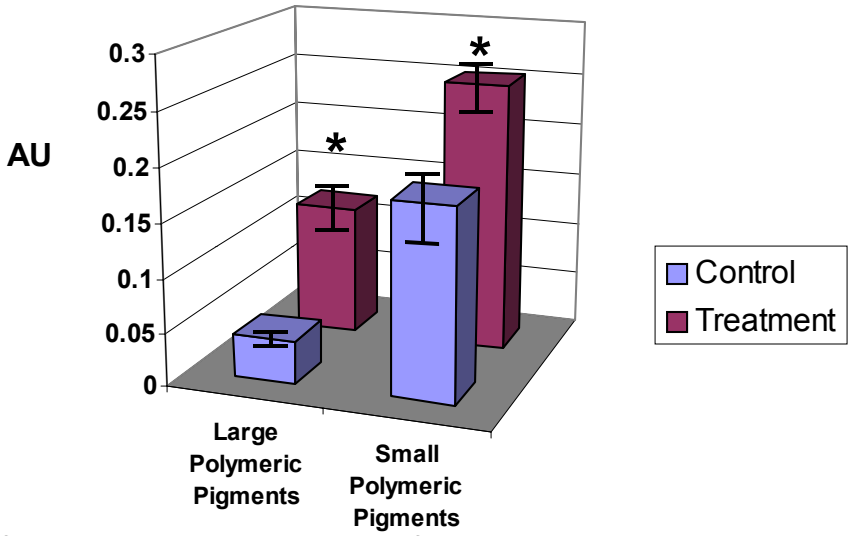
*Indicates significance at P <0.05. Values are the result of duplicate replications; n=4.

Figure 6: Phenol-Free Glycosides (umol/g) of Cabernet Sauvignon Skins Before and After Pre-dejuicing Heat Treatment



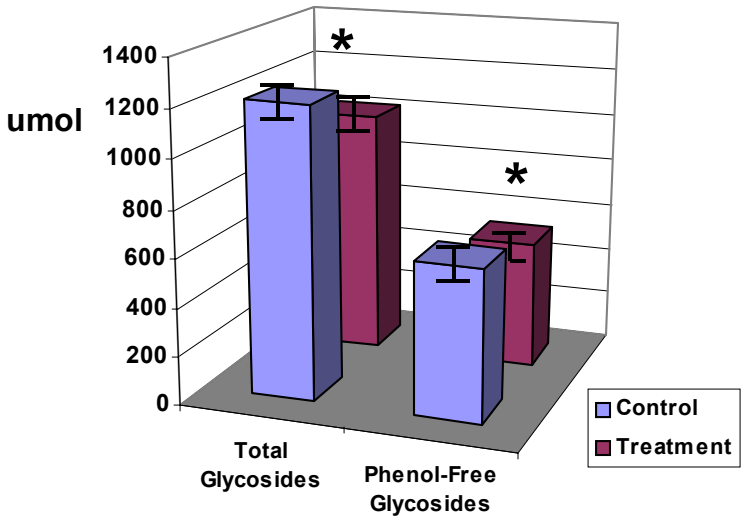
*Indicates significance at P <0.05. Values are the result of duplicate replications; n=4.

Figure 7: Large and Small Polymeric Pigments in Cabernet Sauvignon Wine after Pre-dejuicing Heat Treatment



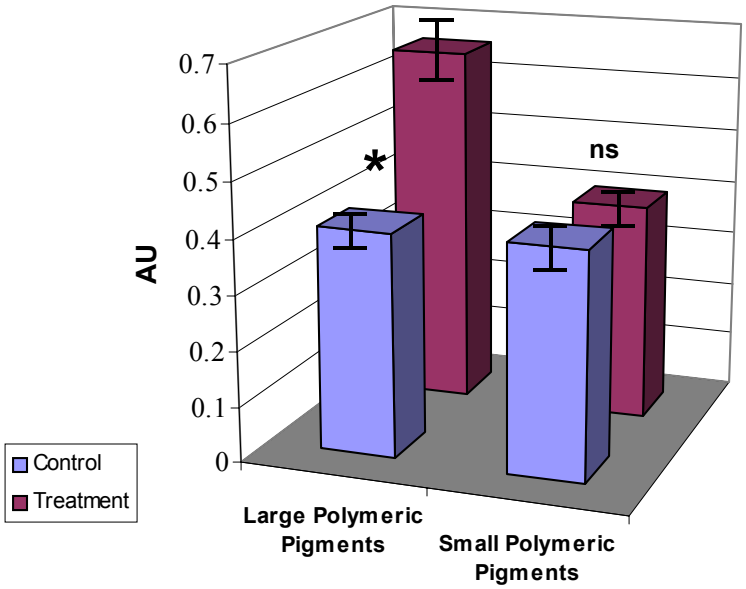
*Indicates significance at P <0.05. Values are the result of duplicate replications; n=4.

Figure 8: Total Glycosides (umol) and Phenol-Free Glycosides (umol) in Post-Bottling Heat Treated Cabernet Sauvignon



*Indicates significance at P <0.05. Values are the result of duplicate replications; n=4.

Figure 9: Large and Small Polymeric Pigments Before and After Post-Bottling Heat Treatment In Cabernet Sauvignon



*Indicates significance at P<0.05. Values are the result of duplicate replications; n=4.

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Quantification of Glycosidase Activities in Selected Strains of *Brettanomyces bruxellensis* and *Oenococcus oeni*

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ABSTRACT

Brettanomyces bruxellensis and lactic acid bacteria are common microorganisms capable of modifying wine aroma and flavor. The activities of β-glucosidase were determined in model systems for fourteen strains of *Brettanomyces bruxellensis* yeast and nine strains of lactic acid bacteria (*Oenococcus oeni*). All *Brettanomyces* strains and seven *Oenococcus* strains exhibited enzymatic activity. *B. bruxellensis* β-glucosidase activity was primarily intracellular; *O. oeni* showed some extracellular activity. Yeasts and bacteria showing activity greater than 1000 nmole mL^{-1} g dry cell $mass^{-1}$ 24 hr^{-1} for *Brettanomyces*, or 100 nmole mL^{-1} g^{-1} dry cell $mass^{-1}$ 24 hr^{-1} for *Oenococcus*, were evaluated for their effect on Viognier grape glycosides. Neither was active on native Viognier grape glycosides.

INTRODUCTION

Wine aroma and flavor are influenced by grape-derived compounds which exist as free volatiles and as sugar-bound glycosides (Abbott *et al*., 1993, Williams *et al*., 1995). Products of grape glycoside hydrolysis include aliphatic residues, monoterpenes, sesquiterpenes, norisoprenoids, or shikimic acid metabolites. (Abbott *et al*., 1993; Sefton *et al*., 1993; Sefton *et al*., 1994; Winterhalter *et al*., 1990). Glycosides may exist as mono- or diglucosides, with sugar moieties occurring as β-D-glucose, 6-O-α-L-rhamnopyranosyl-β-D-glucopyranose, 6-O-α-L-arabinofuranosyl-β-D-glucopyranose and

6-O-α-L-apiofuranosyl-β-D-glucopyranose (Strauss *et al*, 1986; Salles, 1989; Voirin *et al*., 1990). β-D-glucopyranosides appear to predominate (Williams *et al*., 1993). The hydrolysis of grape glycoside may lead to increased wine quality (Winterhalter *et al*., 1990; Francis *et al*, 1992; Abbott *et al*, 1993; Francis *et al*, 1996). Williams *et al*. (1996) demonstrated that grape glycosides were a source of varietal aroma and flavor.

Glycoside hydrolysis may occur enzymatically through β-glucosidases or via acid hydrolysis (Francis *et al*., 1992, 1996, Guanata *et al*, 1985, Williams *et al*., 1982). Enzymatic hydrolysis of diglucoside glycosides occurs as a two-step process. The glucose is first separated from the terminal sugar by a hydrosylase; the glycosidic bond between the aglycone and glucose is then cleaved by the β-glucosidase (Dubourdieu *et al*., 1988; Guanata *et al*., 1982, 1988). In the case of monoglucosides, the β-glucosidase acts directly (Dubourdieu *et al*., 1988). Hydrolases with broad or specific activity may break disaccharide bonds (Guanata *et al.,* 1988).

Acid hydrolysis cleaves glycosides of activated alcohols, producing a carbocation capable of causing aroma and flavor changes (Williams *et al.*, 1982; Gunata *et al.,* 1994; Sefton et al., 1996, 1998). Enzyme hydrolysis cleaves the glycosidic bond without altering the aglycone (Gunata *et al.,* 1984; Winterhalter *et al.,* 1995; Sefton *et al.,* 1998). Endogenous grape β-glucosidases resulted in some hydrolysis during fruit maturation but showed low activity (Cordonnier *et al.,* 1986; Gueguen *et al*., 1997). Enzymes from grapes, molds, and yeasts may release aglycones (Williams *et al.,* 1982). Strains of *S. cerevisiae* have been found to possess β-glucosidase encoding genes (Sanchez-Torres e*t al.,* 1998), and do exhibit some hydrolytic activity (McMahon *et al.,* 1999; Guanata *et al*., 1994), but some non-*Saccharomyces* yeasts display higher hydrolytic activity (Guanata *et*

al., 1994). McMahon *et al.* (1999) found glycosidic activity in five strains of *Brettanomyces bruxellensis*. Strains of *B. bruxellensis* are known to produce β-glucosidases which attack cellobiose, releasing glucose (Fugelsang *et al.,* 1993). Although often deemed as a spoilage organism, *Brettanomyces* may enhance wine aroma and complexity (Fugelsang *et al.,* 1993).

Mono-glucosylated anthocyanins are the primary red pigments in *V. vinifera* grapes (Somers and Verette, 1988), and make up a large portion of the total glycoside concentration (Iland *et al.,* 1996; Yoder *et al*, 1996). Total hydrolysis of anthocyanins in a Virginia Cabernet Sauvignon examined in preliminary work would result in the release of 130 mg/L of glucose. The hydrolytic release of glucose usually results in a corresponding anthocyanindin, which is converted to a colorless pseudobase (Huang, 1955), which may affect color and stability. An increase in glucose concentration coinciding with malolactic fermentation (MLF) has been documented, (Costello *et al.,* 1985; Davis *et al.,* 1986) and may be caused by glycoside hydrolysis (Lafon-Lafourcade, 1983). It is difficult to link bacterial enzyme activity with glycoside hydrolysis, as increased glucose concentrations may be the result of residual grape or yeast hydrolytic enzymes (Davis *et al.,* 1986). However, Grimaldi *et al.* (2001) demonstrated β-glucosidase activity in twelve selected strains of *Oenococcus oeni*.

β-glucosidases can be inhibited by pH, temperature, sugars, ethanol, and phenols (Gunata *et al.*, 1994). The degree of inhibition of glucosidase production and activity is dictated by the organism (Aryan *et al.,* 1987; Delcroix *et al.,* 1994; Leclerc *et al.,* 1984; Rosi *et al.,* 1994). For example, enzymes produced by some strains of *Oenococcus oeni* are inhibited by glucose concentrations as low as 10 g/L, while others show enhanced

hydrolytic activity (Grimaldi *et al.*, 2001). Rosi *et al.* (1994) evaluated a strain of *Debaryomyces hansenii* capable of producing an exocellular β-glucosidase with activity uninhibited by high ethanol and glucose concentrations and whose activity was largely unaffected by acidic pH and low temperatures. β-glucosidases derived from *Saccharomyces cerevisiae* perform optimally at a pH of 5 (Delcroix *et al*., 1993), higher than the usual 3.0-3.5 found in grape juice (Gunata *et al.*, 1994). The acidic conditions in wine may result in denaturation and inhibition of enzymatic hydrolases (Delcroix *et al*., 1994). *S. cerevisiae* loses up to 90% of its β-glucosidase activity after 90 min. incubation (20°C) at pH 3.0 (Gunata *et al.*, 1984, 1994). Optimum temperature for yeast β-glucosidases has been found to be 45-50°C (Delcroix *et al*., 1993). At ethanol contents approaching that of wine (10% v/v), grape and almond β-glucosidases evinced a 60% loss of activity, while glucosidases of *Aspergillus niger, S. cerevisiae and Candida wickerhamii* showed no loss (Gunata *et al*., 1994). Thus, there are a number of factors which may limit the production and/or activity of β-glucosidases in wine. Such activity, however, could have an influence on wine quality and stability.

The objective of this research was to determine the ability of strains of *Brettanomyces bruxellensis* and *Oenococcus oeni* to hydrolyze glycosides in model solutions. Selected strains were assayed for β-D-glycosidase activities, site of production, and hydrolytic activities on native glycosides. The cultures used included 14 strains of *Brettanomyces bruxellensis* and nine strains of *Leconostoc oenos*.

MATERIALS AND METHODS

Cultures: The yeast and bacteria genera and species used in this research are listed in Tables 1 and 2 and were provided by Lallemand, Inc., Montreal, Canada. Yeast strains

were isolated on Yeast Mold Agar (YMA) (Difco; Detroit, MI) plates, pH 5, to obtain pure culture and were maintained on YMA. Bacterial cultures were isolated and maintained on Tomato Rogosa Agar (TRA) as described by Fugelsang (1997).

Growth analysis. *B. bruxellensis* cells were grown in 200 mL Yeast Nitrogen Base (YNB; Difco, Detroit, MI) and *O. oeni* cells in 200 mL Tomato Rogosa Broth (Fugelsang, 1987) at 30°C. Beginning at 24 hours post-inoculation, cultures were agitated for five minutes on a Thermolyne RotoMix™ (Barnstead/Thermolyne, Dubuque, IA) and a 10 mL sample removed and inspected visually for turbidity.

Analyses: The procedure of Blondin *et al.* (1983) as modified by Charoenchai *et al.* (1997) was used to determine hydrolytic enzyme activities on β-D-glucoside. *B. bruxellensis* were cultured as described by McMahon et al (1999). *O. oeni* cells were grown in 10 mL liquid media consisting of Tomato Rogosa Broth (TRB) (Fugelsang, 1987) and 5 g/L arbutin (Sigma, St. Louis, MO), pH 5.0. Cultures were inoculated at 24, 48, or 72 hours (as dictated by growth) and incubated at 30°C until each culture reached log phase. Cultures were centrifuged (5000 X g 10 minutes, 4°C), washed with cold sterile saline (0.7%), and re-centrifuged. The pellets were each transferred to 10 mL filter-sterilized growth medium containing 6.7 g/L Yeast Nitrogen Base (YNB) (for *B. bruxellensis*) or TRB (for *O. oeni*) and 1 mM of the substrate p-nitrophenyl-β-D-glucopyranoside (Sigma; St. Louis, MO). The medium was buffered to pH 3.5 with 0.9 g/L tartaric acid and 0.1 g/L K_2HPO_4. The reaction tubes were incubated for 48 hours at 30 °C. The supernatant was assayed for liberated p-nitrophenyl (pNP): 1.0 mL was mixed with 2.0 mL sodium carbonate buffer (0.2 M, pH 10.2) and measured spectrophotometrically (Genesys 5 ™, Spectronic Instruments Inc., Rochester, NY) at

400 nm. A series of standards was prepared which contained from 0 to 200nM pNP. A substrate blank (buffer and substrate) and sample blanks (cell preparation and buffer) were prepared and subtracted from experimental absorbance readings. All assays were performed in triplicate.

Enzyme activity location: Strains which demonstrated enzyme activity were further analyzed to determine location of activity (whole cells, permeabilized cells, and supernatant) as described by Rosi *et al.* (1994). A loopful of culture was transferred from stock slants to 10 mL of liquid medium (YNB 6.7 g/L, arbutin, 5 g/L, pH 5.0 for yeast; TRB, pH 5.0, for LAB) and incubated at 30°C for 24-72 hours. Once adequate cell density was reached, 0.2 mL of the inoculum was added to 200 mL of liquid medium (YNB 6.7 g/L, arbutin, 5 g/L, pH 5.0 for yeast; TRB, pH 5.0, for LAB) in 500 mL media bottles. Cultures were incubated at 30°C for 24-72 hours until adequate cell density was reached. Cells were prepared and assayed for whole cell (intracellular activity), permeabilized cell (parietal activity), and supernatant (extracellular activity) as described by McMahon *et al.* (1999).

Activity on grape glycosides: 1. *Isolation of glycosides:* Viognier grapes grown in northwestern Virginia were pressed and the juice partially fermented (% alcohol, °Brix.) Viognier glycosides were isolated using C-18 reverse phase Sep-Pak columns (Waters, Milford, MA) activated with 10 mL of methanol followed by 10 mL of deionized water. One hundred mL of undiluted juice were added, and the columns were washed three times with 15 mL of deionized water. Glycosides were eluted with 15 mL methanol. Ten elutions were combined per aliquot, concentrated to dryness, and stored at –20 °C.

Glycoside concentration was estimated by the analysis of glycosyl-glucose (Iland *et al.,* 1996; Williams *et al.,* 1995).

2. Hydrolysis of glycosides: Culture (50 mL) grown in YNB arbutin (yeast) or TRB (LAB) was centrifuged and the pellet resuspended in 50 mL of 100 mM citrate-phosphate buffer (pH 5.0) and added to an aliquot of Viognier glycosides (: M glycosyl-glucose). Pectinolytic enzyme (0.03 g/L) (AR2000, Gist-Brocades, Inc., Seclin Cedex, France) in buffer was used to verify the potential for glycoside hydrolysis. After a 48-hour incubation at 30°C, liberated glycosyl-glucose was measured spectrophotometrically at 340 nm using an enzymatic glucose assay (Boehringher Mannheim, Indianapolis, IN).

Dry Weight: Dry cell weight was determined by filtering 50 mL of culture on pre-weighed membrane filter (0.45 : m; Pall Gelman Science, Ann Arbor, MI). Filters were placed in tared aluminum pans, dried overnight at 100°C, and then reweighed.

Statistical analysis: All data were statistically analyzed using the Tukey-Kramer HSD method in JMP v4 (2000, SAS institute, Cary, NC). Determination of enzyme activities required regression analysis of the series of standards.

RESULTS AND DISCUSSION

All of the 14 *Brettanomyces bruxellensis* strains displayed β-glucosidase activities, ranging from 418 to 250l nmole mL g^{-1} dry cell mass $^{-1}$ per 24 hours (Table 1). With the exception of Vin 5, all *B. bruxellensis* strains exhibited greater intracellular enzymatic activity than parietal, and no extracellular activity (Table 3). McMahon *et al.* (1999), however, found parietal activity to be higher than intracellular in selected *Brettanomyces* strains. The differences between the two studies may be a function of cell growth stage. Grimaldi *et al.* (2001) found β-glucosidase activity in selected yeasts

varied widely based on the phase of growth. In this study cultures were assayed at log phase.

The lack of activity in the supernatant fractions may be the result of young cultures, with a small incidence of autolysis. Yeasts are known to autolyse upon cell death, releasing intercellular compounds (Kunkee and Bisson, 1987). β-glucosidase is located in the periplasmic space of *S. cereviesae* cells, and is released upon cell death (Darriet *et al.,* 1988). During alcoholic fermentation, cell cultures reach maximum autolytic enzyme activity more quickly during than in intact cell cultures (Dubourdieu *et al.,* 1988).

This study suggests that differences in hydrolytic enzyme activity may be a function of strain. Analysis results may also have been influenced by cell wall variation. Results of the procedure used in the study to demonstrate enzyme location (Rosi *et al.*, 1994) are influenced by the ability of cell culture to form a cohesive pellet. Less cohesive cell pellets may have resulted in cell loss during the assay, causing ostensibly lowered concentrations depending on the extent of lost cell mass. McMahon *et al.* (1999) found that the VL-1 strain of *Saccharomyces cerevisiae* evinced low parietal enzymatic activity, possibly due in part to a non-cohesive cell pellet. Strainal differences in cell wall mannan content can result in varying degrees of cell aggregation (Calleja, 1987). In this study, the *Brettanomyces* strain Souche 'O' exhibited the highest total β-glucosidase activity (250l nmole mL g^{-1} dry $cell^{-1}$mass), but activity was below the limit of detection in extracellular and parietal fractions. Empirical observation confirmed that the pellet was non-cohesive. In contrast, some strains of *B. bruxellensis* have high cohesion (Thomas, 1987).

Seven of the nine *Oenococcus oeni* strains showed β-glucosidase activity, ranging from 21 to 178 nmole mL $^{-1}$ dry cell mass^{-1} per 24 hours (Table 2). All strains examined had lower β-glucosidase activity than the *B. bruxellensis* strains. In contrast to *B. bruxellensis*, *Oenococcus oeni* are fastidious and show decreased growth at pH above 5.0 (Fugelsang *et al*., 1994). The use of TRA may have promoted higher titer and subsequent enzyme production than in earlier studies, which demonstrated an absence of glucosidase activity (McMahon *et al.,* 1999). Staggering inoculation techniques used in this study may have more closely approximated log phase at sampling, potentially resulting in higher hydrolytic activity. Grimaldi *et al.* (2001) approximated early and late log phase in modified MRS medium at 26 and 40.5 hr, respectively, consistent with this study's range of 24-48 hr growth. As in this study, activity was found to vary widely due to strain. Different strains of *O. oeni* were used, but activity ranges found by Grimaldi *et al.* (2001) were consistent with those reported here.

No extra- or intracellular β-glucosidase activity was found for the *O. oeni* strains (Table 4). Strains 528, 531, 566, 648 and 649 displayed moderate parietal enzyme activity. Decreases in internal cell pH, caused by sugar fermentation, may cause a decrease in parietal enzyme activity (Maicas *et al*., 1999). Alternatively, the lack of activity in the permeabilized cell fraction may have been due to the release of enzymes, initially leading to higher β-glucosidase concentration, but the enzymes may have been denatured or deported during pellet wash. Cells were permeabilized using a plasmolysis process, which can lead to subsequent autolytic release of enzymes (Breddam and Beenfeldt, 1991). Grimaldi *et al.* (2001) also found that β-glucosidase activity in biomass and supernatant fractions varied widely due to strain and phase of growth.

Viognier glycosides (80 :M total glycosyl-glucose) were used to determine enzyme hydrolysis of a natural substrate in *B. bruxellensis* strains Brux, Souche 'Ave,' Souche 'O,' Vin 4 and Vin 5, and *O. oeni* strains 528, 566, 648, 649, and 655. No glycoside activity was detected for the strains tested (data not reported). These results may be due to the lack of enzymes capable of hydrolyzing disaccharides. Disaccharide hydrolysis occurs in two steps, wherein glucose is first separated from the terminal sugar by a hydrolase and then from the aglycone by a β-glucosidase (Gunata *et al*., 1988). Monosaccharide glycoside hydrolysis can occur directly. Examining yeasts, McMahon *et al.* (1999) found only *Aureobasidium pullulans* effected native glycoside hydrolysis, perhaps due to its arabinosidase and rhamnosidase activity. Strains of *B. bruxellensis* were incapable of either arabinofuranoside and rhamnopyranoside cleavage or native glycoside hydrolysis (McMahon *et al*., 1999). Different cultivars may have differing pools of mono- and diglucosides (Williams *et al*., 1982) and therefore different aglycone pools. Glucosidases evince sugar- and origin-based selectivity. In addition, enzymes from a single organism may have different activities; intracellular and extracellular enzymes have been found to hydrolyze the same type bonds, but display selectivity in specific aglycones (Gueguen *et al.,* 1995).

CONCLUSIONS

The enzymatic liberation of glycoside hydrolysis products may produce aroma, flavor and color changes influencing wine quality. All strains of *Brettanomyces bruxellensis* and *Oenococcus oeni* displayed β-glucosidase activity. *B. bruxellensis* activity was primarily intracellular. *L. oeni* strains showed some extracellular activity,

and little intracellular or parietal activity. No strains studied were able to hydrolyze glycosides isolated from Viognier grapes.

Enzyme activity may be enhanced through the use of aerobic activity, reduced ethanol, elevated pH and temperature, and the removal of glucose as an end product (Rosi *et al.*, 1994); fermentation environments seldom meet these requirements. Some hydrolytic enzyme production is depressed by feedback inhibition through glucose concentrations less than 0.5% (v/v) (Guanata *et al.,* 1994; Rosi *et al.,* 1994). The results of this study suggest that while *B. bruxellensis* and *O. oeni* are capable of producing β-glucosidases in model solutions, the production and hydrolytic activity of those enzymes may be limited in wine. Due to the importance of glycoside hydrolysis on aroma, flavor, color, and color stability,the potential hydrolysis by strains of *Oenococcus oeni* and *Brettanomyces bruxellensis* should be further investigated.

Table 1: Enzyme activities for *Brettanomyces bruxellensis* strains (expressed as nmole of hydrolyzed β-glucoside per mL assay medium per gram dry cell mass per hour).

Brettanomyces bruxellensis strain	Glucopyranoside
211	984 cde
212	418 jk
213	537 hijk
214	914 cdef
215	583 ghj
216	690f ghij
Brux	1231 b
Souche 'Ave'	1476 a
Souche 'O'	250l m
Souche 'M'	878 cdefg
Vin 1	741 fghi
Vin 3	773 defgh
Vin 4	1255 b
Vin 5	1605 a

Values are the averages of duplicate replications. Different letters indicate significance at $P< 0.05$. Limit of detection: 3 nmole/mL

Table 2: Enzyme activities for *Oenococcus oeni* strains (expressed as nmole of hydrolyzed β-glucoside per mL assay medium per gram dry cell mass per hour).

Oenococcus oeni	Glucopyranoside
508	< LOD
528	178 ab
531	21 b
533	< LOD
566	127 ab
648	113 ab
649	126 ab
655	125 ab
656	65 b

Values are the averages of duplicate replications. Different letters indicate significance at $P < 0.05$. Limit of detection (LOD): 3 nmole/mL.

Table 3: β-glucosidase activities by location of enzyme activity (whole cell, permeablized, or supernatant) for *Brettanomyces bruxellensis* strains.

Brettanomyces bruxellensis strain	Whole Cell [1]	Permeabilized [1]	Supernatant [2]
211	27 c	142 e	11 bcd
212	5 d	341 a	9 bcd
213	34 c	105 f	14 bcd
214	19 c	110 f	6 cd
215	< LOD	74 g	11 bcd
216	59 b	321 b	24 a
Brux	26 c	182 d	11 bcd
Souche 'Ave'	14 c	138 e	7 cd
Souche 'O'	< LOD	< LOD	4 d
Souche 'M'	82 a	179 d	9 bcd
Vin 1	32 c	14 g	< LOD
Vin 3	22 c	232 c	9 bcd
Vin 4	65 b	25 g	4 d
Vin 5	4 d	21 g	11 bc

Values are the averages of duplicate replications. Different letters within columns indicate significance at $P<0.05$. Means with the same letter are not significantly different. [1]Activity is expressed as nmole p-nitrophenol (pNP) per mg cells (dry weight) per hour. [2]Activity is expressed as nmole pNP per mL per hour. Limit of detection (LOD): 3 nmol/mL

Table 4: β-glucosidase activities by location of enzyme activity (whole cell, permeablized, or supernatant) for *Oenococcus oeni* strains.

Oenococcus oeni strain	Whole Cell[1]	Permeabilized [1]	Supernatant [2]
528	21	< LOD	< LOD
531	16	< LOD	< LOD
566	113	< LOD	< LOD
648	60	< LOD	< LOD
649	93	< LOD	< LOD
655	< LOD	< LOD	< LOD
656	< LOD	< LOD	< LOD

Values are the averages of duplicate replications. Different letters within columns indicate significance at $P<0.05$. Means with the same letter are not significantly different. [1]Activity is expressed as nmole p-nitrophenol (pNP) per mg cells (dry weight). [2]Activity is expressed as nmole pNP per mL. Limit of detection (LOD): 3 nmol/mL

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SUMMARY

Two separate studies were undertaken to determine the effects of thermal vinification, *Brettanomyces bruxellensis* and *Oenococcus oeni* on grape glycoside concentrations, measured as total and phenol-free glycosyl-glucose. In the first study, pre-dejuicing and post-bottling heat treatments were performed on Cabernet Sauvignon wine. Pre-dejuicing heat treatment resulted in higher levels of total glycosides and phenol-free glycosides, and the ratio of total vs. phenol-free remained constant throughout treatment. Treatment wines also displayed higher concentrations of large and small polymeric pigments than did control wines. Volatile analysis revealed the loss of volatile esters. The data suggests enhanced extraction due to heat treatment, but no evidence of aroma or flavor aglycone release was noted. Anaerobic post-bottling heat treatment resulted in lower concentrations of total and phenolic free glycosides and increased concentrations of polymeric pigments. Increased headspace incidence of aromatic compounds suggested the possibility of glycosidic hydrolysis.

In a second study, fourteen strains of *Brettanomyces bruxellensis* and nine strains of *Oenococcus oeni* were evaluated for β-glucosidase activity in model systems. Assays were performed to determine each strain's overall β-glucosidase activity, location of activity (intracellular, extracellular, or parietal), and activity on Viognier glycosides. All *B. bruxellensis* strains and seven strains of *O. oeni* exhibited activity. *B. bruxellensis* β-glucosidase activity was primarily intracellular; *O. oeni* showed some extracellular activity. No activity was detected on Viognier glycosides.

VITA

Anna Katharine Mansfield was born April 6, 1974 in Charlotte, North Carolina. She received her B.A. in English at Salem College, Winston Salem, NC, in May, 1996. Internship work at local Westbend Vineyards sparked an interest for enological work, so she continued studies in chemistry, biochemistry and cell biology at The University of North Carolina at Asheville from 1996-1998 while working at The Biltmore Estate Winery. Anna Katharine began graduate work at Virginia Polytechnic Institute and State University in July, 1998 in Food Science under Dr. Bruce Zoecklein. She completed her M.S. in June, 2001.

Ode to GG

(The lament of an English major
Attempting to earn a MS)

Such, such, wretch**J**d Sep-pak®
Glycosides poured in thou gaping maw
Rend asunder the phenols from non-phenolic
The red from the red-free
And send the lost aglycones
To endless, dripping, monotony.

Suck, suck, O Sep-pak®!
For in thy C-18
Lies flavor and aroma and color
Or perhaps the suggested potential for all.
Release the secret of thy frit
That I may graduate a'fore fall.