Presence of *Brettanomyces bruxellensis* in North Georgia Wines and Chemical Interaction of Resulting Flavor Metabolites and Acetic Acid

by

DANIELLE WEDRAL

(Under the Direction of Robert L. Shewfelt)

ABSTRACT

Brettanomyces bruxellensis is a particularly troublesome spoilage yeast in wine due to its association with off flavors described as 'burnt plastic,' 'barnyard,' and 'Band-aid®' produced by conversion of phenolic compounds into 4-ethylphenol and 4-ethylguaiacol. The first objective of this research was to assess the occurrence of *B. bruxellensis* induced off-flavors in red V*itis vinifera* wines produced in North Georgia. The eighteen samples of wine were tested by sensory descriptive analysis with confirmation by microbial and chemical analysis. Off-odors associated with *B. bruxellensis* were detected in seven of the eighteen bottle samples. The second objective of this research was to determine the effect of acetic acid on perceived 4-ethylphenol and 4-ethylguaiacol presence. Detection threshold was measured for 4-ethylphenol and 4-ethylguaiacol with and without addition of acetic acid. Acetic acid showed a masking effect on ethylphenol perception. *Brettanomyces* has affected the quality of North Georgia wine.

INDEX WORDS: *Brettanomyces*, Sensory, 4-ethylphenol and 4-ethylguaiacol, Thresholds

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DEDICATION

To my mom and dad, who have been the most profound and important teachers in my life, but also to all my teachers. Everyone can be a teacher, not just those lecturing in the classroom. I have learned though every interaction and experience I have had in Athens, GA and want to dedicate this work to all of you.

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CHAPTER 1

INTRODUCTION

Brettanomyces is a genus of spoilage yeasts that are often referred to as "Brett" and can be found in fermented beverages, mostly recognized in wine and beer. Brett is most notable in history in wild beverage fermentation where the characteristic aroma and flavor are imparted, which often include descriptors like Band-aid®, spices, cloves, earthy, barnyard, and horsy. Over the past 60 years Brett has been found in a variety of products and places, to include wines and winery equipment, beer (especially particular lambic beers), cider, apple cider factories, tequila, and dairy equipment; it has been isolated on five of the seven continents including North America, South America, Europe, Asia and Australia. Countries that have studied and isolated this yeast include some of the top wine producing regions: Italy, France, South Africa, Germany, Uzbekistan, New Zealand, Spain and the United States (Henick-Kling and others 2000; Henschke and others 2007). A Portuguese study analyzed volatiles from wines of different countries and found that more than 25% of red wines had levels higher than 620 µg/L of 4-ethylphenol, the preference threshold above which consumers reject wine (Loureiro and Malfeito-Ferreira 2003; Chatonnet and others 1992; Lesschaeve 2007). Another study performed in Burgundy on Pinot noir wine showed that these yeasts are present in about 50% of the wines undergoing maturating and about 25% in bottles (Gerbaux and others 2000). In a paper exploring the presence of Brett in New York State, *Brettanomyces* has recently been referred to as a 'Global Issue' (Arvik and others 2002) and has been noted for large economic losses in the wine sector worldwide (Fungelsang 1997; Boulton and others 1996).

Brettanomyces spp. are the non-spore forming counterparts of the genus *Dekkera* (Goode 2006). The terms *Brettanomyces* and *Dekkera* are often used interchangeably, but due to *Brettanomyces* being the more common name in the literature, it will be used in this paper. *Brettanomyces bruxellensis* has

been found on the surfaces of grapes as well as in barrels, but the greatest concern is its presence in wine. *B.bruxellensis* is commonly known as Brett in the wine industry and is known for the off flavors the yeast produces.

At low concentrations, compounds that have been associated with Bretty flavors in wine can contribute to wine aroma complexity, producing positive sensory effects reminiscent of grand old-world-style red Bordeaux and Burgundy wines (Mahaney P, 1998). Higher flavor intensities can overwhelm the wine aroma and create an unpleasant experience (Chatonnet P, 1990). These findings show how Brett associated metabolites in wine can be considered either negative or positive depending on concentration and consumer expectation.

The perception of wine flavor reflects a complex mixture of compounds and is highly effected by readily identified undetected compounds. To detect if change has occurred in a flavor profile, threshold testing can be used to determine when the difference begins to change the products acceptability or perceived flavor profile. The yeast *Brettanomyces/Dekkera* is known for its production of 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG). The sensory perception thresholds for 4-EP and 4-EG alone in red wine are 605 µg/L and 110 µg/L, respectively (Chatonnet and others 1992). When both compounds are present in red wine, the sensory perception threshold of 4-EP is lower, approximately 369 µg/L (Chatonnet and others 1992). The sensory perception of these compounds may be affected by other compounds. For example, in a light-bodied red wine with little oak influence, the sensory perception threshold of 4-EP may be ~350 µg/L as opposed to a 1000 µg/L threshold in a full-bodied red with more oak influence (Coulter and others 2003). The assumption that *Brettanomyces* in wine imparts a typical flavor character has been challenged by studies on production of ethylphenol and other typical bi-products. These studies have demonstrated that experts often fail to distinguish or identify contaminated wines. This contradiction could be due to complex chemical and flavor interactions (Conterno and others 2006; Romano and others 2009). Acetic acid and ethylphenol interactions help define Brett character. Due to the ability of other organisms to produce ethylphenols and *Brettanomcyes* to produce acetic acid, it is important to understand perceived flavor when acetic acid and ethylphenols are both present.

Understanding the presence of *Brettanomyces* and its metabolites on impact of wine flavor chemistry will help define possible flavor profiles that may allow a degree of acceptability of this yeast in the wine industry. *Brettanomyces* has been identified worldwide and remains one of the largest economic concerns in regard to spoilage organisms of the wine industry (Boulton and others 1996; Fungelsang 1997). The first objective of this research was to assess the occurrence of *B. bruxellensis* induced off-flavors in red V*itis vinifera* wines produced in North Georgia. The second objective of this research was to determine the effect of acetic acid on perceived 4-ethylphenol and 4-ethylguaiacol presence. The presence of *B. bruxellensis*is has not been previously reported in wines and wineries of the southeastern United States.

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CHAPTER 2

LITERATURE REVIEW

"Wine microbiology entails a complex interaction of a variety of microorganisms that play an essential role on the outcome of the final product and, if the microbiology of wine is disregarded, there will simply be no wine" (Oelofse and others 2008).

BACKGROUND ON *BRETTANOMYCES*

Physical Characteristics of the Organism

Brettanomyces spp. are the non-spore forming counterparts of the genus *Dekkera* (Goode 2006). *Brettanomyces* and *Dekkera* are often used interchangeably and due to *Brettanomyces* being the more common name found in the literature, it will be used in this paper. There are five species jointly belonging to the genera *Brettanomyces/Dekkera*, which include *B. custersianus, B. naardenensis, B. nanus, B. anomalus, and B. bruxellensis* (Kurtzman and Fell 2000). *B.bruxellensis* most commonly affects wines, and it has been found on the surfaces of grapes as well as in barrels. *B.bruxellensis* has an oval to an ellipsoidal shape and reproduces asexually by budding. See figure 2.1 for typical *B.bruxellensis* appearance. Cell morphology changes from elliptic to branched shape after a few months of incubation (Agnolucci and others 2009). The unusual fermentative behavior has made *B.bruxellensis* particularly interesting for scientists to study, namely because of the aroma bi-products and the Custer effect, which is the inhibition of alcoholic fermentation under anaerobic conditions due to high production of acetic acid and redox imbalance (Van Dijken and Scheffers 1986; Henschke and others 2007; Vigentini and others 2008).

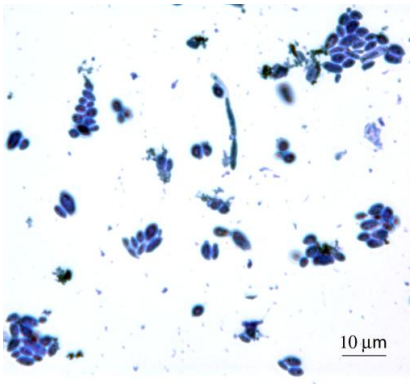

Figure-2.1 Picture of *B. bruxellensis*. Photo by Author.

Organism History

This yeast is often referred to as "Brett" and can be found in fermented beverages, mostly recognized in wine and beer. Brett is most notable in history in wild beverage fermentation where the characteristic aroma and flavor are imparted, which often include descriptors like Band-aid®, spices, cloves, earthy, barnyard, and horsy. Classically, English stock beers that went through secondary fermentation in a cask where *Brettanomyces* was or was added, had the typical 'English stock ale character.' In fact, Claussen suggested "a general rule cannot be given for all cases, but the quality of *Brettanomyces* to be added must be regulated by local circumstances, more especially by the time the beer has been stored and by the temperature of the storing room" (Arvik and Henick-Kling 2002; Henick-Kling and others 2000). The name *Brettanomyces* was first introduced by N Hjelte Claussen of New Carlsberg Brewery to specifically describe the yeast needed to produce English stock beers and later became an official genus in the 1920s when a similar yeast was isolated from Lambic ales of Belgium (Henschke and others 2007). In the 1950s and 1960s the yeast was identified as *Dekkera/Brettanomyces* ssp. and isolated from wine in France, Italy and South Africa, but it wasn't until the 1980s and 1990s that the yeast was characterized for its classic aroma in wines (Henschke and others 2007). Other specific countries from which this yeast was isolated before characterization include Germany, Uzbekistan, New Zealand and Spain (Henick-Kling and others 2000).

Since initial classification and identification, Loureiro and Malfeito-Ferreira (2006) have looked at the presence of *Brettanomyces* in many products and studies over the past thirty to forty years. "Brett" is isolated from a variety of products and places ranging from wines and winery equipment, beer (in particular lambic beers), cider, apple cider factories, tequila, dairy equipment and kombucha tea. "Brett" is often found in these products due to its characteristic long survival periods, therefore growing in products that have storage and potentially long processing periods. For example, *Brettanomyces* is commonly isolated after one year of maturation in lambic beers after *Saccharomyces* is no longer found (Loureiro and Malfeito-Ferreira 2006). Today *Brettanomyces* spoilage usually occurs when wines are fermented or aged in oak barrels (Rayne and Eggers 2008). Aging of the wine is important for this yeast to produce spoilage because of its slow growing nature. The observation of slow growth may be associated with other "wild" yeasts developing early in the fermentation, thereby hindering the detection of *Brettanomyces* (Henick-Kling and others 2000). Modern practices of production, including sanitation, lack of barrels and producing products with low pH, high carbon dioxide and low nitrogen contents, beer, dairy products and the other products mentioned above do not seem to be as affected by *Brettanomyces* as the wine industry.

World Presence and Issue of *Brettanomyces*

In the past Brett was not necessarily considered good or bad, just a characteristic taste. The main factor affecting the sensory properties of this character in *B.bruxellensis* is the occurrence and production of 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG) (Vigentini and others 2008). Small differences in 4-EP concentrations can be very apparent to the consumer with regard to the strength of perceived Brett character. Aroma changes by *B.bruxellensis* can be reliably detected and quantified by trained tasters (Licker and others 1999). At low concentrations, these Brett character compounds can contribute to wine aroma complexity, producing positive sensory effects reminiscent of grand old-world-style red Bordeaux and Burgundy wines (Mahaney P 1998). High concentrations can overwhelm the wine aroma and create unpleasant experiences (Chatonnet P 1990). The presence of Brett character can be considered either negative or positive depending on concentration and expectation of a particular red wine. Today, *B.*

bruxellensis is controversial in that the organism's presence can be considered a positive or negative characteristic for a fermented beverage depending on the person asked and their respective culture. For example in 4-ethylphenol analysis, a New York State group of experts described Brett as 'plastic,' burnt plastic, Band-aid™, cow manure, barnyard and horse sweat, whereas and international group only included burnt plastic and Band-aid™ in their descriptors (Henick-Kling and others 2000).

In general, growth of Brett in wine has a negative connotation in the United States. *B.bruxellensis* has recently been referred to as a 'Global Issue' in a paper exploring the presence of Brett in New York State (Arvik and others 2002). Research on *B.bruxellensis* has been conducted in many parts of the world and the organism has been identified on six of the seven continents including North America, South America, Europe, Africa, Asia and Australia. The current top continents for wine production include Europe, North and South America and Australia, and *Brettanomyces* has been found in wines in a variety of countries on each continent (Conterno and others 2006). A Portuguese study that analyzed wine volatiles from different countries found that more than 25% of red wines had 4-ethylphenol levels higher than the preference threshold of 620 µg/L, above which consumers reject wine (Loureiro and Malfeito-Ferreira 2003; Chatonnet and others 1992; Lesschaeve 2007). These results are not based on random sampling so the percentage may not be accurate, but results indicate that volatile phenols related to *Brettanomyces* are a worldwide concern. Multiple studies have found that *Brettanomyces* is probably the most significant microbiological problem of modern winemaking, causing large economic losses in the wine sector worldwide (Fungelsang 1997; Boulton and others 1996). Another study performed in Burgundy on Pinot noir wine showed that these yeasts are present in about 50% of the wines undergoing maturation and about 25% of bottles (Gerbaux and others 2000). Study of this yeast is not only important for economics of the wine industry, but also may provide a greater understanding of genetics and physiology of stress resistance in microorganisms due to *Brettanomyces* ability to survive in harsh environments (Henschke and others 2007).

Flavor Chemistry

Flavor descriptors that have been associated with *Brettanomyces*-produced flavors include, but are not limited to Band-aid™, plastic, cloves, earthy, barnyard, and horsy. Wines contaminated with *Brettanomyces* often have an undesirable color, which may be due to glycosidic activity or formation of vinylphenolic pyranoanthocyanins (Oelofse and others 2008). The initial detection of these aromas generally takes place during the maturation of wine in barrels (Godoy and others 2008). Brett's character comes from an enzymatic reaction. Hydroxycinnamate decarboxilase converts ferulic and *p*-coumaric acid into 4-vinylguaiacol and 4-vinylphenol, which are respectively reduced to 4-ethylguaiacol (4-EG) and 4-ethylphenol (4-EP) by vinylphenol reductase (Suárez and others 2007). Other yeasts can reduce *p*-coumaric acid to form 4-vinylphenol, but *B. bruxellensis* is unique in the ability to reduce vinylphenols to perceivable amounts of 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG) (Chatonnet and others 1995). 4-EP and 4-EG respectively smell like Band-aids™ and cloves/holiday spices. Cinnamate decarboxylase activity of *B. bruxellensis* is not inhibited by phenolic compounds in grapes, unlike *S. cerevisiae*, so it can produce several milligrams of ethylphenols per liter of wine, which is directly proportional to the population of *Brettanomyces* (Chatonnet and others 1995). Therefore, high polyphenol content makes a wine more susceptible to the enzymatic reactions. Studies have shown poor correlation between amount of 4-EP and amount of yeast colony forming units at any stage in the growth cycle, suggesting that the metabolite may be released upon yeast cell death and autolysis (Fugelsang and Zoecklein 2003). Another possible character compound under study is 4-ethylcatechol (4-EC), which is noted for a medicinal aroma and has the precursor caffeic acid. Due to the requirement to derivatize to be detected by gas chromatography (otherwise 4-EC is not detected in the analysis of other volatile phenols), (Loureiro and Malfeito-Ferreira 2006) 4-EC has not been studied to the extent of the other volatile phenols in wine, but appears to be important due to its lower detection threshold than other ethyl phenols and its medicinal aroma.

Flavor Character

Brett is most noted by the volatile compounds of the bi-products 4-EP and 4-EG. The average values where 4-EP and 4-EG are considered negative are 620 µg/L and 140 µg/L respectively and when they are present together, 4-EP is considered negative at 426 µg/L (Chatonnet and others 1992). 4-EP and 4-EG appear in wines at various amounts and ratios depending on the variety of grape used and wine style. On average they appear at a 10:1 ratio respectively, which corresponds to the respective precursor ratio of *p*-coumaric acid and ferulic acid (Chatonnet and others 1992; Romano and others 2008b). Another study showed average ratios of 4-EP to 4-EG differed with different wine types and was approximately 10:1 for Cabernet Sauvignon, 9:1 for Shiraz, 8:1 for Merlot and 3.5:1 for Pinot Noir. Little work has been done on determining thresholds of these compounds in different varieties, but some indicate thresholds to be higher in Cabernet Sauvignon wines and lower in Tempranillo wines (Suárez and others 2007). Studies have also shown no difference in ethylphenol concentrations in Cabernet Franc, Cabernet Sauvignon, Merlot, Pinot Noir, and Syrah wines (Rayne and Eggers 2008).

The most important traits that influence Brett character are microbial strain, wine pH, sugar content and stage at which contamination occurs (Romano and others 2008). Further studies are currently being conducted on environmental factors for ethyl phenol production. Nitrogen sources enhance production more than residual sugars (Conterno and others 2007). Due to strain differentiation, generalization is difficult, but *p*-coumaric acid appears to have a greater induction effect for cell activity for this yeast, while ferulic acid shows higher utilization (Harris and others 2009). Recent research indicates the primary importance of strain diversity in the development of "Brett" character.

Genetic Diversity and Flavor Character. The spoilage capacity for this yeast is strain dependent (Vigentini and others 2008), and recent studies have emphasized the genetic diversity of *Brettanomyces*. In fact, production of volatile phenols is strain dependent, so this yeast may not alter wine flavor profiles due to volatile phenol production, although using ethyl phenol production as a tool can increase the understanding of this problem in the wine industry (Vigentini and others 2008). Recent studies have shown that *B. bruxellensis* strains have wide variability in their ability to grow in Pinot Noir

wines (Fugelsang and Zoecklein 2003), and individual strains vary in their impact on phenolic profiles (Silva and others 2005). The same and similar genotypes of *B. bruxellensis* have been found worldwide, showing that the yeast may be spread due to wine or winery equipment transport or adapts similarly to the wine making environment (Curtin and others 2007).

Brettanomyces in Contemporary Winemaking

Brettanomyces is often found in fermented beverages including wine because it can take advantage of the cluster effect, which is the ability to complete alcoholic fermentation in the presence of small amounts of oxygen (Arvik and Henick-Kling 2002). *B.bruxellensis* can be present at any stage in wine making, but it is often related to wine aging in oak barrels, stuck or sluggish alcoholic fermentations or malolactic fermentation (Renouf and others 2006), due to its high ethanol and low sugar tolerances (Dias and others 2003b). This yeast can grow at as low as 275 mg/L sugar, and residual sugar in a dry red wine can range from 420 to 1000 mg/L (Rayne and Eggers 2008). In fact, *B. bruxellensis* seems to better adapt than *Saccharomyces cerevisiae* to wine with higher levels of ethanol (Silva and others 2004). Studies have indicated that in red wine the ethanol tolerance for Brett averages 14-14.5% (Loureiro and Malfeito-Ferreira 2006). *Brettanomyces* can also have an inhibitory effect on *Saccharomyces* due to the synthesis of acetic acid (Uscanga and others 2003).

Red wines are particularly susceptible for *B. bruxenellis* infection due to their lower acidity, higher polyphenol content and barrel aging. Therefore *Vitis vinifera* red varietals with higher precursor polyphenol content are more susceptible to the Brett defect. For example, Pinot Noir generally has lower levels of *p*-coumaric giving *B. bruxenellis* less opportunity to produce 4-ethylphenol. *B. bruxellensis's* slow growing nature makes wines that are stored for long periods in wooden barrels particularly susceptible (Suárez and others 2007). Wines that are aged longer in barrels are not only more susceptible to the defect if *B. bruxenellis* is in the barrel, but also are generally considered higher quality wine when put in the barrel, as the best quality red wines undergo longer barrel aging. *B. bruxenellis* has been found at a penetration depth of 8 mm within the wood of barrels (Malfeito-Ferreira 2005). Once *B. bruxellensis* has infected a barrel, the organism cannot be removed by cleaning, shaving or other techniques, although

precautions can be taken to limit growth. Barrel age and type differences have been studied to see if certain barrels were more susceptible to Brett's growth, but no significant differences were found (Garde-Cerdán and others 2008). Malolactic fermentation is another vulnerable period for contamination due to low free sulfur dioxide and residual sugar concentration (Oelofse and others 2008). Brett does not discriminate against barrel type or red *Vitis vinifera* wine type, but contamination is more likely in certain environments.

Controlling Contamination and Flavor Character

Due to the potential to develop undesirable flavors, winemakers try to control the presence of *B.bruxellensis* in wine. Of wild yeast and bacteria, *B.bruxellensis* is often the predominant spoilage organism in the bottle, and therefore attempting to remove or control the yeast before bottling is important (Renouf and others 2007).

There are precautionary steps, from bringing grapes in to bottling, which can help discourage the presence of *B.bruxellensis* in wines. The two main places where *B.bruxellensis* is found are on grape skins, especially damaged grapes, and on winery equipment. Therefore, practicing general sanitation is the first step in prevention (Henick-Kling and others 2000). Cleaning with the antimicrobial dimethyl dicarbonate, commercially known as Velcorin™, can inactivate *B.bruxellensis* that has been introduced with grapes, as well as stopping the spread of the organism in a winery among the equipment and floors. Adequate amounts of sulfur dioxide should be added to fermentation vessels and maintained during fermentation to help inhibit growth of the organism. *Brettanomyces* can enter a "viable-but-not-culturable" state after sulfur dioxide addition, so the yeast may still be present in the wine. These strains are noted to be physically much smaller in size after the addition (Umiker and Edwards 2007). Under 30 mg/L of free sulfur dioxide does stop growth of *B.bruxellensis,* so it is recommended that concentrations above this threshold be achieved throughout the process (Barata and others 2008). New barrels can absorb up to 15 mg/L of free sulfur dioxide over a six month period (Coulter and others 2003). Using 0.5 to 0.8 mg/L molecular sulfur dioxide has been recommended for control (Henick-Kling and others 2000).

Factors to consider while fermenting a wine are use of a starter culture, alcohol concentration, acid concentration, temperature and oxygen exposure. Using a starter culture can cut back on wild fermentation, decreasing the ability for *Brettanomyces* to take over. With regard to alcohol concentration, if the degree of alcohol in wine is increased from 12.5% to 13.5%, accumulation of ethylphenols in wine is inhibited (Garde-Cerdán and others 2008), although Brett has been seen up to 14.5% in Sherry wines (Loureiro and Malfeito-Ferreira 2006). Though fermenting wines to a higher alcohol content may often help inhibit Brett character, ethanol tolerance is also a strain-dependent character (Vigentini and others 2008). Either using grapes high in acid content or adding some weak acids (sorbic, benzoic and fumaric) during the wine making process can help to reduce pH and makes a less inviting environment for Brett. Other research has shown that pH values are not correlated with Brett spoilage, so this recommendation may differ between experts (Oelofse and others 2009). Fermentation vessels and activities associated with these can certainly influence growth of *B.bruxellensis*, but there are several great opportunities to limit the organism's growth within fermentation vessels. Increased maceration temperatures can enhance the extraction of ethylphenols (Gerbaux and others 2002). Oxygen has a positive effect on the growth of *B.bruxellensis*, so wine makers may be discouraged from using the micro-oxygenation technique in wines that are more susceptible to Brett. Topping off barrels to limit the amount of oxygen present at this stage is extremely important in limiting the growth of *Brettanomyces.* 4-EP and 4-EG concentration positively correlate with dissolved oxygen levels and negatively correlate with cellar humidity (Rayne and Eggers 2008). Negative correlations exist between contamination and humidity may be due to the lack of evaporation of wine in barrels, which creates less surface area for oxygen. Reduction in contamination levels and prevention of growth during fermentation are essential because there is no complete removal of bi-products, just reduction in amount

After fermentation and aging, studies have also shown that using proteins to fine wines before introducing them to barrels can greatly reduce *B.bruxellensis* populations (Suárez and others 2007). Filtering has reduced the yeast and bacteria populations by a factor of 10^3, but yeast may pass through a 0.45 µm filtration membrane in a viable, but not culturable, VNC, state (Millet and Lonvaud-Funel 2000).

Filtering and fining can be controversial in that they can remove other flavors, including a wine's specific character, but they can help physically eliminate the organism (Arvik and Henick-Kling 2002). Other effective measures include reverse osmosis and adsorption, using absorbent resin and a membrane with tangential-flow filtration to reduce ethylphenols and ozone sanitation (Oelofse and others 2008). A less common technique, due to money, time and technology, includes using applying pressures of 400-500 MPa for 5-15 minutes at 5-20°C (Suárez and others 2007).

Reducing *B. bruxellensis* or Brett character is most effectively done with simple cellar practices including proper cleaning and hygiene, using appropriate amounts of sulfur dioxide, topping off barrels and general oxygen reduction in the wine/grape must and most importantly not using contaminated barrels. Recent studies have shown that some strains are "fast and early producers" while others are "slow and late producers" making early detection and identification useful as an indicator to use corrective measures earlier in the process (Agnolucci and others 2009). None of these strategies will eliminate this yeast, but they can reduce its effect. Figure 2.2 shows many of the important strategies in controlling *B. bruxellensis* contamination.

Summary of Key Prevention Strategies

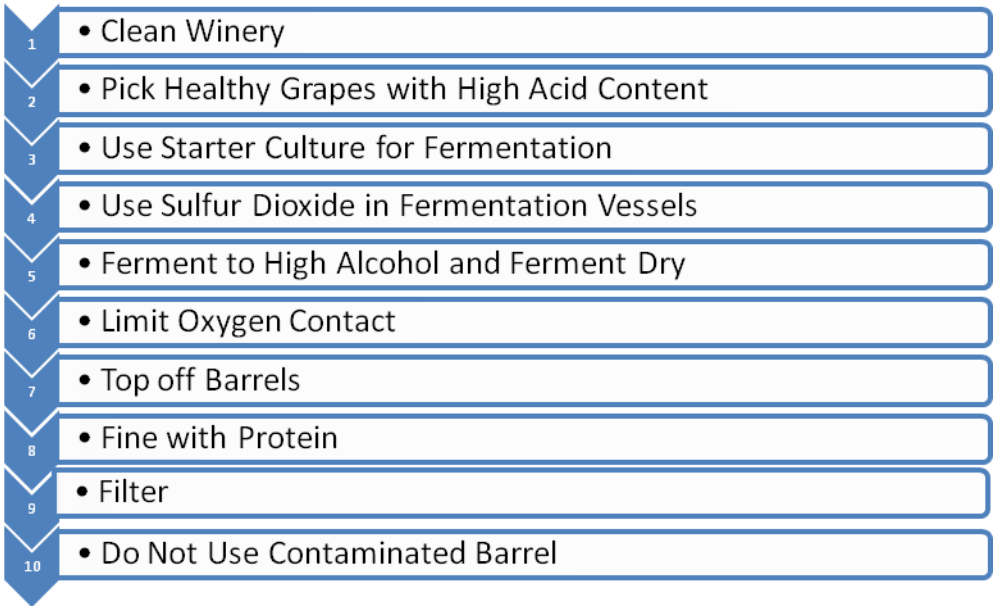

Figure-2.2 Flow Chart of Key Prevention Strategies of *Brettanomyces* or *Brettanomyces* Character Contamination

DETECTION

There are two main ways to detect the presence of *B.bruxellensis*, the first is observing the physical presence of the organism and the second is determining if the organism's metabolites, 4-EP and 4-EG, are present. To determine the presence of the organism, culturing, plating, and polymerase chain reaction (PCR) have generally been utilized. Recently, a fluorescence *in situ* hybridization method using peptide nucleic acid probes (PNA), an artificially synthesized polymer similar to DNA and RNA, can be used. For metabolite detection, a trained sensory panel and/or chemical analysis including gas chromatography coupled with mass spectrometry or olfactory, and the electronic nose can be used.

Neither morphological features, nor other classical methods, are adequate for *Brettanomyces* identification and therefore other techniques must be used as well, for example fluorescence microscopy, PCR, gas chromatography mass spectrometry (GCMS) or sensory analysis with trained tasters (Suárez and others 2007). A combination of microbial and volatile testing, through culturing, GCMS and sensory

descriptive analysis, were the methods used to determine *B. bruxellensis* presence in North Georgia wines.

Microbial Analysis

Brettanomyces bruxellensis can be present at any stage during wine making, but can remain in a viable but not culturable (VNC) state in wine for long periods of time, meaning cells that cannot be observed by standard culture methods continue to metabolize nutrients from their surroundings and are still considered to be alive (Arvik and Henick-Kling 2002). Due to the VNC state and slow growth, in dry wines, *B. bruxellensis* spoilage can take place over several months (Romano and others 2008). The viable population can be 10 times the culturable population (Millet and Lonvaud-Funel 2000). *B.bruxellensis* therefore can be triggered at any time to produce metabolites, but due to their ability to be more resistant to increased alcohol conditions than *Sacchromyces cerevisiae*, wines are particularly susceptible in barrel aging after primary fermentation is complete (Renouf and others 2006).

Culturing. When culturing *B. bruxellensis*, cycloheximide is often used due to its antibiotic effect and inhibition of other wild yeasts (Couto and others 2005). Cycloheximide inhibits many wild yeasts and *Saccharomyces cerevisiae* (Couto and others 2005). *P*-coumaric acid is often used because it is a precursor for 4-ethylphenol production by *B. bruxellensis* (Couto and others 2005). When *p*-coumaric acid is used in culturing, trained people can identify if the 4-EP odor is present to determine if the sample is positive. Sugar can be added because *B. bruxellensis* can still grow, but can help decrease production of acetic acid, which may interfere with the olfactory detection of the 4-ethylphenol odor, making it easier to identify the 4-ethylphenol off odor in the cultures (Couto and others 2005). Sterile filtration is used due to *p*-coumaric acid decomposing with heat (Sigma-Aldrich 2007). Ethanol is used because of *B. bruxellensis* has a higher tolerance to ethanol than *S. cerevisiae* with lower sugar concentrations (Renouf and others 2006; Silva and others 2004). Bromocresol green is used as evidence for acid producing strains (Rodrigues and others 2001). Due to the low growth rate, incubation times of two weeks are common (Rodrigues and others 2001).

Due to the VNC state, culturing and growing this yeast on a differential medium must be supported with other testing for identification. One important result that culturing and growing can provide is the opportunity to quantify the level of contamination in a wine. Although accuracy of this method is questionable due to the organism's slow growth and VNC state, the method is still widely used, generally in conjunction with physiological, chemical or sensory analysis.

PCR analysis is used to identify DNA of a microorganism. Over the years PCR has proved to be reliable, sensitive, and rapid for detecting various microorganisms in wine, however it does not provide any information about the level of contamination (Delaherche and others 2004). Although Real Time (RT) PCR is rapid, quantitative and accurate, there is still concern about PCR inhibitors for example polyphenols or tannins causing difficulty in detecting the organism when present in small amounts (Loureiro and Malfeito-Ferreira 2006). This method of *B.bruxellensis* detection is widely used in literature for detecting presence or absence of the organism in wine. Nonviable cells will contain intact DNA that produces a positive PCR result.

The fluorescence *in situ* hybridization method uses peptide nucleic acid probes that target species-specific sequences of the rRNA. This method combines the benefits of peptide nucleic acid PNA with situ hybridization (Stender and others 2001). PNA utilizes an artificially synthesized polymer similar to RNA and DNA, and has a relatively hydrophobic character resulting in the ability to penetrate hydrophobic cell walls without disrupting cell morphology in a rapid, sensitive and specific way (Stender and others 2001). A drawback to this method, which some scientist also have with RT PCR, is that quantification has not been thoroughly worked out, making it difficult to accurately determine the level of contamination in a particular wine. This method does require a florescent microscope and appears to have a high specificity and sensitivity. Neither this method, *in situ* hybridization, nor PNA alone appear to be widely used in the literature.

Amplified fragment length polymorphism (AFLP) analysis has become the fingerprinting method of choice in Australia for microbial genotyping due to its high sensitivity and reproducibility (Henschke

and others 2007). This method is used to identify minor genetic differences when researching genetic diversity, and allows easy genetic subtyping.

RT PCR, culturing and plating appear to be the most widely used techniques in identifying and quantifying *B. bruxellensis*. Research and testing are currently being conducted with rapid tests using an EIA reagent kit, an immunoassay test that detects Brettanomyces. Antibodies are used as enzymatic reagents for *B. bruxellensis* detection. The 'Z-brett 24' is supposed to provide results in three hours and became available in the fall of 2008.

Metabolite (Ethylphenol) Testing

The main drawback to chemical and sensory analysis testing is that it only determines if Brett has already affected the wine, too late to take preventative actions. For the past two decades, *B. bruxellensis* has been identified by looking for the presence of metabolic compounds, 4-EP and 4-EG. Most recent literature has began to question using the production of 4-EP and 4-EG as a method to determine the presence of Brett in wines, but has not come up with an alternative solution that provides the desired information. In a genetic and physiological study of *B. bruxellensis,* approximately 80% of strains produced 4-EP and 4-EG, and 50% produced high levels of these compounds, showing that testing for the actual presence of this organism by identifying these compounds may not always be accurate (Conterno and others 2006). Testing for 4-EP, 4-EG and other metabolites mainly concerns the presence of the flavor defect Brett produces along with presence of the organism. Until an alternative method is proposed to test the specific flavor defect of Brett, these tests will be used to identify products affected by Brett and intensity of Brett character.

Chemical Analysis

Over the past two decades, extensive research has been conducted to understand the volatile compounds typical of *B. bruxellensis* in wine. The two main metabolites include 4-ethylphenol and 4-ethylguaiacol; others include but are not limited to 4-ethylcatechol, isovaleric acid, guaiacol, ethyl decanoate, *trans*-2-nonenal, isoamyl alcohol, isobutryic acid, 2-phenol ethanol, ethyl-2-methtylbuterate, 1-butanol, and propionic acid. *B. bruxellensis* yeasts can be identified by gas chromatography (GC) by

removing fatty acids from their cell membranes and then derivatising as methyl esters. This method, although accurate, can be time consuming. Where GC is used, headspace trapping systems are often preferred due to preparation time in extraction of samples. Static headspace through purge and trap or a Solid Phase Microextraction (SPME) are predominantly used. GC is often combined with mass spectrometry (GCMS) or olfactory (GCO) methods in identification. Head space mass spectrometry electronic nose (MS e_nose) has also been used, but is not yet common or fully developed.

The static headspace, purge and trap method, first purges where an inert gas is bubbled through a liquid, then the volatiles are moved into a headspace where the volatiles are trapped on an adsorbent material. This method is directly used with thermal desorption and cryogenic trapping for the ability to detect a wide array of compounds. During collection, sampling time should be increased over 30 minutes, because 4-EP and 4-EG are less volatile and more polar components relative to other components in wine and there is a large increase in sensitivity to these compounds after 30 minutes (Whiton and Zoecklein 2000). SPME combines extraction, concentration, and chromatic injection into one step to create a low cost and highly sensitive technique to combine with GC or GCMS. SPME extracts volatiles form liquids or solids by immersion of a fused silica fiber coated with sorbent material in the sample, followed by desorption (Whiton and Zoecklein 2000). Both trapping methods decrease labor intensity and provide accurate analysis.

Gas chromatography mass spectrometry is generally used to selectively separate and characterize compounds based on their spectral and chromatographic properties (Limpawattana 2007). Gas chromatography olfactory takes the gas outlet stream to a nose cone where a human identifies the compound. The GC-O technique is often useful to obtain other insights into the sample, including dilution analysis, detection frequency, posterior intensity and time-intensity methods (van Ruth 2001), but has drawbacks due to using humans as a detector. Humans have different sensitivities, anosmias, become fatigued easily, and can have poor reproducibility making repeatability difficult. GCMS is the dominant technique used for detection of *B. bruxellensis* metabolites.

The MS electronic nose is an instrument that combines with chemometrics as a rapid and low cost technique that only a few studies have used to examine or characterize the aroma of wine (Cynkar 2006). The MS electronic nose essentially tries to understand chemical information by mimicking human senses using sensory arrays and pattern recognition to detect odors and flavors. Although this new method is much less time consuming due to simple analysis preparation and reduced time of analysis than a sensory panel or by headspace GCMS, false negatives and false positives may appear ,and only three levels of contamination can be detected: high, medium and low. The MS electronic nose method has potential to be an extremely useful method in the future after the method has been further developed for better accuracy and precision.

Sensory Analysis

Sensory evaluation is a scientific discipline used to evoke, measure, analyze and interpret reactions to stimuli perceived through senses (ASTM International 2005). The Institute of Food Technologists defines sensory evaluation as "a scientific discipline used to evoke, measure, analyze, and interpret reactions to those characteristics of foods and materials as they are perceived by the senses of sight, smell, taste, touch and hearing" (Dethmers 1981). Sensory evaluation is often applied in characterization and evaluation of foods and beverages with the primary function being to conduct valid and reliable tests that provide data for advisable decisions, for examples in regard to product reformulation, shelf life studies, or in product launches (Meilgaard and others 1999). Products may be evaluated with any of the five senses, but for wine purposes aroma and taste are most important. There are two physiological interactions with odor, via the orthonasal and the retronasal pathways. Orthonasal is the smell in the headspace of the nose. Retronasal is the smell in the headspace of the mouth. "Taste is based on a limited number of chemical compounds which bind to single receptors, but with aroma, there are estimated 10,000 odors and over 900 genes encode the structure of olfactory receptors" (Yeomans 2006). Identifying *B. bruxenellis* contaminated wines by identifying 4-EP and 4-EG is strongly based around orthonasal odor in identifying intensity of Bretty descriptors, but combining flavor is important in taking retronasal smells and taste into account. In sensory analysis, wines are smelled before tasted

because the cognitive interaction between taste and aroma cannot be removed, even with training (Noble 1996).

Generally, odor identification is broken down into two primary dimensions, measuring valence and intensity (Anderson and others 2003). Valence is generally measured qualitatively, where intensity is measured quantitatively. Sensory testing is generally broken down into analytical and affective testing. Analytical tests identify discriminative and descriptive information, where affective testing evaluates acceptance and preference. Analytical tests can be divided into difference testing, threshold testing and descriptive profiling. Difference testing includes Paired-comparison, Duo-Trio, Triangle, Difference from control, Same/Difference test, A-not A, 3- Alternative Forced Choice, and Two-out-of-Five tests, which all measure for significance in similarity or differences of products. Threshold testing measures the concentration range of odor or taste of a substance that is being detected in a practical circumstance (ASTM International 2004), and is generally done by Forced-Choice Ascending Concentration Series Method of Limits. Affective testing is generally used for consumer tests in order to make economic decisions in market sales using a variety of tests, for example, Central Location Test, Home Use Tests and Acceptance tests. Descriptive sensory tests use trained panelists to characterize the qualitative components, sensory attributes, and then measure their intensities. Different methods measuring odor and taste include Flavor Profiling, Quantitative Descriptive Analysis (QDA), and Spectrum™ (Hootman 1992). Products can be judged on appearance, aroma, flavor, texture, or sound characteristics.

Descriptive Sensory Analysis. In descriptive analysis, language can play an important positive role in influencing perceptions for both greater accuracy and objectivity, but descriptive analysis can also be negative by possibly biasing panelists. For example, when perceiving attributes that are not present, providing a list of attributes helps to enhance later recognition that is correct (Lawless 1986). A lexicon is created in order to have one language among the panelists giving words with commonly shared definitions, therefore these words should avoid circularity, not be used in context, have simple phrasing, brief definitions and lack ambiguity (Giboreau and others 2007). Important aspects researchers should consider when creating a lexicon, set of words to adequately describe sensory attributes, are that terms

should be (1) orthoganol, (2) based on underlying structure if it is known, (3) based on a broad reference set, and (4) precisely defined and "primary" rather than "integrated" (Lawless 1986). Reference standards that are reproducible, identify only one term, and can be diluted without changing character are ideal for panelists to discuss and agree on (Rainey 1986).

The Flavor Profile method uses four to six carefully selected panelists who are trained with reference samples and seven-point flavor profile intensities. This method is useful when many different products are being evaluated, but often has lack of consistency and reproducibility due to such few panelists and strong influences from the panel leader. A seven-point system also limits degree of discrimination. Quantitative Descriptive Analysis (QDA®) is friendlier to statistical analysis and also uses standards with evaluation and ratings of intensities of a product which are based off of a 15 cm line scale. The panel leader acts more as a facilitator, and panelists evaluate products alone as to not be influenced by each other after training. Language generation is not generated by evaluators, which limits information gathered. The Spectrum™ Analysis creates a lexicon, set of standards and trains to evaluate these standards on a scale of 0-15. Terminology is created by the evaluators and is based on the evaluator's objectives. The Spectrum™ method gives well chosen reference points in order to reduce variability, yielding a more technical profile (Meilgaard and others 1999). The Spectrum™ technique is also statistically friendly and can be more customized, accurate and precise in judging attributes in a product.

Sensory Analysis for *B. bruxenellis* Metabolites. For sensory profiling of red *Vitis vinifera* wines for Brett character, the Spectrum ™ Method is most applicable due to the ability to customize descriptors. Lexicon development then can be determined by a panel using a wine contaminated with *B. bruxenellis* and attributes agreed on by the panel. The panel leader then can create references to define terms and intensities (Meilgaard and others 1999). *B. bruxenellis* is classified for its production of 4-ethylphenol and 4-ethylguaiacol, but can also produce medium and short chain fatty acids including dedecanoic, isobutryic, isovaleric, and 2-methylbutryic acids, 2-phenolethanol, isoamyl alcohol, *cis*-2-nonenal, *trans*-2-nonenal, B- damascenone, and ethyl decanoate (Fugelsang and Zoecklein 2003). Some

studies believe volatile phenols, isovaleric acid, acetic acid, carboxylic acids, short chain fatty acids and ethyl-decanoate should be further studied for sensory applications (Romano and others 2009). Levels of precursor compounds have been positively correlated with levels of isovaleric acid (Hernández-Orte and others 2008). Recent studies have shown isobutryic and isovaleric acid may be more important than originally thought in defining wines effected by *B. bruxenellis*, and these compounds may produce a masking effect on ethylphenol detection thresholds (Romano and others 2008). These compounds give these perceived flavors of sweaty, dirty socks and cheesy. Descriptive analysis is especially important due to the ability to take many of the perceived Brett characters into account.

Threshold Testing . The perception of a food or beverage flavor embodies a complex mixture of compounds. Many of these compounds are volatile and their number is so great that most go undetected. Wine has many characteristics that contribute to a wide variety of sensory perceptions. In a flavor profile, some of these include organic acids, sugars, alcohols, phenolics, glycerol, esters, aldehyes, ketones, terpenes and other volatile compounds (Rapp and Mandery 1986). Small changes in the compounds present may create large changes in the flavor profile detected by a human. Compounds and substances may naturally occur or be added to food, beverages, and with many other products. In order to detect if change has occurred in a flavor profile, threshold testing can be used to determine when the difference begins to change the products acceptability or perceived flavor profile. Methodology used for threshold testing is important because using different size bottles or training panelists can change results from 10 to 1000 fold (Meilgaard and others 1999). Threshold testing is crucial in wines due to its relation to quality. Quality will only explain interaction effects between compounds where those compounds can be perceived (Ryan and others 2008).

There are four main types of thresholds, the absolute, recognition, difference and terminal. They have been defined by work (Meilgaard and others 1999), as the following:

- Absolute detection threshold- the lowest stimulus capable of producing a sensation.
- Recognition threshold- the level of a stimulus at which the specific stimulus can be recognized and identified. (This level is generally higher than the absolute threshold.)

- Difference threshold- the extent of change in the stimulus necessary to produce a noticeable difference.
- Terminal threshold- that magnitude of stimulus above which there is no increase in perceived intensity.

The absolute or detection threshold is often used to determine when an added or natural substance is able to change the aroma or flavor of a product. Some of the variety of methods that determine thresholds based on psychophysical design are the method of limits, the method of average error, and the frequency method, but the method of limits is generally used and recommended (ASTM International 2004) due to its rapidity, practical value, and minimum tasting/smelling effort that allows for a larger panel and more reliable results (Meilgaard and others 1999). Other methods may be more accurate if executed correctly, but the method of limits requires five or more times as many tastings and would have reduced coverage of compounds or individuals by as much (Meilgaard 1993).

A common way to determine thresholds is using the Ascending Forced Choice Method of Limits, which allows for quick and reliable results of group thresholds in a wide range of stimulus concentrations (ASTM International 2004). This method measures group thresholds, which are important to use because of the variability of each individual in a panel member who are from different cultures, may have different thresholds of perception, and product experience leading to different discrimination abilities (McEwan and others 2002). In order to deal with this issue, individual differences in sensory threshold for a given compound can be described by the log standard deviation from the geometric mean of the panel using the ASTM Method E679, the Ascending Method of Limits (Meilgaard 1993).

Threshold Testing in Wine. Reliable reporting of a single value for thresholds of flavor compounds in wine can be misleading. Reports recommend providing a range of thresholds that reflect influences of wine style, evaluation mode, and are relevant within-subject variables (Yu and Pickering 2008). Perceptual interactions occur due to complex chemical compositions that can dramatically impact the final product flavor, and these interactions remain difficult to predict in wine even when using synthetic solutions (Atanasova and others 2005). Creation of a wine matrix can be particularly difficult

to create due to the interactions affected by alcohol (Ryan and others 2008), which is variable wine to wine. Although great diversity exists in volatile phenol production, *Brettanomyces* has different tendencies in synthetic media and in a wine matrix, making testing more complex. Therefore, future studies need to incorporate more wine matrices to better understand this yeast (Oelofse and others 2009). For example, the sorption capacity of 4-ethylphenol is significantly higher from alcoholic fermentation than YPD culture (Pradelles and others 2008). Strong increases in odor threshold are shown when ethanol is present, exhibiting inhibition of odor activity of wine volatiles by ethanol (Grosch 2001). Perceptual interactions also may lead to dominance of one perceived quality of one component, which is not proportionally related to higher intensity to the component alone (Atanasova and others 2005). Odors with low Odor Activity Values (OAVs) can skew the perception of other odors with higher OAVs, which is the ratio of a concentration of an odor to the odors threshold (Ryan and others 2008). Threshold testing is conducted to evaluate product quality for Brett metabolites and therefore is important for understanding the organism's effect on flavor profile.

Threshold for 4-ethylphenol and 4-ethylguaiacol. The sensory perception thresholds for 4-EP and 4-EG alone in red wine are 605 µg/L and 110 µg/L respectively (Chatonnet and others 1992). When both compounds are present in red wine, the sensory perception threshold of 4-EP is lower, 369 µg/L (Chatonnet and others 1992). The sensory perception of these compounds may be affected by other compounds. For example, in a light-bodied red wine with little oak influence, the sensory perception threshold of 4-EP may be ~350 µg/L as opposed to a 1000 µg/L threshold in a full-bodied red and more oak influence (Coulter and others 2003).

Recent studies have shown that *B. bruxellensis* is not the only organism that is able to produce 4-ethylphenol and 4-ethylguaiacol, and that not all strains produce these compounds. One study stated 20% of stains did not produce these compounds (Conterno and others 2006). Winemaking strains of *S. cerevisiae* differ in their ability to decarboxylate hydroxycinnamic acids to vinylphenols, therefore the idea that *Brettanomyces/Dekkera* yeasts differ in this characteristic as well seems plausible (Curtin and others 2007; Shinohara and others 2000). A few organisms including *Lactobacillus brevis* and

Pediococcus pentosaceus can decarboxylate *p*-coumaric acid to form 4-vinylphenol, but few, including *Brettanomyces* and *Lactobacillus plantarum*, are capable of producing ethylphenols (Chatonnet and others 1995). Lactic acid bacteria were tested for the ability to produce volatile phenols and 37% of the strains tested could produce volatile phenols from *p*-coumaric acid and 9% could produce 4-ethylphenol (Couto and others 2006). What differentiates *Brettanomyces* is the high production of ethylphenols whereas *L. plantarum* can only produce in very low concentrations. For example, *Pichia gulliermondii* is capable of converting *p*-coumaric acid to 4-ethylphenol from grape juice, grapes and winery equipment (Dias and others 2003a), but has shown low to no conversion rates in wine, making *Brettanomyces/Dekkera* yeasts the sole agent for the ethylphenol off flavor (Barata and others 2006; Vigentini and others 2008).

Brettanomyces typical flavor character has also been recently challenged by studies mostly regarding ethylphenol production in different strains along with the typical bi-products. Studies have shown that experts looking for contaminated wines are often not accurate in their findings, which could be due to other chemical and flavor interactions (Conterno and others 2006; Romano and others 2009). These studies question the existence of a "typical" Brett flavor. For example, isobutyric acid and isovaleric acid have masking effects on the detection threshold of ethylphenols also affecting the perception of 4-EP and 4-EG (Romano and others 2009).

Due to the ability of other organisms to produce ethylphenols and *Brettanomcyes* to produce acetic acid, it is important to understand perceived flavor when acetic acid and ethylphenols are both present. *Brettanomyces* spp. are considered important producers of acetic acid, and under oxidative conditions, a high acetic acid level indicates possible contamination of this yeast (Benito and others 2009). Growth of this yeast is not significantly affected by 4-EP production (Weissberg and others 2008), providing reason to concentrate on other important metabolites. As mentioned, *Brettanomyces* spp. have also been shown to produce substantial amounts of acetic acid, and this accumulation can begin at the start of fermentation using a simple substrate like sucrose (Uscanga and others 2006). In the presence of oxygen *Brettanomyce* produces greater amounts of acetic acid and the lower amounts of ethanol (Uscanga

and others 2003). Oxygen supply can continue to create and/or stop production because increased acetic acid can inhibit glucose metabolism by this yeast (Uscanga and others 2003). Certain strains of *Brettanomyces* can produce 25 g acetic acid/l from 100 g glucose/l, which is significant enough to look at this yeast as a method of optimizing acetic acid production (Freer and others 2003). For these reasons it is important to understand acetic acid and ethylphenol interactions when defining Brett character.

IMPORTANCE OF THIS STUDY

The presence of *B. bruxellensis*is has not been previously reported in wines and wineries of the southeastern United States. "Despite the extensive work conducted on these compounds in wine, relatively few studies have documented their concentrations in commercial vintages from some of the major wine making regions, including Australia and France" (Rayne and Eggers 2008). *B. bruxellensis*is has affected wines worldwide. The presence of this organism and flavor characteristics will help define the issue of which the wine industry of Georgia must confront.

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CHAPTER 3

BRETTANOMYCES-INDUCED OFF-FLAVORS IN RED *VITIS VINIFERA* WINES OF GEORGIA[1]

[1] *Brettanomyces*- induced off-flavors in red *Vitis vinifera* wines of Georgia, Wedral D, Shewfelt R, Kays S, Frank J. To be submitted to *American Journal of Enology and Viticulture*.

ABSTRACT

Brettanomyces bruxellensis is a particularly troublesome spoilage yeast in wine due to its association with off flavors described as 'burnt plastic,' 'barnyard,' and 'Band-aid®' produced by conversion of phenolic compounds into 4-ethylphenol and 4-ethylguaiacol. The objective of this research was to assess the occurrence of *B. bruxellensis* induced off-flavors in red V*itis vinifera* wines produced in North Georgia. The eighteen samples of wine were tested by sensory descriptive analysis with confirmation by microbial (selective enrichment medium and plating for determining the presence of the yeast) and chemical analysis (tenax headspace trapping, short path thermal desorption, and gas chromatography mass spectrometry for presence of the metabolites). Off-odors associated with *B. bruxellensis* were detected by sensory analysis in seven of the eighteen bottle samples. *Brettanomyces*-like yeasts were isolated from four of the positive samples. The Band-aid® descriptor exhibited the largest standard deviation in the tested wines and was the strongest indicator in sensory analysis in determining if the organism was present. GC-MS analysis verified the presence of *B. bruxellensis* metabolites in samples with *Brettanomyces*-associated off odors. Wines with stronger off character did not necessarily have different intensities of other and more positively associated characteristics. The presence of *B. bruxellensis*is has not been previously reported in wines and wineries of the southeastern United States.

INRODUCTION

Brettanomyces spp. are the non-spore forming counterparts of the genus *Dekkera* (Goode 2006). *Brettanomyces* and *Dekkera* are often used interchangeably. Due to *Brettanomyces* being the more common name in literature for *Brettanomyces/Dekkera* spp., it will be used in this paper. *Brettanomyces bruxellensis* has been isolated from the surfaces of grapes as well as in barrels. *B. bruxellensis* is commonly known as Brett and is known for the metabolite-associated flavors produced by the yeast.

Flavors that have been associated with Brett include Band-aid ®, spices, cloves, earthy, barnyard, and horsey. Brett's character comes from enzymatic reactions. High polyphenol content in grapes and juice make a wine more susceptible to the enzymatic reactions of hydroxycinnamate decarboxilase converting ferulic and *p*-coumaric acid into 4-vinylguaiacol and 4-vinylphenol, which are respectively reduced to 4-ethylguaiacol (4-EG) and 4-ethylphenol (4-EP) by vinylphenol reductase (Suárez and others 2007). Other yeasts can reduce p-coumaric acid to form 4-vinylphenols, but *B. bruxellensis* is unique in the ability to reduce vinylphenols to higher amounts of 4-ethylphenol and 4-ethylguaiacol (Chatonnet and others 1995).

At low concentrations, these Brett character compounds can contribute to complexity of a wine aroma, producing positive sensory effects reminiscent of grand old-world-style red Bordeaux and Burgundy wines (Mahaney P 1998). High concentrations can overwhelm the wine aroma and create an unpleasant experience (Chatonnet P 1990). These findings show how Brett can be considered both negative and positive depending on concentration and expectation of a particular red wine. The objective of this study is to assess the presence of the yeast *B. bruxellensis* and *B.bruxellensis* character in red *Vitis vinifera* North Georgia wines.

MATERIALS and METHODS

Microbiological Detection

For determining yeast presence and identification, wine was cultured in two different media and then grown on selective plates. The first medium used was Yeast Nitrogen Based Medium (YNB)

containing 6.7 g Yeast Nitrogen Base extract (Difco™, Becton Dickinson and Co., Sparks, MD, U.S.A.), 100 mg coumaric acid,10 mg cycloheximide and 5 g glucose per L of distilled water) (Dias and others 2003). This medium was sterilized by membrane filtration. The second medium was Glucose Yeast extract Peptone (GYP) medium containing 20 g glucose, 5 g yeast extract, 10 g peptone, 2 g agar per 1 L of distilled water; pH was adjusted to 5 and 5 g calcium carbonate was added at 4°C (Rodrigues and others 2001). One mL of wine was added to 10 mL of culture medium and incubated for 5 days at 25°C. This process was done in duplicate. A loop full of media was then streaked onto selective plates, *Dekkera/Brettanomyces* Differential Medium (DBDM) and incubated at 25°C for two weeks. The medium contained 6.7 g YNB extract, 6 mL ethanol (v/v), 100 mg coumaric acid, 10 mg cycloheximide, 22 mg bromocresol purple, 20 g agar per 1 L of deionized water; pH was adjusted to 5.4 (Rodrigues and others 2001). All components were sterilized by membrane filtration except for the agar, which was autoclaved.

Colonies were identified by using a smell test after culturing. Odor was evaluated by using three people trained in identifying 4-ethylphenol. Microscopic analysis of colonies and broth was used to cell morphology, as *Brettanomyces* spp. have ogival or missile-shaped cells with multilateral budding (Rodrigues and others 2001). Figure 3.3 shows typical *B.bruxellensis* shape and size from a barrel sample obtained for this study. A control *Brettanomyces bruxellensis* strain, isolated from Cabernet Sauvignon wine, was obtained from the University of California Davis. The yeast was UCD VEN 2082 strain from a 1989 vintage and phylogenetic group b, but may also appear as New York State Agriculture and Experiment Station CE120 (Conterno and others 2006). Phylogenetic group b had 81% of its strains coming from the Americas (Conterno and others 2006).

Gas chromatography-mass spectrometry analysis for wine

Volatile compounds were detected in wine by using fresh bottles of wine and using a sparge and tenax trap system (Purge and Trap System, Scientific Instruments Services, Ringoes, N.J., U.S.A.) with 10 ml of room temperature freshly opened wine collecting for one hour onto a 3 mm 150 mg tenax packed with Alltech tenax ta 60/80. Sample collection was one hour. Ten ml of carvone was used as the

internal standard. The volatiles were thermally desorbed using an automated short path thermal desorption model system (Model TD-5, Scientific Instrument Services, Ringoes, N.J., U.S.A.) on the injector port of the gas chromatograph/mass spectrometer (GCMS, Model 6890N/5973, Agilent, Palo Alto, Calif., U.S.A.). The volatiles were separated using a fused silica capillary column (length 30m, inner diameter 0.25mm, and film thickness 0.25 µm). The stationary phase was a non-polar phenyl arylene polymer.

After physically injecting the sample, the purge duration was 30 seconds followed by an injection duration for 30 seconds. The collected samples were desorbed at 150°C for 5 min, ramped 30°C/min to 200 and held for 5 min, and ramped 30°C/min to 250 and held for 5 min. The analytes collected on the GC column using a CO_2 cooled cryofocus trap (-40°C) (SIS 2" Cryo-Trap, Scientific Instrument Services, Ringoes, N.J., U.S.A.). After desorption, the cryofocus trap was rapidly heated to 200°C and the analytes separated using temperature programming. Desorption volatile compounds were injected onto a column inlet at 250°C using helium flow rate of 1.0 ml/min. The split ratio was 2:1 The column temperature was programmed at 40°C and then ramped 10°C/min to 120°C and held for 1 min, then ramped 5°C/min to 240°C and held for 1 min, and ramped 10°C/min to 300°C and held for 5 min. The operational MS conditions were as follows: ion source, 230°C; electron energy 70 eV; multiplier voltage 1247 V; GC-MS interface zone 280°C; and scanning mass range (m/z) of 35 to 350 mass units.

A general standard was made for 10 samples, but 4-EP and 4-EG were dominantly studied. See Table 3.1 for list of standards. Analysis for each sample was done in replicates of three.

Volatile component identification and quantification

Wine volatiles were positively identified by comparing retention time and mass spectrum with authentic standards. Compounds were initially identified by comparison with the mass spectrum Wiley library (7^{th} ed., Wiley, N.Y., U.S.A) and the National Institute of Standards and Technology of mass spectral database. Two main volatile components of *Brettanomyces* contamination, 4-ethylphenol and 4-ethylguaiacol, were selected along with eight minor compounds. The two major compounds were

quantified by creating a calibration curve with three concentrations 125, 250 and 500 ppm in hexane for each compound using authentic standards. The other eight compounds were dissolved in hexane at 500 ppm with the two major compounds and used for identification of compound, but not quantification.

Sensory panel

A trained panel consisting of 12 members from the University of Georgia Food Science and Technology Department, tested 16 red *Vitis vinifera* wines from North Georgia wineries. Panelists were pre-screened using a questionnaire according to Meilgaard, Civille, and Carr (2007) on the bias of their health, age, lack of pregnancy or breast feeding, food habits, ability to participate in this project. The panel composed of non smokers, 5 females and 7 males ranging from 21 to 58 years of age. The panel took place in a controlled sensory panel room (20°C) containing partitioned booths equipped with fluorescent lights at the Food Processing Research and Development Laboratory of the Department of Food Science and Technology at the University of Georgia, Athens, GA. Panelists were introduced to the facility and protocol prior to testing.

Sensory procedure

Panelists were introduced and trained on the principles of descriptive analysis using the Spectrum™ method, which is based on a universal intensity line scale. Based on a wine contaminated by *Brettanomyces*, the panel created descriptors and definitions of attributes in the wine. The panel then agreed on a lexicon of twelve descriptors including Band-aid®, vanilla, woody, barnyard, earthy, rancid, metallic, sour, smokey, pepper, holiday spice and dark fruit. After panel agreement, standard references were chosen based on previous published references along with references specifically created by the panel (See Table 3.2). Five one-hour training sessions were devoted to training the panelists on wine aroma descriptors.

Three samples were given in a setting with one to two sittings a day. Using reference standards, panelists for smell were asked to mark the intensity of smell for reach attribute trained on, on a 150 mm

line scale. Panelists were also asked to mark the intensity for flavor through mouth. No standards were provided. Evaluation was done in duplicate. Panelists recorded the scores for each attribute on paper ballots accordingly. Panelists were asked to rinse their palates with unsalted crackers and water in between samples. After the testing or training session was complete panelists received chocolate and candy treats as rewards for their participation.

Wine was evaluated in standard wine glasses at room temperature with approximately 2 oz pours. The test was done in a randomized blind manner using 3-digit codes to label samples. The sample order, which was different for each assessor was chosen to minimize potential order and carry-over effects (McEwan and others 2002). Each sample was evaluated in duplicate on separate testing days.

Statistical Analysis of Results

Analysis of variance (ANOVA) with Fisher's least significant difference (LSD) was conducted on Statistical Analysis Systems program (SAS) version 9.1 (SAS Inst., Cary, N.C., U.S.A.) for the Sensory Band-aid® attribute. The LSD test was used to determine significant amounts of Brett character. The ANOVA was to determine correlation of orthonasal aroma and flavor perception in mouth of 4-ethylphenol along with orthonasal aroma of 4-ethylphenol and chemical amount of 4-ethylphenol determined by Gas Chromatography Mass Spectrometry.

RESULTS and DISCUSSION

Brett character was judged by sensory descriptive analysis. Wines with the strongest sensory Brett character were 1, 6, 12 and 16 (Figure 3.1). These samples were determined to have strongest character by showing significantly higher perceived intensities of the Band-aid® descriptor, which contained 4-ethylphenol, by orthonasal aroma and flavor perception in mouth measured. Least Squares Difference (LSD) was used to determine differences in samples based on the intensity measured on a 15 mm intensity line scale used in the Spectrum™ method.

Actual levels of 4-ethylphenol were measured by gas chromatography, which showed that wines 8, 12 and 15-18 contained *B. bruxellensis* metabolites (see Table 3.3). Sample 8 had metabolites present below threshold levels, while samples 12, 15 and 18 had results around threshold levels and samples 16 and 17 had results above threshold levels. Samples 12 and 16 both had Brett character and metabolites around or above threshold, showing more apparent contamination. Wines 1 and 6 contained perceived Brett character, but no metabolites; these results may appear due to false sensory perceptions including the dumping effect. The dumping effect is when lack of appropriate scale causes panelists to misuse other scales to report their experience (Lawless and Heymann 1999). Samples 15, 17 and 18, which had metabolites present, but were not strongly perceived by sensory analysis, appeared to be influenced by the masking effect. Other metabolites of *B.bruxellensis* included fatty acids along with different processing practices. For example, level of oak aging can greatly influence threshold of ethylphenols (Coulter and others 2003; Romano and others 2009). One wine that sensory and GCMS data did not align had a mix of *Vitis aestivalis* and *Vitis vinifera* grapes. The different flavor profile and intensity of *V. aestivalis* grapes may also provide a masking effect on ethylphenols.

The degree of effect by *B.bruxellensis* on a wine was indicated by levels of 4-ethylphenol and 4-ethylguaiacol along with sensory perception of these compounds. Low effect is below detection threshold levels both by chemical value and sensory results, medium is around detection threshold levels, and heavy is well above detection threshold levels for these compounds. Wine 8, 15 and 18 have been affected at a low level, wines 12 and 17 a medium and 16 heavily (Table 3.4).

The othonasal Band-aid ® descriptor and the amount of 4-ethylphenol by GCMS were used to measure the main *B.bruxellensis* metabolite presence, 4-ethylphenol. Although intensity of contamination was not always similarly represented in testing, a statistically significant correlation ($\alpha < 0.0001$) was found between the orthonasal aroma and flavor perception in mouth of 4-ethylphenol and ($\alpha = 0.0194$) between orthonasal aroma and gas chromatography results. Correlation between orthonasal aroma and flavor perception in mouth shows that Brett character effects both orthonasal and retronasal aroma (Table 3.5). Correlation between sensory analysis and GCMS results proves the panel was well trained and

provides support that the Spectrum™ method, if properly used, is an effective way to measure contamination of *B.bruxellensis*.

Eight out of twenty barrel samples had *B.bruxellensis* present (Table 3.6). Figure 3.2 shows microscope photos of *B.bruxellensis* in barrel H sample that were used for yeast identification.

Conclusion

Four out of seven wineries had Brett character in bottles. Three out of five wineries barrel samples were contaminated. Winery F seems to be the only winery seriously affected. Winery G, E and D should take precautionary measures. Lower levels and even medium levels of Brett character can be seen as positive, but based on practices, the amount of Brett character can easily increase and possibly become a problem.

Wineries with Brett in barrels appeared to have Brett in at least one bottle sample. Similar to the rest of the world, North Georgia wines have been affected by *B.bruxellensis*, but they have not been severely affected.

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Table-3.1 Volatile Standards Used to Find *B.bruxellensis* Metabolites in Contaminated Wines Used in Gas Chromatography Analysis

Common Chemical Name	Mass Spectrometry Name
Isovaleric acid	Butanoic acid, 3-methyl-
Guaiacol	Phenol, 2-methoxy
2-Nonenal	2-Nonenal (Z)-
Carvone (standard)	2-Cyclohexen-1-one, 2 methyl-50(1methylethenyl)
4-Ethylguaiacol	Phenol, 4-ethyl-2-methoxy
4-Ethylcatechol	4-Ethylcatechol
Ethyl decanoate	Decanoic acid, ethyl ester
1- Butanol	1- Butanol
4-Ethylphenol	Phenol, 4-ethyl
Isoamyl alcohol	1- Butanol, 3-methyl-

Table-3.2 Sensory Descriptors, Definitions and References for Wine Descriptive Analysis Aroma Evaluation

Descriptor	Standard	Definition	Intensity
Band-aid®	4-ethylphenol	Aromatics associated with band-aids®	7
Vanilla	sugar cookie (Kroger)	Aromatics associated with vanillin	7
Woody	Toothpicks	wood or oak barrels	4
Barnyard	white pepper	Smell of barnyard and livestock (hay, manure, feed, moldy, urine, etc.)	10
Earthy	mushrooms	Aromatics associated with decaying vegetal matters, damp dark soil, earth, and must	10
Rancid	oxidized vegetable oil	Aromatics associated with oxidized fats and oils	6
Metallic	iron tablet dissolved in water	Aromatics associated with chemical feeling stimulated by smell of metal	4
Sour	apple sauce	Aromatics associated with smell of acid	4
Smokey	liquid smoke	Aromatics associated with any type of smoke flavor	13
Pepper	black pepper	Aromatics associated with black pepper	9
Holiday Spice	mix of cloves, allspice, nutmeg and cinnomon	Aromatics associated with holiday spices (used in desserts) and 4-ethylguaiacol	11
Dark Fruit	mix of frozen plum, dark cherry and strawberries	Aromatics associated with dark fruit including dark berries, cherries, and plums	7

Table-3.3 Level of Ethylphenols Measured by Gas Chromatography

Wine	4-ethylphenol (µg/L)	4-ethylguaiacol (µg/L)
8	81	not measurable
12	300	not measurable
15	323	73
16	618	119
17	1049	146
18	201	148

In red wine, thresholds for 4-ethylphenol and 4-ethylguaiacol are 605 µg/L and 110 µg/L respectively and when in combination 4-ethylphenol+4ethylguaiacol (10:1) 369 µg/L[2]

[2] Chatonnet P, Dubourdieu D, Boidron J & Pons M. 1992. The origin of ethylphenols in wines. J Sci Food Agric 60:165-178.

Table-3.4 Wine and Winery Results of Effect by *B.bruxellensis* in Bottle Samples [3]

Winery	Wine	Presence or Absence of Organism	Presence of Metabolites	Perceived Presence by Sensory Analysis
A	1	-	-	+
	2	-	-	-
B	3	-	-	-
	4	-	-	-
	5	-	-	-
C	6	-	-	+
	7	-	-	-
D	8	+	Low	-
	9	-	-	-
	10	-	-	-
E	11	-	-	-
	12	-	Medium	+
	13	-	-	-
	14	-	-	-
F	15	-	Low	-
	16	-	Heavy	+
G	17	-	Medium	-
	18	-	Low	-

[3] (+) refers to a sample contaminated with *B.bruxellensis*, (-) refers to a sample not contaminated, low levels of contamination have 4-ethylphenol below threshold level, medium contain 4-EP around threshold levels, and high contain 4-EP above threshold levels

Table-3.5 Correlation of 4-Ethylphenol Levels in Chemical Analysis and Sensory Data

Column1	Taste	Odor	GCMS
Taste	r=1	r=0.72	r=0.33
		$\alpha<0.0001$	$\alpha=0.19$
Odor	r=0.72	r=1	r=0.56
	$\alpha<0.0001$		$\alpha=0.019$
GCMS	r=0.33	r=0.56	r=1
	$\alpha=0.19$	$\alpha=0.019$	

Table-3.6 Wine and Winery Results of Effect by *B.bruxellensis* in Barrel Samples

Winery	Barrel Sample	Presence or Absence of Organism
C	a	-
	b	-
	c	-
	d	-
D	e	Slight
	f	+
	g	Undetermined
E	l	-
	m	-
	n	-
	o	-
	p	Undetermined
F	h	+
	i	+
	j	+
	k	-
G	q	+
	r	-
	s	+
	t	+

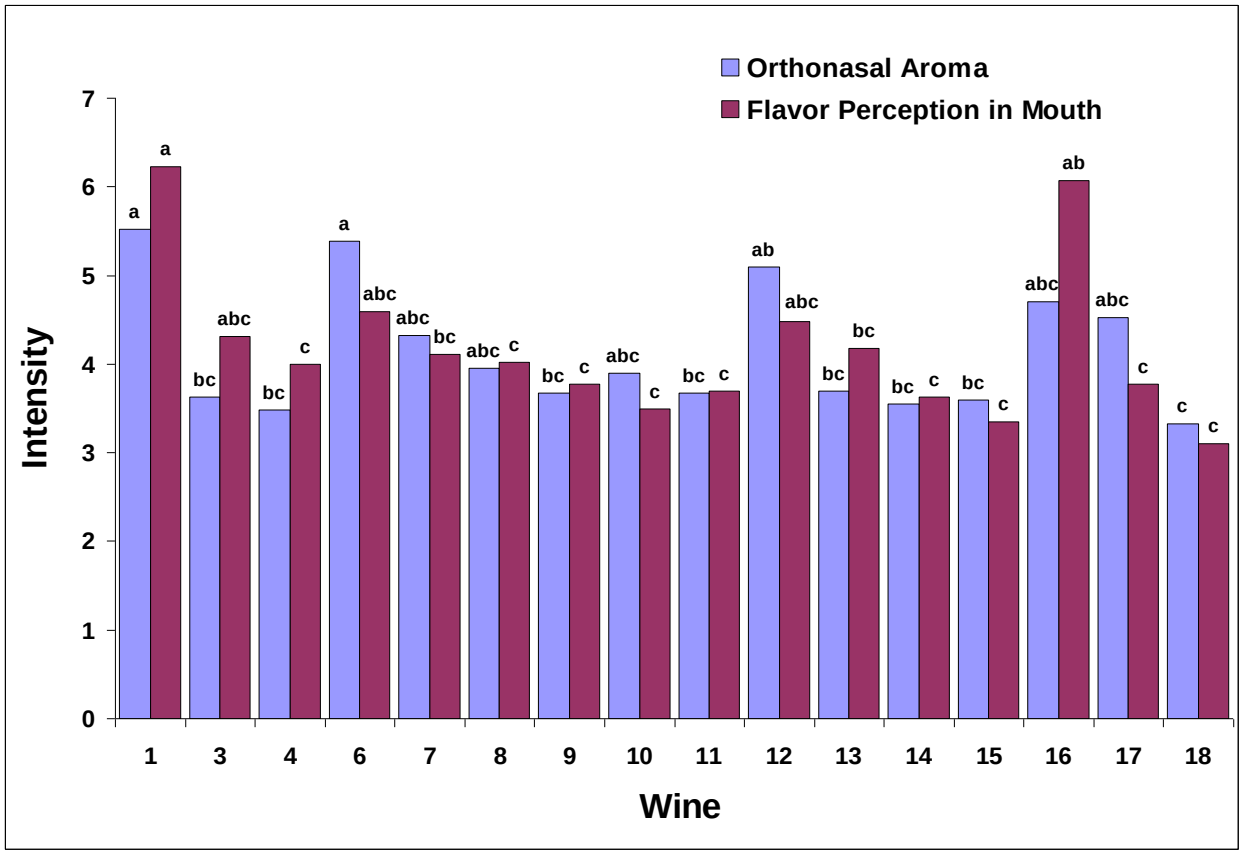

Figure-3.1 Least Squares Difference Sensory Results for Perceived *B.bruxellensis* Metabolites in Wines. Means with the same letters are not significantly different at $p \leq 0.05$.

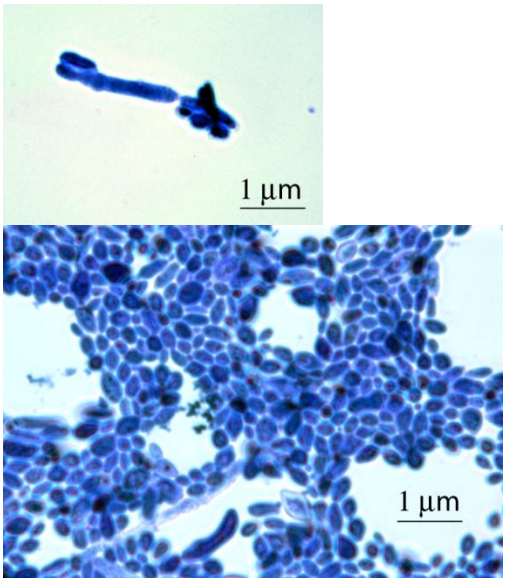

Figure-3.2 *B.bruxellensis* Barrel H Contamination. Photos by Author.

CHAPTER 4

THRESHOLD VALUES FOR 4-ETHYLPHENOL AND 4-ETHYLGUAIACOL WHEN ACETIC ACID IS INTRODUCED[4]

[4] Threshold values for 4-ethylphenol and 4-ethylguaiacol when acetic acid is introduced, Wedral D, Shewfelt R, Frank J. To be submitted to *American Journal of Enology and Viticulture*..

ABSTRACT

Brettanomyces is a particularly troublesome spoilage yeast in wine due to its association with off flavors described as 'burnt plastic,' 'barn yard,' and 'Band-aid®' produced by conversion of phenolic compounds into 4-ethylphenol and 4-ethylguaiacol. *Brettanomyces* also can produce detectable amounts of acetic acid. The objective of this research was to determine the effect of acetic acid on perceived presence of 4-ethylphenol and 4-ethylguaiacol. Detection threshold was measured for 4-ethylphenol and 4-ethylguaiacol with and without addition of acetic acid, following ASTM E 679 guidelines. Acetic acid showed a masking effect on the ethylphenol content in wine. This effect may contribute to the lack of correspondence between ethylphenol concentration and perceived Brett character in previous studies that sought to identify *Brettanomyces* by sensory analysis.

INTRODUCTION

The perception of flavor embodies a complex mixture of compounds. Many of these compounds are volatile, and many of these compounds are undetected. Wine has many factors that contribute to the wide variety of sensory perceptions. In a flavor profile, these include organic acids, sugars, alcohols, phenolics, glycerol, esters, aldehyes, keytones, terpenes and other volatile compounds (Rapp A 1986). To detect if change has occurred in a flavor profile, threshold testing is used to determine when the difference begins to change the products perceived flavor profile.

The yeast *Brettanomyces* is associated with its production of 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG). The sensory perception thresholds for 4-EP and 4-EG alone in red wine are 605 µg/L and 110 µg/L, respectively (Chatonnet and others 1992). When both compounds are present in red wine, the sensory perception threshold of 4-EP is lower, approximately 369 µg/L (Chatonnet and others 1992).. When both compounds are present in red wine, the sensory perception threshold of 4-EP is lower. The sensory perception of these compounds may be affected by other compounds. For example, in a light-bodied red wine with little oak influence, the sensory perception threshold of 4-EP may be ~350 µg/L, as opposed to a 1000 µg/L threshold in a full-bodied red and more oak influence (Coulter and others 2003).

The assumption that *Brettanomyces* imparts a typical flavor character to wine has been recently challenged by studies, mostly those regarding ethylphenol production in different strains along with the typical bi-products. Studies have shown that experts looking for contaminated wines are often not accurate in their findings. This observation could be due to unknown chemical and flavor interactions (Conterno and others 2006; Romano and others 2009). These studies question the concept of a typical Brett aroma and flavors.

Due to the ability of other organisms to produce ethylphenols and *Brettanomcyes* to produce acetic acid, it is important to understand flavor when acetic acid and ethylphenols are both present. *Brettanomyces* is considered an important producer of acetic acid, and under oxidative conditions, a high acetic acid level can indicate possible contamination (Benito and others 2009). For these reasons it is

important to understand interaction of acetic acid and ethylphenols when defining Brett character. The objective of this study is to determine the effect of acetic acid on presence of 4-EP and 4-EG in red *Vitis vinifera* wines. Acetic acid may enhance or hide the *Brettanomyces* character in red wines.

MATERIALS and METHODS

Sensory Panel

The sensory panel consisted of 30 judges of graduate students, faculty and staff belonging to the Food Science, Horticulture and Microbiology Departments at the University of Georgia. The panel was composed of 16 females and 14 males. Sensory evaluation took place in a controlled sensory panel room (20°C) containing partitioned booths equipped with fluorescent lights at the Food Processing Research and Development Laboratory of the Department of Food Science and Technology at the University of Georgia, Athens, GA.

Sensory Procedure

North Georgia red *Vitis vinifera* wines, fermented from grapes grown in Georgia, were tested by a trained sensory panel and by Gas Chromotography Mass Spectrometry for *Brettanomyces* character and metabolites 4-ethylphenol and 4-ethylguaiacol. A 2004 wine sample wine with no 4-ethylphenol or 4-ethylguaiacol presence tested by GCMS and little to none of this character in sensory evaluation was used to determine the olfactory detection thresholds.

Detection threshold was determined for 4-ethylphenol and 4-ethylguaiacol with and without addition of acetic acid. Detection thresholds were calculated following ASTM E 679 guidelines. Six sets of three-alternative forced-choice (3-AFC) tests were performed. Each series contained three samples where one or two was positive and the other one or two was the control. The positive sample(s) were in ascending order (17-34-68-137-275-550 µg/l) concentration with 4-ethylphenol: 4-ethylguaiacol in a 10:1 concentration ratio. Panelists were asked to choose which sample was different from the other two. This method was repeated with the next strongest concentration. After completing this study in duplicate with

4-EP and 4-EG alone, acetic acid was added at a concentration of 25mL/L to the ethylphenol solutions. Samples were evaluated orthonasally and consisted of 10 ml wine within a 35 ml screw cap bottle with a 1.5 cm neck diameter. Coffee beans were available to panelists to clear nasal passageways between samples.

The wine samples were freshly made and introduced to bottles and capped at least one hour before the experiment in order to allow for equilibration. All tests were performed in a room with individual tasting booths and in duplicate.

Statistical Analysis

Individual best-estimate values of threshold was derived from the pattern of correct/incorrect responses produced separately by each panelist (ASTM International 2004). The individual best-estimate threshold was calculated as the geometric mean of the last concentration, with an incorrect response, and the first series of two to three correct responses in a row. Group thresholds were derived by geometrical averaging of the individual BETs.

Results and Discussion

The best estimate threshold for 4-EP and 4-EG alone is 165 µg/L (4-EP level). The best estimate threshold for 4-EP and 4-EG with the addition of acetic acid is 287 µg/L (4-EP level). Figure 4.1 and Table 4.1 show the group BETs by proportion correct.

Similar to recent work (Romano and others 2009), the calculated detection threshold of the ethylphenol was approximately four times lower than reported in previous literature (Chatonnet and others 1992), which originally seemed to be accepted by the scientific community. Although recent threshold reports challenge original studies, wine complexity can easily influence threshold results. Acetic acid raised the detection threshold of ethylphenols by approximately 75%, indicating that it has a masking effect on *Brettanomyces* character and ethylphenol detection by humans. Acetic acid detection threshold will change for different wines with different characters, but it is recommended that levels not

be above 0.7 g/L (Jackson 2002). With an acetic acid level below detection threshold (~ 25 mg/L), the ethylphenol content must increase by 75% to detect a change in wine odor due to ethylphenols. If *Brettanomyces* is producing acetic acid, it will be more difficult to determine if *Brettanomyces* is present by sensory analysis of ethylphenols. Because a common metabolite of *Brettanomyces* is acetic acid, sensory evaluation for *Brettanomyces* should not just take ethylphenols into account for presence of this yeast.

These results show that depending on amount of acetic acid production, due to a variety of environmental and genetic factors, the *Brettanomyces* typical character, consisting of 4-ethylphenol and 4-ethylguaiacol, are perceived differently. When evaluating typical *Brettanomyces* character in a wine, other compounds that *Brettanomyces* produces need to be taken into account. In character testing, different wine matrixes need to be considered due to the current diversity of wine matrixes, to streamline results. *B. bruxellensis* yeast volatile aroma compounds need to be characterized with regard to genetic diversity to identify a typical or lack of typical character.

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Table 4.1: Average Proportion of Answers Correct by Panelists When Detecting Ethylphenol Thresholds

4-EP µg/L	17	34	68	137	275	550
4-EP and 4-EG alone	0.050	0.134	0.334	0.450	0.517	0.750
4-EP and 4-EG with acetic acid	0.015	0.033	0.066	0.188	0.413	0.715

In red wine, thresholds for 4-ethylphenol and 4-ethylguaiacol are 605 µg/L and 110 µg/L respectively and when in combination 4-ethylphenol+4ethylguaiacol (10:1) 369 µg/L[5]

[5] Chatonnet P, Dubourdieu D, Boidron J & Pons M. 1992. The origin of ethylphenols in wines. J Sci Food Agric 60:165-178.

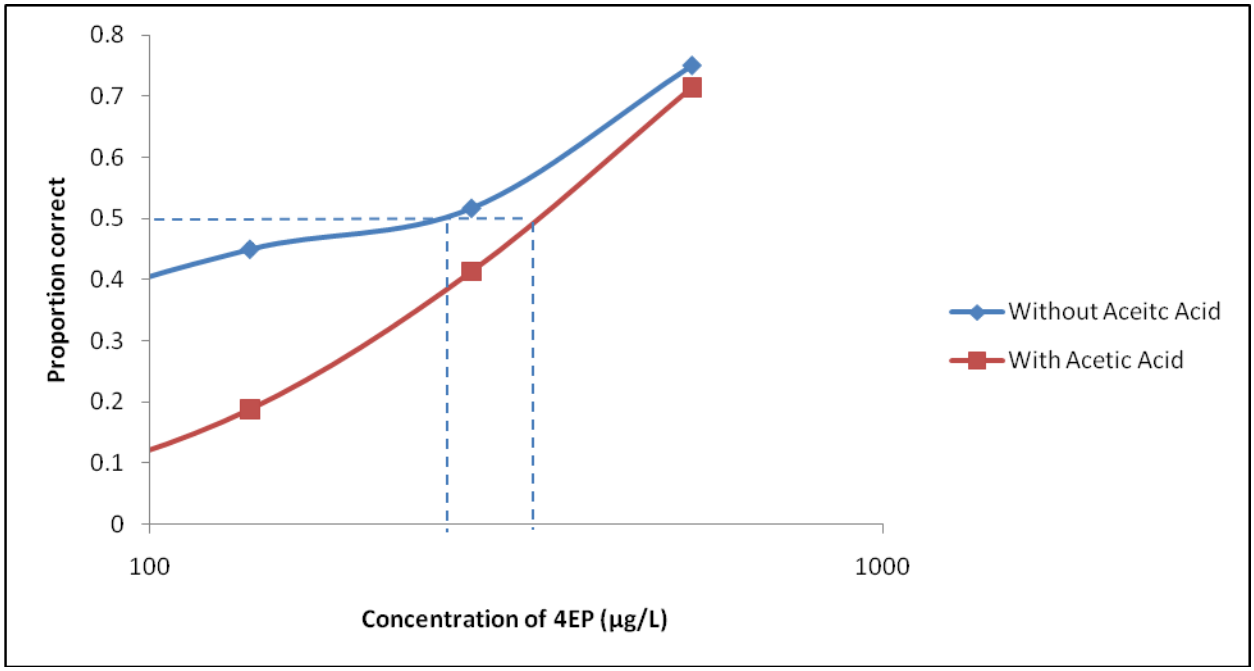

Figure 4.1: Proportion Correct of Answers by Panelists vs. Concentration of 4-EP (µg/L)

Summary of Threshold Report
Report: Odor Threshold Determination of 4-ethylphenol and 4-ethylguaiacol in wine alone and with addition of acetic acid

Procedure: ASTM E 679

Equipment: Brown glass 35 ml screw cap bottle with a 1.5 cm neck diameter with 10 ml wine

Sample: 4-ethylphenol and 4-ethylguaiacol and acetic acid

Scale Steps: 6

Dilution factor: 1/2x and 2x 4-EP (17-34-68-137-275-550 µg/l) and 4-EG (1.7-3.4-6.8-13.7-27.5-55.0 µg/l)

Number of Subjects: 30

Temperature: ~68

Done in Duplicate

Best estimate threshold: For 4-EP and 4-EG alone

Zol= 164.65

Log 10 Zol=2.22

Standard log deviation=0.582

Best estimate threshold: For 4-EP and 4-EG with Acetic acid

Zol= 287

Log 10 Zol= 2.46

Standard log deviation= 0.406

Note: Z represents a dilution factor proposed to designate a dimensionless measure of sample dilution needed to reach some target effect. "ol" represents the dilution at which the odor reaches a limit that corresponds to the best-estimate threshold.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Similar to the rest of the world, North Georgia wines have been affected by *B.bruxellensis*. Four out of seven wineries had Brett character in bottles. Three out of five wineries' barrel samples were contaminated. Only one winery seems to be seriously affected, while others should take precautionary measures. Lower levels and even medium levels of Brett character can be seen as positive, but based on practices, the amount of Brett character can easily increase and possibly become a problem.

Flavor profiles of wines with different levels of contamination showed that levels of ethylphenols affect other flavor profile characteristics differently depending on strain type and style of wine.
For naturally occurring *B. bruxellensis* contaminated samples the amount of Brett character perceived and levels of acetic acid are negatively correlated. The Band-aid® aroma and acetic acid level on GC were negatively correlated with a low (α=0.1215) significance level.

In spiked samples, the calculated detection threshold of the ethylphenols was approximately 2.2 times lower than reported in literature (Chatonnet and others 1992), but wine complexity can easily influence these results. Acetic acid raised the detection threshold by approximately 75%, supporting the premise that it appears to have a masking effect on Brett character and ethylphenol concentration.

A better understanding of Brett character in wine is needed. First *B. bruxellensis* yeast volatile aroma compounds need to be characterized with regard to genetic diversity. Genetic diversity has caused re-evaluation of flavor profile and questions the ability to identify Brett by sensory evaluation alone. Understanding the flavor profile of a wine based on different levels of the yeasts different metabolites would give a greater understanding of Brett character. For example understanding that acetic acid masks ethylphenols shows that volatile acidity could be taken into account when identifying Brett character. Choices in processing techniques may also be changed to achieve desired masking effects. Using

information about how flavor profile can change in a *B. bruxellensis* contaminated wine will allow scientists to determine if *B. bruxellensis* can be defined to have a typical character and if the flavor profile can be more accurately defined. Also, past flavor-profile evaluations have used a variety of wine matrixes including different alcohol and chemical contents mimicking a wine, therefore studies also need to be done using similar matrixes in order to have consistent results. *B.bruxellensis* typical flavor needs to be re-evaluated because there is a possibility no longer one typical flavor exists.

Reference

Chatonnet P, Dubourdieu D, Boidron J & Pons M. 1992. The origin of ethylphenols in wines. J Sci Food Agric 60:165-178.