HEALTH BENEFITS AND QUALITY OF TEXAS RED WINES

A Dissertation

by

GABRIELA DEL CARMEN ANGEL MORALES

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2011

Major Subject: Food Science and Technology

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ABSTRACT

Health Benefits and Quality of Texas Red Wines.

(August 2011)

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The overall objective of this work was to investigate the reduction of 3-alkyl-2-methoxypyrazines that can affect the quality of Texas wines and to study the health benefits of Texas wines.

The first objective was to demonstrate the anti-inflammatory potential effect of red wine polyphenols from Black Spanish wine (*Vitis aestivalis)* in colonic human fibroblast cells. Results show that polyphenols from Black Spanish wine decreased activation of NF-kB transcription factor and target pro-inflammatory cytokines and cell adhesion molecules. Induction of microRNA-126 by wine extract was found to be one of the underlying molecular mechanism by which wine extract decreased vascular cell adhesion molecule (VCAM-1) and inflammation in colon cells.

The second objective was to investigate the role of the green june beetle (GJB) as exogenous source of MPs. Results demonstrated GJB as source of 3-isopropyl-2-methoxypyrazine (IPMP), where one GJB could elevate MPs above sensory perceptible levels in 4.3 gallons of wine. The incorporation of GJB to the winemaking process may

contribute negatively to the sensory properties of Texas wines and therefore should be strictly controlled.

The third objective was to explore the effect of micro-oxygenation treatment and accelerated aging techniques relevant for the state of Texas in the reduction of MPs levels as determined by SPME-GC-MS. Results show that MPs were not affected by MOX or oak interaction.

The fourth research objective was to explore the potential effect of three commercial available yeast strains, BM45, K1, and D80, on MPs levels in Black Spanish wines. MB45 strain resulted in the highest amount of MPs. Conversely K1 and D80 yeast strains reduced IBMP levels in comparison with the control. In addition we evaluated MPs levels of wines fermented with a chemical defined grape juice medium. Data suggest that BM45 and D80 yeast strains reduced IBMP but K1 yeast did not show any effect in comparison with the control. In addition MPs were evaluated in the yeast mannoproteins fraction. This data demonstrate for the first time the interaction of yeast mannoproteins with IBMP.

This work will provide valuable information regarding the potential health benefits of *Vitis aestivalis* grapes and reduction of MPs and thus improving the quality of Texas wines.

To my family

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

The presented research addresses aspects centering on the evaluation of quality and health benefits of red wine, where the investigated research objectives are relevant to wine production in the State of Texas. The wine industry in the State of Texas has been continuously growing and is faced with some region-specific challenges.

Epidemiologic studies indicate that individuals with the habit of daily moderate wine consumption experience significant reduction in all-cause and particularly cardiovascular mortality when compared to individuals who abstain from or who drink alcohol to excess. The chemopreventive properties of red wine have largely been attributed to polyphenolic compounds present in grapes and wines; however, several mechanisms involved in the prevention of inflammation by polyphenolics are still unclear, and new mechanisms are being proposed. For this reason, this study investigated the anti-inflammatory effects of a Texas wine and also examined microRNA-based underlying mechanisms.

Wine quality worldwide and in Texas has been affected by elevated concentrations of 3-Alkyl-2-methoxypyrazines (MPs) that add a green vegetative aroma and can mask over fruity/floral aromas in wine.

In recent years micro-oxygenation (MOX) has been used in accelerated aging of wines and several studies have reported positive effects of MOX on quality aspects of wine such as improved palatability, enhanced color stability and intensity. Some studies

This dissertation follows the style of *Journal of Agricultural and Food Chemistry*.

investigate the effects of MOX on the sensory perception of MPs, however the effects of MOX on actual MPs concentrations have not been investigated intensively. Also different yeast strands have been reported in the potential reduction of MPs during wine fermentation, however, actual concentrations of MPs have not been investigated extensively. For this reason, the presented studies investigated the influence of MOX and other techniques of acelerated aging, different yeast strands, and materials, which may bind MPs, on MPs concentrations by SPME-GC-MS.

The provided information serves as an overview to provide valuable information regarding wine quality and health benefits of wine to the Texas grape and wine industry and beyond.

CHAPTER II

LITERATURE REVIEW

Wine and the Texas Wine Industry

Grapes along with some other horticulture crops are major sources of economic income in many countries, either as a table fruits or as processed products like wines. According to the statistics from FAO, world wine production in 2009 was 6.69 x 107 tons (1). Currently, around 87% of the grapes harvested are used in wine production, the other 23% are sold as tables grapes or as grape-derived products (2). Wine production and consumption has shown a steady increase worldwide over the last decade, and United States is no the exception. Moreover, while French wines are still popular options in the US, market share has been switched to lower-priced wines from other regions such as Italy, Chile, Spain, South Africa, and Australia (3).

Currently, the United States is the fourth highest wine producing country in the world following Italy, France and Spain (4). Within the US the major wine producing states are California, Washington, Oregon, New York, Virginia, Pennsylvania, Ohio, and Texas (5). The wine industry in Texas is one of the oldest. Texas was the first state to establish vineyards in North America, as European settlers move to new places, they brought grapevine cuttings with them, allowing the development of the wine industry in the 1800's (6). The Texas winery industry has increased in the last 5 years. The number of bonded wineries in the state of Texas has grown from 113 in 2005 to 226 in 2010, and continuing to expand with almost all of the growth coming from the addition of small

wineries. Currently Texas has 226 commercial wineries and approximately 3,700 acres of producing vineyard farmland with eight American Viticulture Areas (AVAs) (7).

Wine production in Texas in the last seven years has grown more than 30% to over 2.3 million gallons, ranking Texas as fifth in the United States of America in wine production behind California, Washington, New York, and Oregon. Texas also became the fourth largest consumer of wine in the United States (8).

Due to specific growing conditions in Texas that result in reduced fruit production and on occasion vine death, some Texas grape growers and wine makers choose to work with non-viniferous or non-European grapevines and fruit such as Cynthiana, Blanc du Bois, Black Spanish and Muscadines. These grapes are more resistant to specific challenges presented by growing conditions in Texas. Moreover Texas growers cultivated European grapes as well such as Cabernet Sauvignon, Merlot, Sangiovese, Syrah or Shiraz, Tempranillo, Cabernet Franc, Chardonnay, Chenin Blanc, among others Vitis vinifera varieties (9).

The wine and grape industry in Texas has contributed greatly to the economic strength of the state. According to the TTB data, wine produced from local grapes totaled 462,739 gallons in 2008, and total wine production was estimated to be 2 million gallons (5).

Wine, grapes and related industries account for nearly 9,000 jobs in Texas, with an associated payroll in excess of $298 million. The retail value of Texas wine in 2007 is estimated at $98.5 million. Winery revenue totals $55 million, including an estimated $24.6 million (44%) in winery direct sales (sales by wineries direct to consumers).

Winery direct sales include sales to consumers in the winery tasting rooms, wine clubs, winery mailing lists and e-commerce or Internet sales (10).

Texas government has enabled the Texas Agrilife Extension Service and Grayson County College to develop world-class education and research certificate program for viticulture and enology. In addition the Texas Department of Agriculture has developed the Texas Wine Regional Partnership Grant to enhance the growth, visibility and awareness of the Texas wine industry. The Texas Legislature created the Texas Wine Marketing Assistance Program (TWMAP) in 2001 under the Texas Department of Agriculture (TDA). TWMAP is charged with assisting the Texas wine industry in promoting and marketing Texas wines and educating the public about the Texas wine industry.

In addition, Senate Bill SB1370, which allocates monies received from incremental excise and sales tax revenues on wine into education and research programs, support Texas' wine industry growth. Additional funds have been made available to Texas A&M University, Texas Agrilife Extension, Texas Tech University, Texas Wine Marketing Research Institute, Grayson County College, Texas Agricultural Experiment Station and the Texas Department of Agriculture for non-certificate education and research programs on enology, viticulture and pest management.

North American Grapes

In the Vitaceae family, the Vitis genus, from the agronomic and economic point of view is by far one of the most important (11). Currently, there are nearly 60 different

inter-fertile species that exist in the Northern Hemisphere. Vitis vinifera is the most widely used in the wine industry (11).

Cultivation of grapes historically is linked to the consumption of wine. Selective cultivation and breeding increased the production of a bigger fruit size with higher sugar content (11). Vinifera grape varieties have thin skin, sweet flesh, and high sugar content, making them suitable for the production of high quality wines (12).

In the North American continent there are several native varieties of grapes. The domestication of these has played an important role in the development of the eastern American wine industry. In fact, the American wine industry may not be possible in places where Vitis vinifera L. cannot grow due to environmental conditions such as severe weather or endemic diseases (13).

Some North American grape varieties include *Vitis labrusca*, *Vitis rotundifolia*, *Vitis riparia,* and *Vitis aestivalis*, which have economic relevance for the Texas wine industry. Unfortunately, there is not enough scientific information available for most American grape varieties, since the majority is not suitable for making good wines.

In the state of Texas the white species cultivated include Blanc Du Bois, Chardonnay, Chenin Blanc, Sauvignon Blanc, Muscat Blanc, Pinot Blanc, and Riesling. Whereas, the red varieties include Cabernet Sauvignon, Merlot, Black Spanish, Norton, Sangiovese, Syrah, and Cabernet Franc (6).

Vitis aestivalis

Although this variety has excellent features for optimal vinification, it is not the most widely used wine grape. Unlike others grape varieties such as V. *rotundifolia* and V. *labrusca*; it has an adequate sugar content. The most representative cultivars are Norton and Cynthiana, which are grown mainly in Missouri and Arkansas (12, 14). Even though these cultivars are considered as two separate ones, it is thought that they stem from the same vine, and have acquired two names throughoutt the course of propagation and distribution (14). In recent years, the Norton grape has increased in popularity in the Midwestern and Southern states, due to its adaptability and resistance to fungal diseases (15).

Wine Chemistry

Wine is a complex mixture of indigenous components and those obtained by chemical and biochemical transformation during the winemaking process or during wine aging. Wine composition varies widely and is influenced by the grape (variety, quality) and by the winemaking conditions. Many of the wine components (e.g. carbohydrates, proteins, and polyphenols) come from the skins, pulp of grape and from the cell wall of the yeast. Most of these compounds will be eliminated by clarification and stabilization treatments of the wine (16).

Grape and Wine Polyphenols

Phenolic acids are aromatic secondary plant metabolites, widely spread throughout the plant kingdom (17). Structurally, phenolic compounds are derivatives of benzene with one or more hydroxyl substituent, often accompanied by functional substitutions such as esters, methyl esters, glycosides and others (18).

Based on their structural properties, phenolic compounds may be divided into various groups, including phenolic acids, flavonoids, tannins, and other phenylpropanoid derivatives (19). Phenolic acids are characterized by one carboxylic acid functional group and two constitutive carbon frameworks, the hydroxycinnamic and hydroxybenzoic structures, commonly substituted with one or more hydroxyl groups (17). Phenolic compounds are the most important constituents of wine, in terms on their high concentration and also because they play an important role in the organolopetic properties of wine.

During the winemaking process, grape compounds are transferred to the must and to the wine, which contain several polyphenols at different degree of polymerization. The simplest compounds are mono-, di-, and tri-phenols (phenol, pyrocatechol, resorcinol, hydroquinone, phloroglucinol).

Phenolic aldehydes such as vanillin, p-hydroxybenzaldehyde, syringic aldehyde, coniferyl-aldehyde, benzoic acids such as gentisic acid, gallic acid, vanillic acid, salicilic acid, and syringic acid. Moreover some hydroxycinnamic acids such as caffeic, ferulic, and p-coumaric acids are present in grape, must and wines (20).

More-complex grape polyphenols contain two or more aromatic rings (cumarines, benzopyrones, and flavilium ions) to form flavanols, flavonols and anthocyanins (20). These molecules are present in the grape mainly in the monoglycoside form, with the sugar residue linked to the hydroxyl group in position C-3 of the O-containing ring. Some flavonols vary in color from white to yellow and are closely related in structure to the flavones. They are represented mainly by kaempferol, quercetin and myricetin, while simple O-methylated derivatives such as isorhamnetin (quercetin 3'-methylether) are also common (Fig. 1). These compounds form co-pigments with anthocyanins (in red wines); they, together with oxidation products of tannins, are mainly responsible for the color of white grapes and wines (21).

Among wine polyphenols, anthocyanins and tannins are very important since they are responsible for the color, structure and mouth-feel of red wines (22).
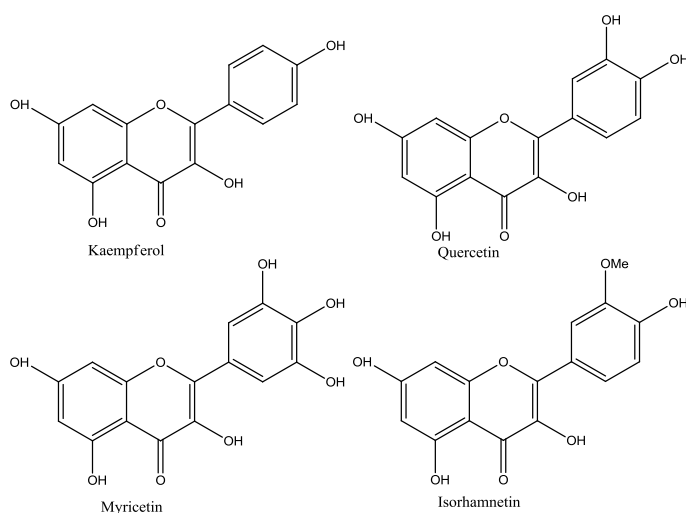
Figure 1 Structures of four common flavonol aglycones encountered in plant tissues

Anthocyanins

Anthocyanins are one of the most important plant pigments. They belong to the flavonoid group and are mainly located in the cell vacuole of fruits and flowers (23). In grapes, anthocyanins are located in the grape skins, with the exception of some varieties that also contain anthocyanins in the pulp (24, 25). Anthocyanidins are unconjugated and are composed of an aromatic ring A bonded to a heterocyclic ring C that contains oxygen (flavylium cation), which is also bonded by a carbon-carbon bond to a third aromatic ring B (26).

Currently, there are more than 23 naturally occurring anthocyanidins identified, along with more than 600 anthocyanins (24, 26, 27). However only six anthocyanidins (Figure 8) are commonly found in higher plants: delphinidin, cyanidin, pelargonidin, petunidin, peonidin, and malvidin. They differ from each other by the number and position of hydroxyl and methoxyl groups located in the B-ring of the molecule (26, 28).

In grapes, five anthocyanidins have been identified: delphinidin, cyanidin, petunidin, peonidin, and malvidin (Figure 2). Similar anthocyanins may be present in grapes and wines, the concentrations of each depends on the climatic conditions, production area, cultivar, maturity and processing conditions among other variables (24, 29). From the five anthocyanidins present, malvidin is by far the most abundant in red grape varieties, representing between 50 to 90% of total anthocyanidins in some grape varieties.

Anthocyanins contain in their skeleton the benzopyrilium ion as the base molecule, which is responsible for the color of red berry varieties and red wines. They are un-stable and participate in reactions during fermentation and maturation to form more

complex pigments, such as, procyanidinsand proantho-cyanidins, which mainly arise from the interaction between anthocyanins and other phenolics compounds, especially flavan-3-ols such as catechin, gallocatechin, and epicatechin. All these reactions result in the formation of more stable compounds that stabilize wine color since they partly resist discoloration by SO2 and provide better color stability at wine pH. (30).

Name	R₁	R₂
Delphinidin	OH	OH
Cyanidin	OH	H
Petunidin	OCH_3	OH
Peonidin	OCH_3	H
Malvidin	OCH_3	OCH_3
R₃		
p-coumaroyl	acetyl	caffeoyl
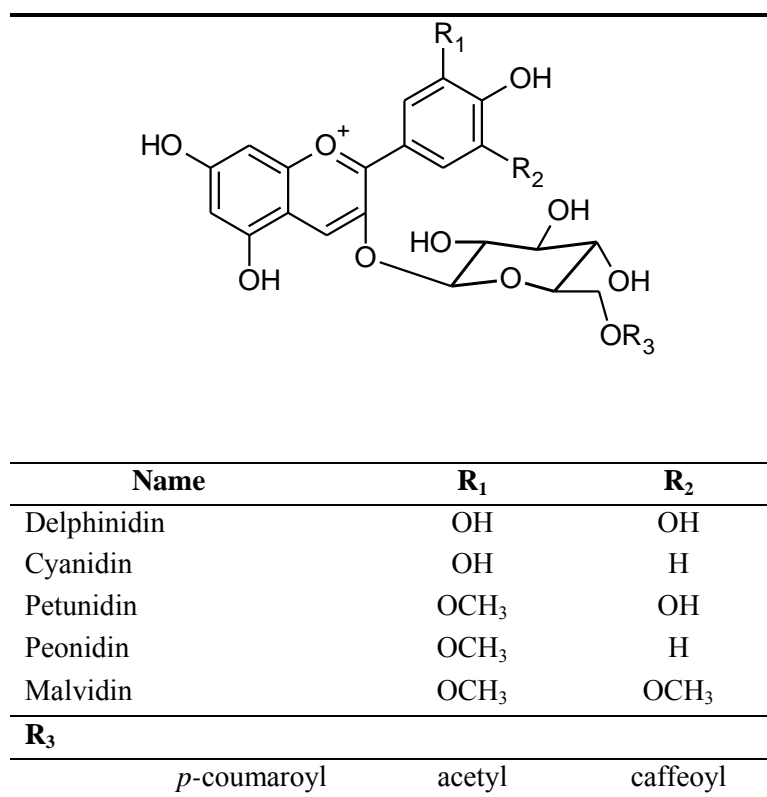
Figure 2 Anthocyanins occurring in wines (19)

Tannins

Tannins are an important group of polyphelic compounds present in wine. These compounds are water-soluble phenolic compounds of relatively high molecular weight (500 to 3000 Daltons), that have the ability to precipitate alkaloids, gelatin and other proteins (31). Tannins are commonly classified based on their structural characteristics into hydrolysable tannins, consisting of polyesters of gallic or ellagic acid, and condensed or non-hydrolysable tannins, commonly referred to as proanthocyanidins, and composed of flavan-3-ol nuclei polymers (32).

Figure 6 shows the proanthocyanidins of (+)-catechin, (-)-epicatechin, and (-)-epicatechin-3-O-gallate present in grapes and wine. These compounds can form dimmers, trimers, and tretramers of flavanol subunits usually linked by carbon-carbon bonds in the C_4–C_6 or C_4–C_8 positions (24, 33).

Hydrolyzable tannins are not naturally present in grapes but they are found in wines. The presence in wine is due to the aging process when wines are stored in oak barrels (24, 25). In the wine industry, these tannins are the only ones legally authorized as wine additives (25).
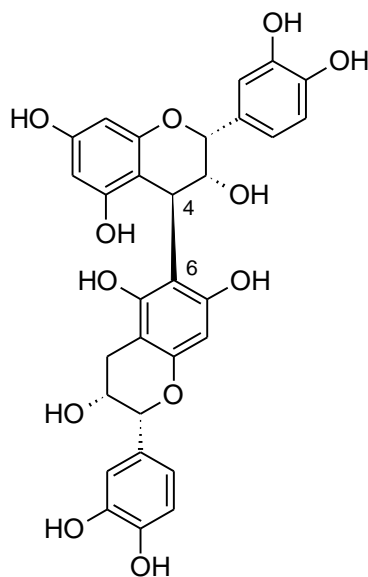
Figure 3 Structure of simple dimeric proanthocyanidins in condensed tannins

Stilbenes

Stilbenes, a non-flavonoid family of more complex polyphenolics, are also present in grapes and wines. Stilbenes are phytoalexins synthesized in leaves, skins and roots as a defense mechanism against fungal infections or abiotic stressors like UV radiation (19, 24, 25). The content of stilbenes in wines varies considerably and depends on several factors such as grape variety, environmental conditions, microbial diseases, enological methods, etc (24, 34). Resveratrol belongs to the family of stilbenes and is also produced by the grapes and a few other plant species. This group is characterized by two benzene rings bonded by an ethane chain. Resveratrol is mainly extracted during maceration and fermentation of wines with concentrations ranging from 0.2 to 13 mg/L for red wines, and 0.1 to 0.8 mg/L in white wines (24, 25).

Volatile Compounds

Wine volatiles may contribute to pleasant aroma notes, or to unpleasant aromas, which are considered flaws. Wine volatiles include a variety of compounds such as isomyl acetate, hexyl acetate, phenylethyl acetate, and the fatty acid group, which includes hexanoic, actanoic, decanoic and dodecanoic acid and fusel alcohol acetates such as isobutyl, isamyl and β-phenylethyl acetates. The group containing ethyl esters and the fusel alcohol acetates are responsible for the fruity aroma and flavor in wines. (35). These compounds contribute to the specific characteristic aroma of the wine.

Methoxypyrazines

A significant problem associated with Texas wine quality is caused by the high concentration of undesirable volatile compounds like 3-Alkyl-2-methoxypyrazines (MPs). This and similar compounds can mask the fruity /floral aromas in wine, replacing them with a green and vegetative flavors and aromas (36, 37).

MPs play an important role in the flavor chemistry. These compounds are widely distributed in the plant kingdom, and can reach total concentrations in excess of 1000 pg/g in the vegetative tissue and unripe fruits of several plants, including bell pepper, potatoes, asparagus, and peas. MPs are responsible for the vegetative, herbaceous, capsium-like aromas (38). They can contribute positively to certain varietal wine aroma profiles. MPs have been found in Cabernet Aauvignon, Sauvignon Blanc, Cabernet Franc and Merlot among others (39, 40). However when present a high level, MPs may be considered as a negative sensory attribute, as their aroma may not be suitably

balanced by other aroma compounds in the wine. The most commonly found MP in wines are, 3-Isobutyl-2-methoxypyrazine (IBMP), 3-sec-butyl-2-methoxypyrazine (SBMP), and 3-isopropyl-2-methoxypyrazine (IPMP) as shown in the chemical structure in Figure 4 (40, 41).

isopropyl methoxypyrazine
(IPMP)

isobutyl methoxypyrazine
(IBMP)

Sec-butyl methoxypyrazine
(SBMP)
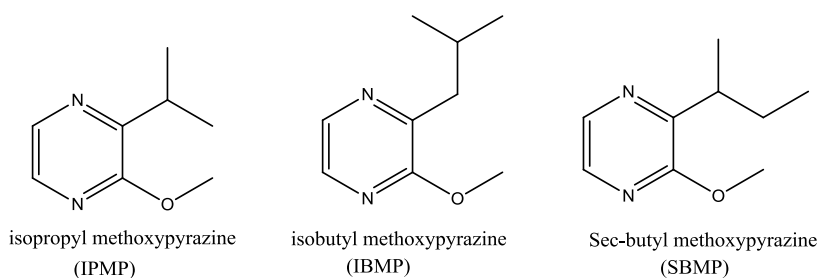
Figure 4 Grape derived methoxypyrazines that contribute to wine flavor

These compounds are associated with wines from cooler climates and under ripe, low quality fruit. MPs are reported to accumulate pre-veraison, and then to decrease noticeably between veraison and maturity. Several environmental factors have been correlated with final concentrations of MPs in grapes, including vine growth, temperature, and cluster light exposure. It has been demonstrated that higher humidity in the pre-veraison months may result in higher MPs content in the grapes at harvest. Further studies have demonstrated the influence of irrigation and vine density on the methoxypyrazine content in musts and wines. They found that samples from irrigated vines had significantly higher average contents of IBMP than samples from non-irrigated plants. Average levels of this compound were also higher in samples from vines with the

higher vine density (42). Other studies have demonstrated that levels of MPs in grapes are significantly lower in mature fruit and as well in fruit grown in warmer climates due to increased light exposure on fruit (42-44).

Recently lady beetles were identified as a second source of elevated MPs in wine. This flaw is referred to as "ladybug taint" (LBT). LBT is a wine defects resulting from the undesired incorporation of lady beetles (Coleoptera: Coccinellidae), particularly Harmonia axyridis (commonly called the Multicolored Asian Lady Beetle, MALB), into the fermentation process. MALB can be found in vineyards in large numbers around the time of commercial grape harvest (45, 46) and are common in many winemaking regions of the world, including Italy, France, Spain, Greece, South Africa, Argentina, Brazil, Canada and United States (46). MALB was incorporated to the vineyards as a biological control agent. Along with beneficial impacts as a biological control, MALB have adverse impacts in the winery industry due to their role as an important source of IPMP. "Lady Bug" Taint is responsible for millions of dollars in lost revenue from downgraded or discarded wine worldwide.

Methoxpyrazines Byosinthesis

Plants synthesize MPs as secondary products of amino acid metabolism and are therefore, wine flavor compounds directly derived from the grape.

The biosynthetic pathway proposed for these compounds involves formation of the amide of the appropriate amino acid, formation of a pyrazinone, and methylation (47). However the complete biosynthetic pathway leading to the formation of MPs is still

unknown. A number of pathways have been proposed and all agree that the pathway involves an amino acid and an unknown 1,2-dicarbonyl compound leading to the formation of a 3-alkyl-2-hydroxypyrazine (HP) intermediate, which is enzymatically methylated to form MP (*47*). Several studies have suggested that the amino acids valine, leucine and isoleucine are each precursors to IPMP, IBMP and SBMP, respectively because of similarities in the alkyl side chains (47). Feeding experiments in bacterial strains that accumulate IPMP have shown that the addition of 13C-L-valine results in the production of 13C containing IBMP, thus confirming that amino acids are a precursor to MPs (48).

Currently the mechanism by which the amino acid is converted to the HP intermediate remains unclear. It has been proposed that the respective amino acid gains a second nitrogen through an unknown amidation reaction and then undergoes a condensation reaction with a 1,2-dicarbonyl compound such as glyoxal to produce HP as shown in Figure 5 (47).
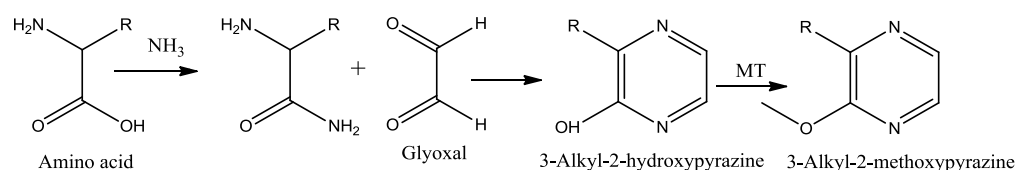
Figure 5 Proposed biogenesis pathway of methoxypyrazines

The presence of 2-hydroxy-3-isobutylpyrazine (IBHP) and 2-hydroxy-3-isopropylpyrazine (IPHP) was reported for the first time in grapes and plants and S-adenosyl-L-methionine dependent O-methyltransferase (OMT) activity has been purified from grapes as well. This study reported levels of HP in the range of 5 and 20-fold higher than MP levels in unripe grape varieties such as, Semillon, Merlot and Sauvignon Blanc. On the other hand, the ratio of HP/MP reported in this study was 1.3 to 2.1 in Cabernet Sauvignon that also accumulates MPs (49). This study predicted the final step of MP biosynthesis exists in wine grapes by the pathway involving the methylation of HP to MP by the activity of OMT as shown in Figure 6 (49, 50).
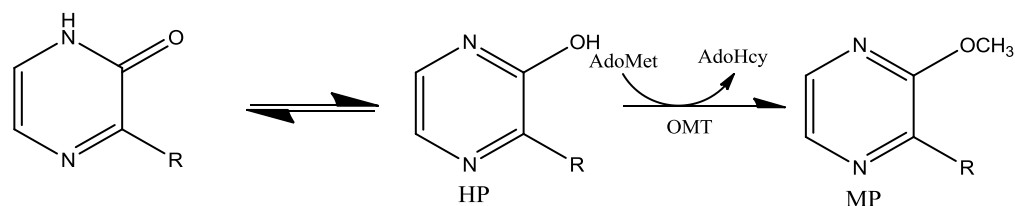
Figure 6 Enzymatic 0-methylation of HP in grapes.

Sequencing of the N-terminus of the purified methyltransferase enzyme (50) enabled the identification of a grape cDNA that encodes this enzyme (51). While this gene is yet to be functionally characterized, a number of results imply that this gene is involved in the pathway of MPs synthesis. The peak of expression of this gene during development of Cabernet Sauvignon berries correlates well with the peak of IBMP accumulation. It was also shown that the expression of this gene is higher in cooler conditions than in

warmer conditions (51), which supports that vines grown in cool climates produce grapes with greater levels of MP than vines from warmer climates. An understanding of the biosynthesis of methoxypyrazines in grape berries will enable the development of biotechnological or conventional breeding strategies to manipulate this trait in grape varieties or to develop management regimes to control its accumulation in fruit.

Different Technologies to Reduce Methoxypyrazines

Three decades have passed since MPs were first found in grapes and wines (52). Since then the wine industry has been trying to reduce the MPs in wine with the help of scientists. However this has not been an easy task due to MP's extraordinary low threshold of ng/L in wines (40, 41, 53).

Although sensory evaluation is crucial for analyzing wine flavor and quality, instrumental measurements can plan an important role in quantifying MPs, therefore knowing the concentration in the grapes. Growers would be able to blend the must with grapes that contain a low concentration of MP. Therefore MPs will be reduced and will not mask the fruity and floral wine aromas, thus improving wine quality.

To remove MPs in the winery, it is important to understand how environmental or physiological conditions influence MPs levels in the vineyard. Several studies have reported that IBMP has a peak in concentration occurring in berries approximately 0 to 14 days before veraison. This period is followed by a rapid decline in IBMP during maturation (39, 40).

In addition, the incorporation of the steam during the fermentation process might provide a sensory effect to the wines causing ¨green flavor¨ to the wines (43, 54).

MPs are efficiently extracted by conventional red wine practices and their concentrations in wine are strongly correlated to their concentrations in grapes (55). Efforts to reduce MPs levels have included both viticulture and enological interventions. Several studies have evaluated the efficacy of vinification and cellaring practices in reducing MPs (56, 57) and have generally concluded that remediation of MPs results in other nonselective changes to the wine. Viticultural management strategies that reduce MPs in the vineyard have been reported (58). This study demonstrated that basal early leaf removal (10 to 40 day after anthesis) reduced IBMP up to 60% compared with the control (no leaf removal) in mature Cabernet franc berries. Thus management practices that reduce initial accumulation of MPs in grapes pre-veraison have demonstrated a greater reduction in final MPs concentrations at harvest, out performing intervention later in the season. This effect might be due to the increase of sunlight reaching the berries. Recent investigation has shown that cellar conditions with temperature control up to 22 C and light exposure during storage influenced MPs composition. In this study IBMP concentration on wine storage over 12 months under light and temperature conditions of 22 C were consistently affected (56).

Moreover, attempts to decrease MPs concentrations in wine using common cellar practices such as bentonite fining, oak contact, and pectinases have had limited success (57). Other practices such as thermovinification and activated charcoal can reduce IBMP, but lack selectivity and thus may remove desirable components from the wine.

Furthermore, the effects of closure and packaging type used on wines have been studied. In this study authors found that IBMP, IPMP and SBMP were decreased in red wines stored for 18 months in tetrapak by 45, 32 and 26% respectively. Authors speculated that the decrease in MPs might result from their migration to the aluminum surface layer of the container, resulting in adsorption on the surfucuial oxide layer. Furthermore, authors found a reduction of 20% of all MPs in red wines closed with natural cork, which suggested that a migration of MPs occurs from the natural corks into the wine (56).

Another potential strategy to reduce MPs in wine may be the use of selected yeast strain, which have been shown to influence many volatile compounds across a range of chemical species (53, 59, 60). As an example, several very odorous thiols that are absent from grapes and must have been developed in Sauvignon blanc wines during yeast fermentation. Thiols like 4-mercapto-4-methylpentan-2-one (4MMP), 4-mercapto-4-methylpentan-2-ol, 3-mercaptohexanol, 3-mercaptohexyl acetate (60).

A recent study evaluated the effect of multiple yeast co-inoculations on Sauvignon Blanc wine aroma composition. They correlated methoxypyrazines compounds, with the fresh green attribute. From the results of the sensory descriptive analysis the fresh green flavor among others were significantly different attributes for the different yeast strains inoculated. These substantial differences among strains for different aromas, demonstrated a strong evidence that the differences in sensory properties among the wines were due to strain effects (61).

Another study investigated for the first time to our knowledge, the capacity of yeast to affect MPs concentration in wine using analytical techniques. They examined strain effects on IBMP in Cabernet Sauvignon and Cabernet Franc wines and found differences of up to 37%. Lalvin BM-45 andLalvin D80 produced wine with the lowest levels of IBMP concentrations. Further studies were done to investigated the effect of commercial *Sachcaromyces cerevisiae* strains on IPMP concentration on Cabernet Sauvignon (59). This study showed that wines treated with yeast strains such as, EC1118, ICV-21 and ICV-D80 were not significantly decreased on IPMP concentration. However for the wine treated with BM-45 yeast strain, they found a significantly higher amount of IPMP than wines fermented using the other yeast strains. Authors suggested that this might be caused by an external contamination of their sample.

Nevertheless this study should be extended to investigate possible strain effects on other MPs found in wine, particularly IBMP with HS-SPME-GC-MS, which provides a rapid and sensitive tool to analyze MPs in wines. In addition, they evaluated the sensory descriptive impact of these different yeast strains on MPs. They found that wine produced with yeast EC1118 scored significantly higher than control wines (no IPMP added) for earthy/peanut/must aroma, green pepper aroma, nutty/peanut flavor and canned green vegetable (six sensory attributes associate with MPs). In contrast, juice fermented with D21 yeast produced wines with the lowest intensity of the sensory attributes associated with MPs. Authors suggested that these results were due to a masking effect from others aromas. This is in agreement with other studies, which demonstrated the masking effect of oak on "ladybug taint" (LBT) -MPs (57).

Another possible strategy to reduce MPs in wine is the use of mannoproteins. Mannoproteins produced by yeast are the second most abundant family of polysaccharides in wine, representing ca. 35% of the total polysaccharides (62, 63).

Yeast mannoproteins are highly glycosylated proteins containing over 90% sugars, mainly mannose, which are located in the outermost layer of the yeast cell wall. Most of them act as structural components, giving the cell wall its active properties and being partially responsible for its permeability (64). In *Saccharomyces cerevisiae*, the glycan portion of mannoproteins is mainly composed of mannose, with some neutral oligosaccharides that contain *N*-acetylglucosamine and acidic sugars containing mannosylphosphate (65).

There are two groups of yeast mannoproteins present in wine. The first one are those secreted into wine by yeast during alcoholic fermentation at levels close to 100-150 mg/L. They possess highly varied sizes extending from 5000 at more than 80,000 Da. The second group of mannoproteins deals with those released into wine due to autolysis of yeast during ageing on lees, probably through the cleavage of linkages between mannoproteins, glucans and chitin (62).

These macromolecules play an important role in winemaking; for example, they are involved in the improvement of tartaric acid stability, reduction of protein haze, decrease in astringency of red wines, inhibition of tannin aggregation, stimulation of malolactic fermentation and the stabilization of wine color and phenolic compounds (63, 66-68).

Scientific researchers have studied the binding effect of volatile compounds on a yeast-wall, such as bentonite mixture in fining experiments. They found that binding of

β-ionone was higher (about 30%) than the three other volatile compounds studied (n-hexanol, ethyl hexanoate, isoamyl acetate) (69). Further studies confirmed that these interactions were mainly hydrophobic in nature, although binding was dependent on the type of aroma compound and nature of the substrate (66, 67). There are few studies that focus on the interaction between mannoproteins from yeast cell walls and aroma compounds (63, 66, 67, 69-71).

These studies have shown the importance of the physico-chemical nature of the volatile compounds and the conformational and compositional structure of these macromolecules. Moreover they observed different effects depending on the yeast strain that produced the mannoproteins (63). Authors suggested that the glycosidic and the peptidic bounds of these macromolecules are responsible for the interaction. However a greater degree of interactions was observed with hydrophobic compounds in a study performed with crude mannoproteins extracts (63, 66, 67, 71).

Another study demonstrated that industrial yeast derivatives (yeast extracts and autolysates) were able to modify the aroma composition by affecting the volatility of indigenous wine aroma compounds or by adding new aroma compounds such as ''yeast-like'' odors (72). Up to now, enologists' experience claims that mannoproteins added to wine at concentrations of 1 g/L might reduce the vegetative aroma in wine due to the binding properties of mannoproteins with MPs. However the amounts of MPs by analytical techniques such as SPME-GC-MS have not been conducted to demonstrate the above mentioned techniques.

Analytical Techniques to Measure Methoxypyrazines

A major problem with analysis of MPs in grapes and wines has been the lack of sensitivity of the available analytical techniques. Some authors have published different techniques to isolate and concentrate MPs. These techniques include liquid-liquid extraction (LLE), solid-phase extraction (SPE) and more recently solid phase micro-extraction (SPME) with the use of a deuterium isotope as internal standard.

LLE is the simplest and most widely used technique for analyzing aroma compounds of foodstuff. Dichloromethane, diethyl ether and a mixture between diethyl ether and hexane are the solvents that have been used to extract MPs in grapes, must and wine. Vacuum distillation and N_2 stream are the techniques that have been applied to concentrate MPs. However this technique is particularly unreliable because it requires a large concentration factor. Additionally, other compounds present in the sample appear as interferences (73).

Solid-phase microextraction (SPME) is a new, solvent-free sample preparation technique, commonly used in trace analysis in complex matrices such as foodstuffs. This technique has been developed to combine sampling and sample preparation in one step. SPME can be a fast, sensitive, solventless and economical method of sample preparation before analysis using gas chromatography and in some cases, high-performance liquid chromatography (74). The sensitivity of the technique depends mainly on the value of the distribution constant of analytes partitioned between the sample and the stationary phase of a fibre (Kfs). The type of fibre affects the amount and character of sorbed species (75).

Recently scientists have been using SPME-GC to analysis MPs in grapes, must and wines without using an isotopically labeled internal standard which can lead to a greater degree of uncertainties (76). To address this limitation and also to permit automation, an analytical method using SPME with stable isotope labeled internal standards coupled with GC-MS is used in this work. This method has the advantage that both the target and the standard molecule behave conservatively throughout the entire extraction process. This method focuses on the quantification of IBMP and IPMP using their deuterium labeled analogue as the internal standard for each target compound. IBMP and IPMP in grapes, must and wines can be reliably and accurately quantified down to sensory threshold levels (2.5 ng/L).

Yeast Nutrients

Saccharomyces species can grow on a minimal range of organic and inorganic nutrients, with hexose sugars as a source of carbon and energy under anaerobic conditions. However sources of nitrogen, phosphate, sulfate, and various minerals K+, Mg2+ and ZN2+ and trace elements, provide the necessary nutrients to growth and reproduction. In wine fermentations, *Saccharomyces* species have been shown to produce limited growth in the complete absence of oxygen. Exposure of fermentation to oxygen is normally limited, to prevent unwanted oxidation reactions. However, small additions stimulate growth and fermentation.

Grape must typically contains sufficient essential nutrients to enable the adequate growth of yeast, which is necessary to complete the fermentation of sugars, however

supplementation experiments reveal that most nutrients are not sufficient; sugars are major exceptions. Glucose and fructose are fermented to ethanol and CO_2 to provide energy and carbon compounds for yeast growth. Sugar concentration determined the final ethanol concentration of wines. The catabolism of sugars leads to formation of non-volatile compounds, polyols and carboxylic acids, the volatile fatty acids, and volatile sulfur compounds; polymers like mannoproteins are also produced.

Saccharomyces cerevisiae yeast requires a relatively high level of nutrients to complete the fermentation of grape must, typically producing 12-15% v/v ethanol. Yeast assimilable nitrogen (YAN) has been identified as a key nutrient that is often suboptimal in many grapes must worldwide (77-81). A minimal concentration of more than 140 mg/L is often quoted as necessary for the fermentation of low-solids (filtered), low temperature (< 15 °C), anaerobic must of moderate sugar level (20%) (77).

Importance of YAN for Wine Production

Yeasts assimilate a variety of nitrogen compounds, predominantly primary amino acids, ammonium ions and small peptides. These compounds are rapidly accumulated by yeast in the early stages of fermentation, during which they fill the biosynthetic pools of amino acids needed for protein synthesis and growth, while the surplus is stored in cell vacuole (78, 82). The types and concentration of nitrogen during fermentation regulates yeast growth and metabolisms and therefore affect the production of non-volatiles and volatiles compounds, which many of these have a sensory implications (77). The non-volatile compounds, glycerol and the carboxylic acids, malic acid, α-ketoglutaric acid,

and succini acid, have all been reported to vary according to nitrogen source and concentration (77, 78, 83). The most important volatile compounds include higher alcohols, short to medium chain fatty acid (CMFAs), and their ethyl esters and acetate esters. Ethyl esters are important for wine quality because they elicit pleasant aromas. Higher alcohols can impart alcohol or solvent odours of floral in the case of 2-phenylethanol, while MCFAs have soupy, unpleasant odours (61, 84).

Insufficient YAN is often associated with lower biomass yield, which in turn, slows fermentation rate with increased risk of sluggish or stuck fermentation, increased production of undesirable thiols (eg. Hydrogen sulfide) and higher alcohols and low production of esters and long chain volatile fatty acids (77, 80).

High YAN leads to increased biomass and increased formation of ethyl acetate, acetic acid, and volatile acidity. Increased concentrations of haze-causing proteins, urea and ethyl carbamate and biogenic amines are also associated with high YAN musts (77).

Intermediate must YAN leads to the best balance between desirable and undesirable chemical and sensory wine attributes. This can be achieved in the winery by the use of nitrogen supplements, such as diammonium phosphate (DAP) and the choice of fermentation conditions (77, 78).

Components of YAN

The primary amino acids constitute a major source of YAN for yeast, however they vary in efficiency as nitrogen sources (80). Table 1 list the individual amino acids commonly found in the whole grape and grape juice. This table also illustrates that the

concentration of each amino acids vary significantly. The amino acid concentration and composition can vary according to the grape cultivar, rootstock, site and seasonal conditions and level of maturity (77, 78, 80, 85).

Viticultural practices such as rate and timing of nitrogen application, soil management techniques and even *Botrytis* infection are important determinants of grape amino acid composition and concentrations (80). Total amino acids are important in terms of vine metabolism; therefore it is the assimilable portion of the total amino acid components that is oenological significance. Assimilable amino acids as a percentage of YAN increase during ripening. However this trend was unaffected during the application of nitrogen in the vineyard (86, 87).

The assimilable amino acid component can account for 51-92% of juice YAN at harvest (85). L-arginine and L-proline generally make up the greatest proportion of the total amino acid concentration present in the grape (87). Arginine is an important nitrogen source for yeast, but its accumulation by yeast is regulated by the presence of more preferred nitrogen sources such as ammonium (77, 80).

DAP addition therefore, inhibits arginine utilization until the ammoniun has been metabolized. Arginine serves as a nitrogen source for yeast due to its catabolism by arginase to form L-ornithine and urea. The excretion of urea, which results for arginine metabolism by some strains has implications for ethyl carbamate formation in wine (80). L-proline is only utilised to a limited extent by yeast in the normal anaerobic environment of alcoholic fermentation due to the need for oxygen equivalents by proline oxidase, which catalyses the first step in its catabolism (77).

Phosphate is an essential nutrient for energy metabolism and consequently regulates many metabolic pathways, however there is no information available about how phosphate affects flavor metabolism. Similarly, metal ions primarily function as cofactors in enzymatic reactions but the link between availability and flavor metabolism is unknown. Conversely, certain vitamins, particularity bioctin, and pantothenic acid, are known to affect the formation of aroma compounds due to their variable content in must.

Recent research has focused on how much and when to add the N supplement in relation to controlling fermentation rate. However, little is known about the impact of N on the flavor profile of wine. The metabolic pathways for the assimilation of nitrogenous compounds that are present in grape juice by yeast are well known but the regulation of these pathways is still being determined.

New Technologies for Wine Production

Wine technology and production techniques have changed in the last century, and in the last three decades the use of micro-oxygenation and acceleration of aging, alone or in combination, have shown to be a feasible option for wineries to modify the chemical profile and sensory characteristics of wines in a short period of time and even improve the quality of wines (88-92).

Micro-oxygenation

During the winemaking process, oxygen is required and plays an important role in wine maturation. At early stages oxygen aids in the fermentation, and later on helps to

stabilize color and develop aromas during aging (22). Despite these beneficial effects of oxygen, an excess may lead to wine oxidation having negative effects such as phenol oxidation, higher astringency, and adverse microbial activity (93).

Micro-oxygenation (MOX) is a technique that consist in introducing small and measured amounts of oxygen into wines with the objective of improving wine color, aroma and texture and involves the use of specialized equipment to regulate the oxygen doses applied (94). Typically dosage rates are relatively small, ranging from 2-90 mg of O2/L of wine/month, this value depends on the type of wine and the stage of maturation (95).

MOX does not usually include the passive oxygen exposure that occurs during barrel ageing, pumping over, topping up and racking, whereby oxygen exposure may be intentional but not measured. Micro-oxygenation can be applied at any stage of winemaking however, several studies have demonstrated that MOX has shown more effective results in improving wine structure before malolactic fermentation when tannins and anthocyanins are still mostly in simple monomeric form (91, 93). Tannin and anthocyanin reactions occur independently of MOX, so when MOX is applied after malolactic fermentation, it has to act on substrates that have already undergone some polymerization and condensation (94, 96, 97).

Micro-oxygenation was developed in France in the mid 1990s in an attempt to replicate barrel conditions for wine matured in large stainless steel and cement vessels, although nowadays it is not only an alternative to oak barrel ageing, but it's a tool for

winemakers to enhance color stability, as well as reduce the herbaceous aromas and improve mouth feel (92, 93).

This technique has been applied in recent years all around the world, such as in Spain, France, Italy, Australia, New Zealand, The United States and Chile.

MOX provides several benefits to the winemaking process. MOX has an impact on the phenolic composition of wines, and thus an indirect influence on the astringency and color stability (97). The reactions in which oxygen can participate include oxidation, condensation, and polymerization of different compounds. As a result, new compounds and pigments are formed giving to the wine its characteristic structure and color stability (98). The yeast is another target of adding oxygen during alcoholic fermentation. Recent work demonstrated that the correct addition of oxygen during alcoholic fermentation may give to yeast a higher resistance to ethanol and as well causes yeast to assimilates more nutrients such as N2. While in the absence of oxygen, medium chain fatty acids accumulate in the yeasts and can be secreted into the wine, contributing to suck fermentation (94).

Health Benefits of Dietary Polyphenols

Several epidemiological studies strongly suggest that consumption of fruits, vegetables, and grains is associated with the prevention of chronic degenerative diseases such as cardiovascular diseases, cancer, diabetes, and Alzheimer's disease (99). Fruit and vegetables are the main dietary sources of polyphenols for humans, along with tea and wine. It's known that polyphenols have antioxidant properties, which may have one

or more of the following functions: free radical scavengers, reducing agents, potential complexes of prooxidant metals, and quenchers of the formation of singlet- oxygen. Due to their antioxidants properties, dietary polyphenols are able to contribute to the control of oxidative reactions and provide protection in vivo (100). In addition, polyphenols have shown to inhibit the enzymes responsible for radical production, including xanthine oxidase and protein kinase C, cyclooxygenase, lipoxygenase, microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, and NADH oxidase, and to efficiently chelate trace metals that play important roles in the generation of reactive oxygen species (100).

Recent studies suggest have shown that polyphenols from fruit, vegetables and wine promote protective effects against several types of cancer cell lines, as well as in animal models (101). In general, several studies have demonstrated that polyphenolic compounds are effective in reducing cell proliferation, inducing apoptosis, modulating cell signals through different pathways, and arresting cells in specific stages of the cell cycle (102).

Health Benefits of Grapes and Wine

Several studies have demonstrated that red wine provides health benefits against chronic degenerative diseases such as cardiovascular diseases, diabetes and cancer (103, 104). The cardiovascular benefits of red wine became the focal point after the observation of the "French Paradox". The "French Paradox" correlated that in France there is a high intake of saturated fat but low mortality from coronary heart disease

(CHD) as opposed to most other countries. This paradox may be attributable in part to high wine consumption (105).

Wine is rich in polyphenolic compounds including flavonoids (catechin and epicatechin) and hydroxy stilbenes such as resveratrol and monomeric catechols like caffeic acids. Most of these compounds possess antioxidant, anti-inflammatory and anti-cancer effects (104). The health benefits of red wine acts through various mechanisms such as chemical antioxidant action, metal chelators and modulators of cell signaling pathways (104, 106).

Low-density lipoproteins (LDL) play an important role in the formation of atherosclerotic plaques and in the endothelial inflammatory pathway. Polyphenols from grapes and wine promote an inhibition of LDL oxidation in vivo by several different mechanisms. These mechanisms involve polyphenols that act as free scavengers, metal chelators and an increase antioxidant activity by sparing vitamin E and carotenoinds in the LDL particle (104).

Numerous studies have demonstrated that resveratrol is a potent cardioprotective compound (107). Recent studies demonstrated that resveratrol increases the expression in human vascular endothelial cells of endothelial nitric oxide synthase, an enzyme responsible for synthesizing the potent vasodilator nitric oxide. As well, it decreases the expression of the potent vasoconstrictor endothelin (108). Oxidative stress has been shown to induce endothelial dysfunction and the development of atherosclerosis. Evidence showed that 1–100 lmol/L resveratrol significantly inhibited intracellular and extracellular ROS production. It has been postulated that by enhancing the intracellular

free radical scavenger glutathione, resveratrol maintains cell viability and inhibits oxidation (109).

In addition, resveratrol posses anti-inflammatory properties by reducing the expression of cytokines such as interleukin-6 (IL-6) and interleukin-8 (IL-8) which are circulating cytokines that have been identified as a marker of inflammation in atherosclerotic plaques (108).

Molecular Aspects of Vascular Inflammation

Vascular inflammation is one of the causes for the initiation and progression of atherosclerosis and related complications such as acute thrombotic complications (110). Endothelial cells form a monolayer covering the inner surface of blood vessels and play an important role in the regulation of vascular inflammation (111).

Recent studies have demonstrated that chronic inflammation, induced by pathogenic bacterial/viral infection or non-pathogenic factors, such as oxidized low-density lipoprotein, plays a major role in the development of atherosclerosis (110, 112). During the early stage of atherosclerosis formation, endothelial cells produce cell adhesion molecules (CAMs) such as intracellular cell adhesion molecule-1 (ICAM), vascular cell adhesion molecule-1 (VCAM-1), and endothelial-selectin (E-selectin). Under this condition numerous leukocytes adhere to the vascular endothelium, transmigrate the endothelium causing endothelial dysfunction and tissue injury (110). Atherosclerotic lesions are also characterized by increased expression of several families of macrophage scavenger receptors, also implicated in uptake of modified lipoproteins, and activation of

the Toll-like receptor (TLR) cell signal (110, 113) The TLR, named after Drosophila genes, belong to the family of patter recognition receptors that recognize microbial structures and products. Bacterial lypopolysaccharide (LPS) from gram-negative bacteria, is a potent initiator of inflammatory responses and serves as an indicator of bacterial infections. Thus, LPS is commonly used as a model for endothelial inflammation. Although TLR-4 have been indentified as the main LPS receptor. When human cells are exposed to LPS, they bind to TLR-4 cell signaling pathway and then initiates the activation of down-stream mediators such as IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF-6) and activation of the pro-inflammatory transcription factor NF-kB that target the expression of proinflammatory cytokines such as tumor necrosis factor (TNF- α) and interlukin-6 (IL-6) (114).

There is scientific evidence confirming the role of reactive oxygen species (ROS) on inflammation and activation of downstream cellular events, including signal transduction, proliferative response, and gene expression (115). Indeed, elevated production of ROS is linked to multiple disorders, including diabetes, atherosclerosis, and cardiovascular complications (116). In addition, inflammation is a pathology that leads to atherosclerotic plaque formation and consequent blockage of arteries (Figure 7).

The underlying mechanisms involve the increased expression of pro-inflammatory cytokines such as tumor necrosis factor (TNF-α), the IL-6 family of cytokines, IL-8 and other chemokines. The effect of all these molecules cause activation of nuclear factor kappa B (NFKB) and in turns induces genes involved in inflammatory responses (117).

Polyphenolics have been shown to act at the molecular level to improve endothelial

function and inhibit platelet aggregation (99).
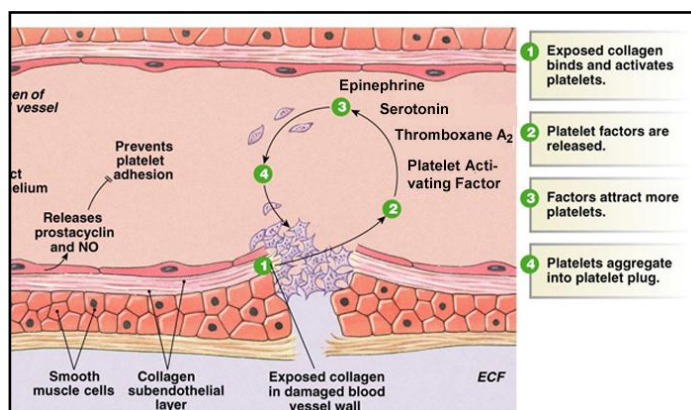
Figure 7 Platelet aggregation

Some of the endothelial protective mechanisms of plant polyphenolics are related to induction of antioxidant defenses through modulation of expression and activity of mitochondrial antioxidant enzymes to inhibition of endothelial cell adhesion molecules mediated by interference with the NF-kB-dependent transcription pathway to regulation of blood pressure through inhibition of angiotensin-converting enzyme activity, induction of prostacyclin and suppression of endothelin-1, a potent endothelium-derived vaso constrictor (118, 119).

Role of MicroRNAs in Inflammation

Currently, microRNAs (miRNAS) are being investigated for their role as post-transcriptional regulators of pro-inflammatory genes. miRNAS are an evolutionary

conserved class of endogenous ~22 nucleotides noncoding RNAs involved in posttranscriptional gene repression (120). miRNA suppress protein synthesis by inhibiting the translation of protein from mRNA or by promoting the degradation of mRNA, thereby silencing gene expression (121). miRNA are regulators of gene expression and recently have been discovered to be involved in various physiological and pathological process such as vascular inflammation. miRNA-126 and miRNA-146a are the most abundant miRNAs expressed in endothelial cells related to inflammation (122, 123). Urbich (124) demonstrated that miR-126 inhibited the expression of VCAM-1, thus, decreasing miR-126 in endothelial cells increases TNF-α –stimulated VCAM-1 expression and enhances leukocyte adherence to endothelial cells. miRNA-146a has been directly implicated in mechanisms by which innate immune response is regulated. It acts as a negative feedback loop and attenuates chronic inflammation by suppressing the expression of the toll-like and cytokine receptors downstream components (IRAK1 and TRAF6, whose activation leads to NF-kB activation (120). However, despite numerous studies that show the induction of miRNA-146a expression by pro-inflammatory mediators, there is still little information regarding its role and mechanism of action.

CHAPTER III

RED WINE POLYPHENOLS ATTENUATES INFLAMMATION IN CCD-18CO COLON CELLS AND TARGET MICRORNA-126

Summary

Chronic intestinal inflammation is a significant risk factor for colon cancer. and microRNAs are emerging as a potential factor relevant to inflammation. Polyphenolic compounds from fruit and vegetables have been demonstrated to have anti-inflammatory properties, but their role in microRNA regulation in inflammation has not extensively been investigated. The overall goal of this research was to assess the chemopreventive potential of polyphenolics extracted from red wine in human colon cells and the involvement of microRNAs as underlying mechanism. Results show that wine polyphenolics decreased the mRNA expression of lipopolysaccharide (LPS)-induced ICAM, VCAM, PECAM and NF-kB in a dose dependent manner (0-100 μg GAE/mL) by 0.79, 0.66, 0.68 and 0.80 fold, respectively. Protein expression was also decreased in a dose-dependent manner as determined by Western Blotting. miR-126, which has a target region within the mRNA of VCAM, was increased by 2.79-fold by the wine extract at 100 ug/mL.The potential role of miR-126 was confirmed by transfecting cells with the inhibitor of miR-126. The inhibitor down-regulated miR-126to 0.71-fold of control cells and mRNA levels of ICAM, VCAM, PECAM and NF-kB were up-regulated by 3.25, 1.49, 2.91 and 1.75-fold respectively. This indicates the potential role of miRNA-126 in the anti-inflammatory properties of wine extract in CCD-18Co.

Introduction

Inflammatory bowel disease (IBD) is a chronic disease characterized by uncontrolled inflammation of the intestinal mucosa which can the gastrointestinal tract (125). Pathophysiological bases of this disease involve genetic factors, immune dysregulation, barrier dysfunction, and a loss of immune tolerance towards the enteric flora (126). Increase of inflammatory mediators, including reactive oxygen species such as nitric oxide, prostaglandins and inflammatory cytokines play an important role in immune dysregulation (125).

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic inflammatory disorders of the intestines, collectively designated IBD (127). The increasing incidence of inflammatory bowel disease over the last decades has been associated with changes in dietary pattern. Although a clear relationship between wine's polyphenols and inflammatory bowel disease has not been established yet.

Additionally, polyphenolics from fruit and vegetables have been shown to exert anti-inflammatory effects mediated through the inhibition of nuclear factor-kappaB (NF-kB). These protective effects may have implications in the prevention of colon carcinogenesis, since sustained pro-inflammatory signals in colonic epithelial cells and ongoing mucosal inflammation can result in IBD and colorectal cancer (128).

In IBD, cytokines, chemokines and cell adhesion molecules (CAMs) play an important role in the pathogenic process of IBD. Fibroblast cells provide a framework that actively modulates several T-cell functions, including proliferation, survival and cytokine production. Previous studies have demonstrated that CAMs molecules such

intracellular cell adhesion molecule-1 (ICAM), and vascular cell adhesion molecule-1 (VCAM-1) are expressed on the surface of fibroblasts (129). Recent studies have demonstrated the increased expression of ICAM-1 in CD in the intestinal mucosa, submucosa and muscle layers, and this may contribute to local interactions with lymphocytes that have penetrated the deeper layers of the intestinal wall as it is characteristic of CD (130). It has been demonstrated that downregulation of CAMs molecules and cytokines plays an important role on the inhibition of various forms of experimental immune and inflammatory responses in colon fibroblast cells (131).

In addition, some studies have shown that fibroblasts grown from histological normal human duodenal mucosa expressed gene expression for granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukins (IL-) IL-1a, IL-1b, IL-6, IL-8 and IL-10 when stimulated with bacterial lypopolysaccharide (LPS) or IL-1α (132). LPS and virus-proteins can increase cytokine secretion, suggesting a role in host defense. Moreover, LPS stimulation of myofibroblasts have demonstrated to be involved in intestinal fibrosis and disturbance of epithelial cell barrier function (133).

The transcription factor NF-kB is a major regulator of pro-inflammatory cytokines (134). Activation of NF-kB by inflammatory cytokines or microbial pathogens has been demonstrated (135, 136). Patients with IBD have been detected an elevated levels of tumor necrosis factor (TNF-α), IL-6 and ICAM-1 and VCAM-1 expression molecules (130). There is strong scientific evidence that demonstrated the suppression of CAMs molecules expression could diminish leukocyte trafficking into inflamed bowel tissue (137).

miRNAS are an evolutionary conserved class of endogenous ~22 nucleotides noncoding RNAs involved in posttranscriptional gene repression (120). Currently, microRNAs (miRNAS) are being investigated for their role as post-transcriptional regulators of pro-inflammatory genes. miRNA suppress protein synthesis by inhibiting the translation of protein from mRNA or by promoting the degradation of mRNA, thereby silencing gene expression. miRNA are regulators of gene expression and recently have been discovered that are involved in various physiological and pathological process such as vascular inflammation (138). Urbich research (124) demonstrated that miR-126 inhibited the expression of VCAM-1, thus, decreasing miR-126 in endothelial cells increases TNF- α – stimulated VCAM-1 expression and enhances leukocyte adherence to endothelial cells.

Several studies have addressed the anti-inflammatory properties and health benefits of fruit and vegetables polyphenolics, there are no reports showing how wine polyphenols from Black Spanish grapes exert a chemopreventive protection against inflammatory colon diseases. The objective of this research was study the effect of wine polyphenolics on the mitigation of cellular damage on non malignant colon cells, with relevance to chronic inflammatorydiseases and the involvement of microRNA-126 as potential underlying mechanism.

Material and Methods

Chemicals, Antibodies, and Reagents

The Folin-Ciocalteu reagent, dichlorofluorescein diacetate (DCFH-DA), and lipopolysaccharide (LPS) were purchased from Fisher Scientific (Pittsburgh, PA). Dimethyl sulfoxide (DMSO) was obtained from Sigma (St Louis, MO). Bradford reagent was obtained from BioRad (Hercules, CA), antibodies against NF-kB p65, and phospho-NF-kB p65, ICAM-1, PECAM-1, IL-6, TNF-α, and GAPDH were obtained from Cell Signaling Technology (Beverly, MA); VCAM-1 was obtained from Santa Cruz Biotechnology, Inc.(Santa Cruz, CA).

Red Wine Polyphenolics Extraction

A red wine, Port Barrel Reserve made of Black Spanish grapes, was provided by Messina Hof (Bryan, Tx). Wine polyphenolics were extracted using a C18 cartride (Waters, Inc., Mildford, MS). The wine (5X diluted with acidified water) was applied to the C18 cartridge under vaccum and polyphenolics were eluted with acidified methanol after washing the cartridge with acidified water. The methanol was evaporated in a rotavapor (Büchi Laborthechnik AG, Flawil, Switzerland) at 40 °C. The extract was storage at -80 ° C and dissolved in dimethyl sulfoxide (DMSO) prior use.

Determination of Total Soluble Phenolics

Total soluble phenolics were determinated by Folin-Ciocalteu assay using a microplate reader FLUOstar (BMG LAbthec Inc., Durhman, NC) with absorbance read

at 726 nm and quantified by linear regression using gallic acid standard, and expressed as gallic acid equivalents (GAE) (*139*).

Cell Lines

The human non-cancer colon CCD-18Co cells were obtained from ATCC (Manassas, VA). CCD-18Co cells were cultured using high glucose Dulbecco's Modified Eagle Medium, supplemented with 1% penicillin/streptomycin, 1% non-essential amino acids (10mM), 1% sodium pyruvate (100 mM) and 20% of fetal bovine serum (Invitrogen, Carlsbad, CA). Cells were maintained at 37 ºC with a humidified 5% CO_2 atmosphere.

For cell culture assays, the wine extract was dissolved in dimethyl sulfoxide (DMSO). The extractwas then diluted to a known concentration of total polyphenolics and normalized to contain a maximum of 0.2% DMSO in the culture medium, which did not show any effect compared to untreated control cells. A control with 0.2% DMSO was included in all assays.

Cell Viability

Cell viability was assessed using the CellTiter 96® Aqueous One Solution (Promega, Madison, WI) following the manufacturer's protocol. CCD-18Co cells were seeded at a density of 3,000 and 5,000 cells per well, respectively in a 96-well plate and incubated for 24 h to allow cell attachment prior to incubation with extracts for 48 h. The absorbance ratio between cell culture treated with the extracts and the untreated controls x 100, represents cell viability (percentage of control). Absorbance was measured at 490

nm with a 96-well plate reader (BMG Labtech Inc., Durham, NC). Cell counting was assessed using an electronic particle counter (Z2™ Series, Beckman Coulter, Inc, Fullerton, CA) on CCD-18Co after a 48 h treatment with the wine extract to confirm that cell growth was not inhibited.

Generation of Reactive Oxygen Species (ROS)

Cells seeded in a 96-well plate (3,000/well) for 24 h to allow cell attachment, prior to incubation with wine extract (25 to 100 μg GAE/mL) for 24h. ROS generation was induced with 1 μg/L LPS for 4 h and detected using 2', 7' -dichlorofluorescein diacetate (DCFH-DA) (116).The fluorescence signal was monitored after 30 min at 520 nm emission and 480 nm excitation with a FLUOstar Omega plate reader (BMG Labtech Inc, Durhan, NC). Relative fluorescence units (RFU) were normalized to control cells not treated with wine extract.

Real time PCR analysis of mRNAs and microRNAs

Total RNA was isolated according to the manufacturer's recommended protocol using the RNeasy Mini kit (Qiagen, Valencia, CA) for mRNA analysis and the mirVana^{TM} miRNA isolation kit (Applied Biosystems, Foster City, Ca) for micro-RNA analysis. Samples were evaluated for nucleic acid quality and quantity using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at 260 and 280 nm. Isolated RNA was used to synthesize cDNA using a Reverse Transcription Kit (Invitrogen Corp., Grand Island, NY) according to the manufacturer's

protocol. PCR-RT was carried out with the SYBR Green PCR Master Mix from Applied Biosystems (Foster City, Ca) on an ABI Prism 7700 Sequence Detection System (Applied Biosystems Inc, Foster City, CA). Primers were designed using Primer Express software (Applied Biosystems, Foster City, CA). Each primer was homology-searched by an NCBI BLAST search to ensure that it was specific for the target mRNA transcript. The pairs of forward and reverse primers were purchased from Integrated DNA Technologies, Inc. (San Diego, CA). Product specificity was examined by dissociation curve analysis. The sequences of the primers used were:

IL-6: F: 5'-AGGGCTCTTCGGCAAATGTA-3',

IL-6: R: 5'-GAAGGAATGCCCATTAACAACAA-3'

NF-kB: F: 5'-TGGGAATGGTGAGGTCACTCT-3'

NF-kB: R: 5'- TCCTGAACTCCAGCACTCTCTTC-3'

VCAM-1: F: 5'-ACAGAAGAAGTGGCCCTCCAT -3'

VCAM-1: R: 5'-TGGCATCCGTCAGGAAGTG -3'

ICAM-1: F: 5'-TGGCCCTCCATAGACATGTGT -3'

ICAM-1: R: 5'- TGGCATCCGTCAGGAAGTG -3'

TNF-α: F: 5'- TGTGTGGCTGCAGGAAGAAC -3'

TNF-α: R: 5'- GCAATTGAAGCACTGGAAAAGG -3'

Quantification of miR-NU6B and miR-126a were performed using the Taqman®MicroRNA reverse transcription kit (Applied Biosystems, Foster City, CA). For the PCR reaction, the reverse transcription product was diluted 1:15 and amplified

using TapMan®2X Universal PCR Master Mix (No AmpErase®UNG) (Applied Biosystems, Foster City, CA) according to the manufacturer's specifications. To determine relative miR-146a expression, miR-NU6B small nuclear RNA was used as an endogenous control.

Protein Expression

Cells seeded in 10 cm plates ($1x10^6$) were allowed to stabilize for 24h before their treatment with the wine extract (25 to 100 μg GAE/mL) for 30 min followed by LPS (1 ug/mL). Cell lysates were obtained after 24 h and analyzed by western blots. For western blot analysis, cells were washed and lyses with non-denaturing buffer (10 mM Tris-HCl, 10 mM NaH_2PO_4, 130 mM NaCl, 1% (v/v) Triton X-100, 10 mM sodium pyrophosphate, (pH 7.5), and 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Protein content was determined using the Bradford reagent (Bio-Rad, Hercules, CA) following the manufacturer's protocol. Cell lysates (60 μg protein diluted with Laemmli's loading buffer (Invitrogen, Carlsbad, CA) and boiled for 5 min) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis at 100 V for 2 h. Proteins were transferred by wet blotting onto a 0.2 μm PVDF membrane (Bio-Rad, Hercules, CA). Membranes were blocked using 5% non-fat milk in 0.1% Tween-PBS (T-PBS) for 30 min and incubated with primary antibody (1:1000) in 5% bovine serum albumin in T-PBS overnight at 4°C with gentle shaking. This was followed by incubation with the secondary antibody (1:2000) in 5% milk T-PBS for 2 h. Reactive

bands were visualized with a luminal reagent (Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) after 1 min of incubation.

Transfection Assay

Transfection with 0 to 100 nmol/mL as-miRNA-126a (Dharmacon) was performed using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according the manufacturer's protocol when cells were 60% confluent. The controls for the miR-126a experiments used an equal amount of a nonspecific oligonucleotide. After transfection for 5 h, the transfection mix was replaced with complete medium. Cells were collected at 24 hrs after transfection for RNA extraction.

Luciferase Assay

Cells were plated in 12 well plates at 1×10^5 per well and incubated with 50 and 100 μg GAE/mL wine extract for 18 hr, then lysed with 200 μL of 1X reporter lysis buffer (Promega, Madison, WI) and 30 uL was used for the luciferase assays. Lumicount microwell plate reader (Packard Instrument, Dowers Grove, IL) was used to quantify luciferase activities, which were normalized to protein concentrations for each sample.

Statistical Analysis

Quantitative data represent mean values with the respective standard deviation (SD) or standard error of the mean (SE) corresponding to 3 or more replicates. Data from each

chemical analysis were analyzed by one-way-analysis of variance (ANOVA) using JMP version 8.0 (SAS Institute Inc., Cary, NC). Mean separations were deemed significant at $p \leq 0.05$ using a Tukey-Cramer HSD comparison for all pairs.

Results and Discussions

Chemical Analysis

The concentration of total soluble phenolics was 28000 µg GAE/mL. The wine had 1692 ug GAE/mL. The content of total soluble phenolics was within the same concentration-range of other red wines prepared from different varieties with an average of 1600 µg GAE/mL as previously reported (101, 140).

Effect of Wine Extract on Normal Colon Cell Growth and Protection Against the Generation of Reactive Oxygen Species

The net growth of the CCD-18Co cells was assessed by cell counting after wine extract treatment for 48 h. Wine extract did not inhibit the growth of cells at concentration up to 100 µg GAE/mL. The LPS-induced generation of ROS was significantly reduced by wine extract at a concentration of 100 µg GAE/mL by 68% when compared to the control (Figure 8). Previous studies have investigated the effects of botanical extracts on the generation of ROS in human fibroblast cells and have demonstrated protective effects against oxidative damage (141). Natural antioxidants such the polyphenolics present in wine extract and fruit and vegetables have been

demonstrated to possess radical scavenging properties, which can neutralize free radicals and reduce oxidative damage (104, 109, 142, 143). In this study the polyphenolic extract from red wine reduced the generation of intracellular ROS likely based on their antioxidant capacity (144).
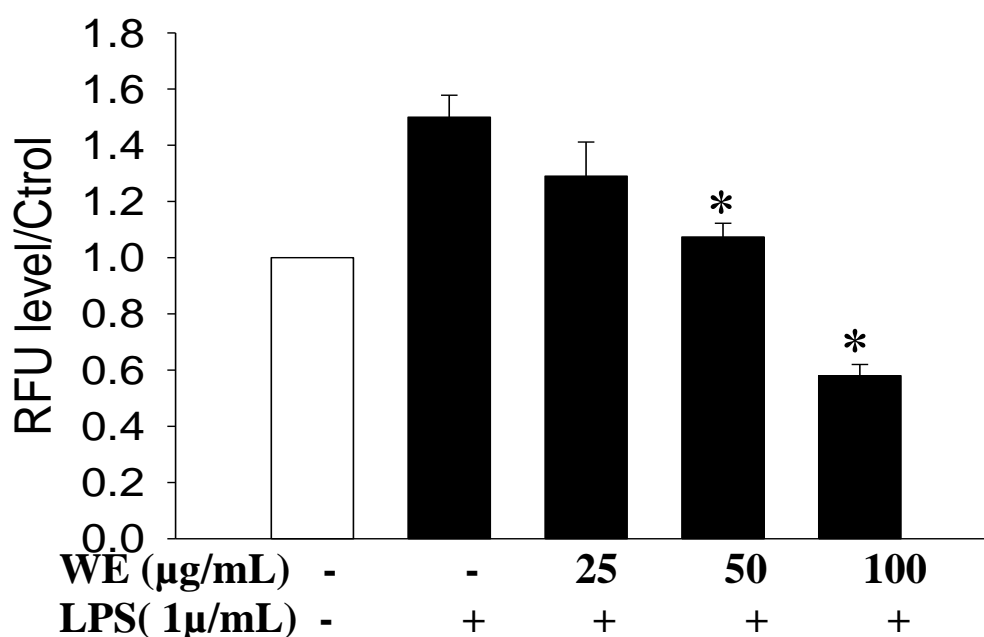
Figure 8 LPS-induced generation of reactive oxygen species in CCD18 cells treated with wine extract. Each experiment was performed at least three times, and results are expressed as means ± SEM. Statistically significant differences from LPS-induced cells are indicated by* ,P< 0.05).

Effect of Wine Extract on LPS-Induced Inflammation

The effect of wine extract on LPS-induced inflammation was investigated as a model to assess their potential role in the protection against inflammatory bowel diseases.

The LPS-induced NF-KB was modulated by wine extract at gene and protein expression levels. At gene expression level (Figure 9) NF-kB was up-regualted by LPS by 1.95-fold; this effect was significantly reversed by wine extract at 100 μg GAE/mL down to 0.43-fold of LPS-challenged cells. Likewise, the wine extract decreased the protein-level of the active phospho- NF-kB p65 uni at 100 μg GAE/mL. These findings suggest that the wine extract decreased NF-kB activation which plays an important role in the initiation and progression of chronic diseases (145).
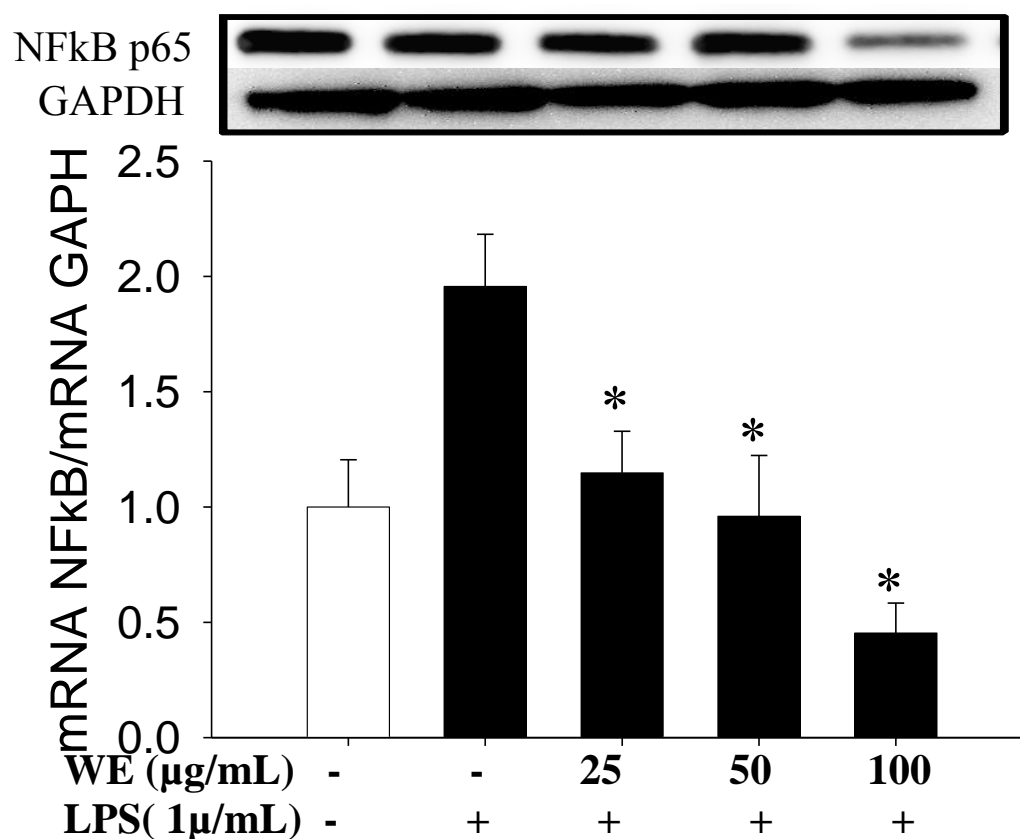
Figure 9 Gene and protein expression of NF-kB in CCD-18Co cells after 16 hr of incubation with wine extract. Each experiment was performed at least three times, and results are expressed as means ± SEM. Statistically significant difference from LPS-induced cells,* indicates significantly changes at P< 0.05).

The reduction of NF-kB activation and inflammatory cytokines has also been reported for polyphenolics in fruits, vegetables and teas (125, 136, 146, 147). NF-kB activation leads to up regulation of a wide range of pro-inflammatory genes included cytokines and CAMs molecules (148).

LPS-induced pro-inflammatory cytokine/chemokines TNF-α and IL-6 were decreased by wine extract at gene expression levels (Figure 10). TNF-α was induced by LPS up to 1.93-fold; this effect was reversed by wine extract at 100 μg GAE/mL down to 0.48-fold of LPS-challenged cells. Whereas gene expression of IL-6 up-regulated by LPS up to 1.95-fold, this effect was reversed by wine extract down to 0.58-fold of LPS-challenged cells at 100 μg GAE/mL. Overall, these results indicate the anti-inflammatory effects of wine extract.

Cell adhesion molecules play a role in the infiltration of leukocytes into the bowel wall, which is a landmark of IBD. Down-regulation of adhesion molecules can interfere the recruitment of leucocytes in colon cells therefore preventing the development of IBD (149).

Up-regulation of ICAM-1 and VCAM-1 has been shown in actively inflamed mucosa in patients with inflammatory bowel disease (IBD). ICAM-1 and VCAM-1 play an important role in the reduction of leucocyte-endothelial interactions because its been demonstrated that both.

The cell adhesion related genes ICAM-1, VCAM-1, and PECAM were up-regulated by LPS up to 1.98, 1.52, and 1.84-fold respectively, and with wine extract at 100 μg GAE/mL reversed mRNA levels of ICAM-1 down to 0.40-fold; VCAM-1 to 0.44-fold and PECAM to 0.37-fold of LPS-challenged cells, respectively (Figure 11).

The down-regulation of CAM's by wine extract indicates that the extract may be able to prevent transendothelial migration of lymphocytes, thus decreasing inflammatory responses in the intestinal mucosa (130).
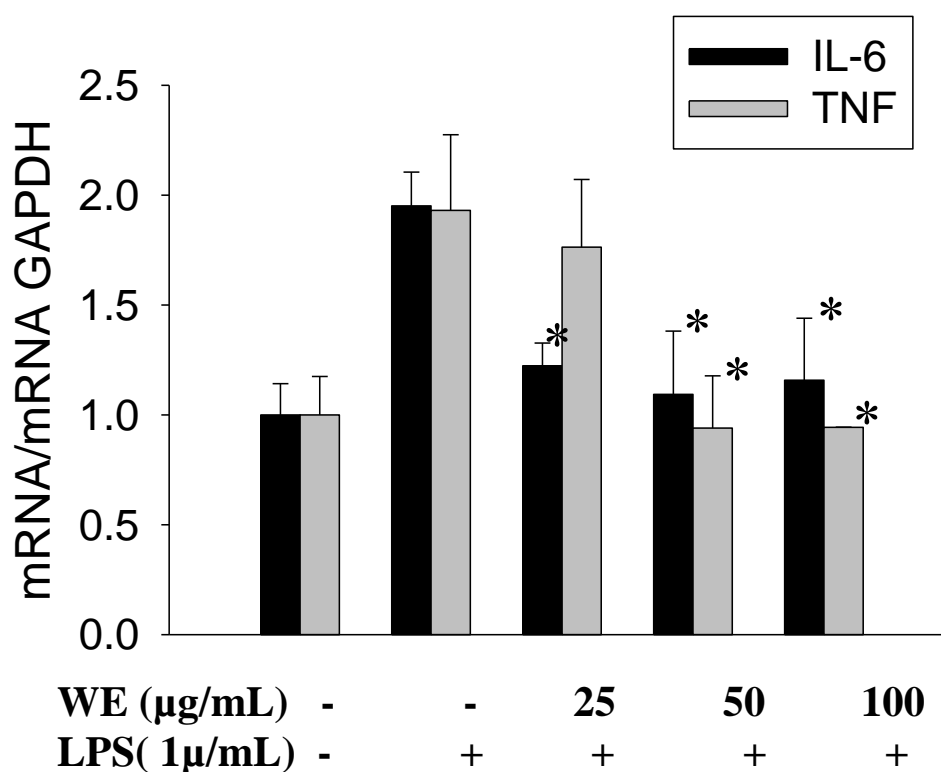
Figure 10 Gene expression of IL-6 and TNF-α gene expression in CCD-18Co cells after 16 hr incubation with wine extract. Each experiment was performed at least three times, and results are expressed as means ± SEM. Statistically significant difference from LPS-induced cells,* indicates significantly changes at P< 0.05).

Extensive research has demonstrated the inhibitory effects of grape polyphenolics in expression of intercellular adhesion molecules *in vitro* in non-cancer colon cell line CCD-18Co and in cancer cell lines, including Molt-4 leukemia, A-549 lung, MDA-MB-231 breast, and SW-480 colon cancer cells (150-153) and *in vivo* studies with Wistar rats and hyperlipidemia mices (154-156). Consequently, anti-adhesion molecules represent a target for botanical-based therapies in the reduction of inflammation in IBD.
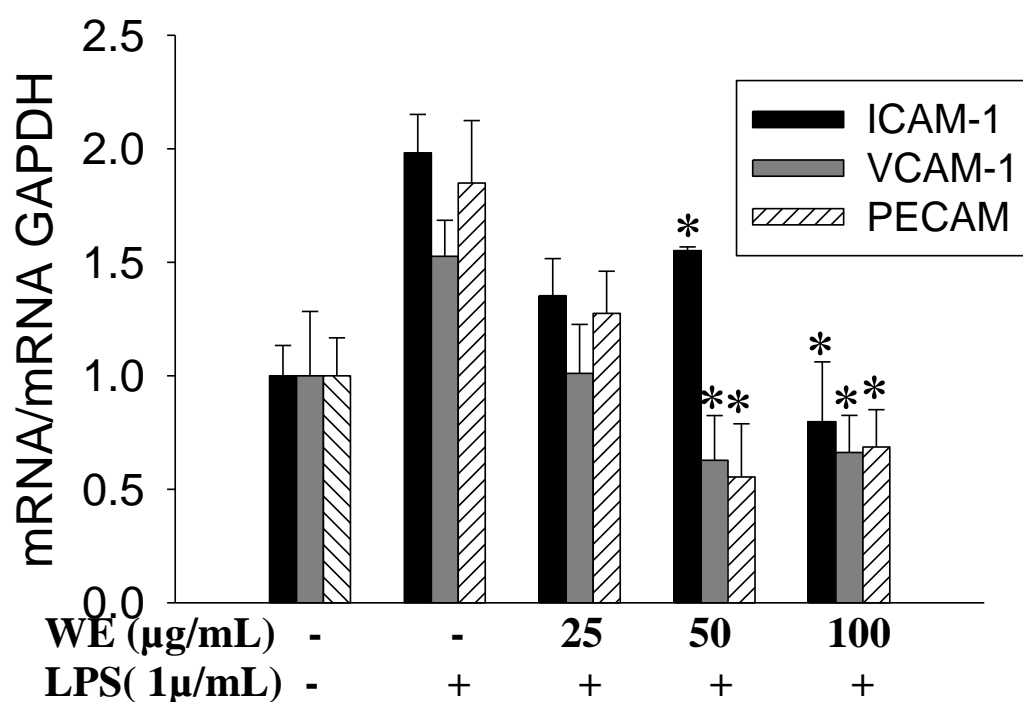
Figure 11 Modulation of cell adhesion markers on LPS-challenged CCD18Co after 16 hr of incubation with wine extract. Each experiment was performed at least three times, and results are expressed as means ± SEM. Statistically significant difference from LPS-induced cells,* indicates significantly changes at P< 0.05).

Moreover, protein expression of NF-kB target genes ICAM-1, VCAM-1, and PECAM were decreased by treatment with wine extract at 100 µg GAE/ml (Figure 12).
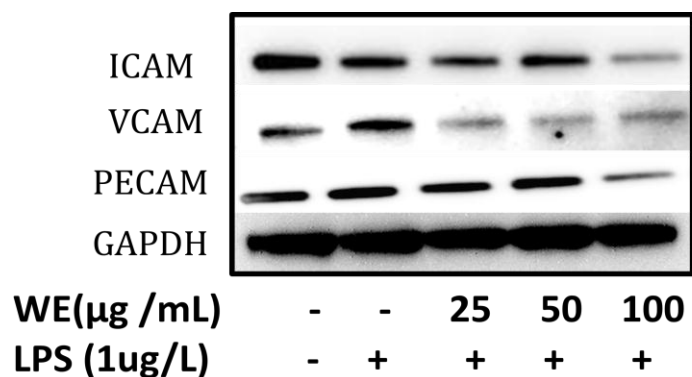
Figure 12 Protein expression of ICAM, VCAM and PECAM 16 hr after incubation with wine extract. Each experiment was performed at least three times.

Effect of Wine Extract on Gene Expression of miRNA-126a

miRNA-126 has been demonstrated to play an important role on regulating endothelial expression of VCAM-1 (157). However no previous studies have been reported the role of miRNA-126 in colon cells, nor their role in the anti-inflammatory effects of botanical extracts. Previous studies have demonstrated that VCAM-1 is involved in adhesion and transmigration of leukocytes expressing the α4-integrin ligand. This ligand plays a critical role in the pathogenesis of IBD. α4-integrin ligand inhibition results from interruption of VCAM-1 interaction. Thus, VCAM-1 plays an important role in IBD-derived chronic inflammation (148).

Results showed that LPS significantly decreased the expression of miRNA-126 and wine extract reversed this effects and induced miRNA-126 up to 2.79-fold of LPS-treated cells (Figure 13).
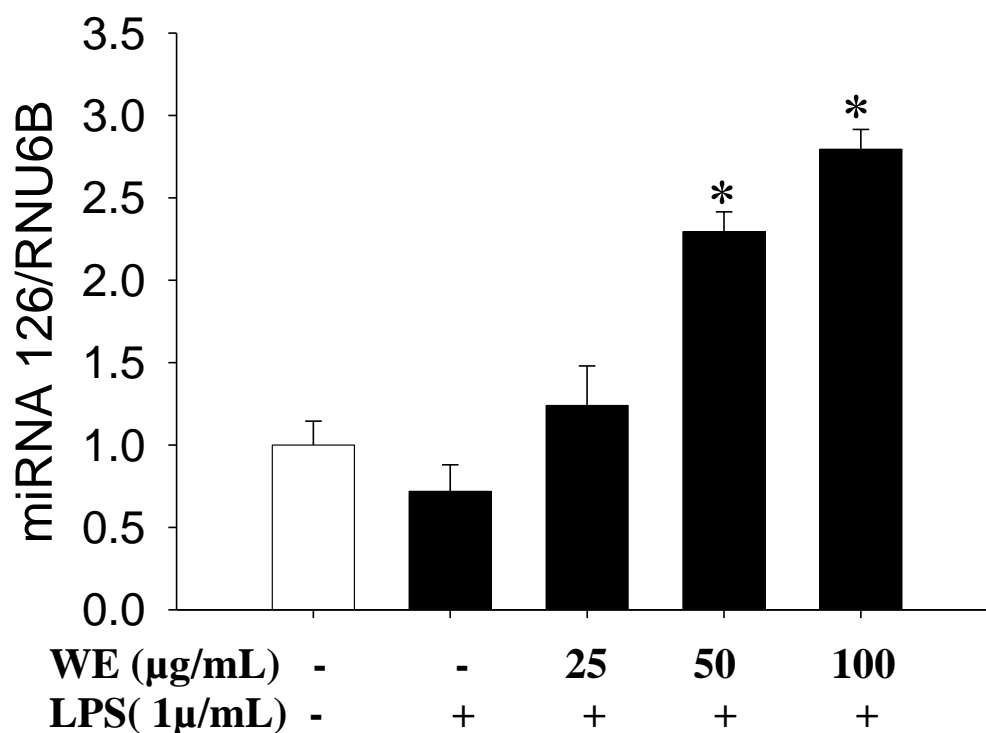
Figure 13 Effect of miRNA-126a on LPS-challenged CCD-18Co cells. Each experiment was performed at least three times, and results are expressed as means ± SEM. Statistically significant difference from LPS-induced cells,* indicates significantly changes at P< 0.05).

To demonstrate that wine extract induced the expression of miRNA-126 as one of the underlying mechanisms that decreased VCAM-1 and protect human colon cells from chronic inflammation, CCD-18Co cells were transfected with the antisense inhibitor miRNA-126a. Results showed that the inhibitor of miRNA-126 decreased the expression of endogenous miRNA-126a down to 0.71-fold while the wine extract significantly increased the levels of miRNA 126 up to 1.53-fold of control cells at 100 μg GAE/mL (Figure 14).
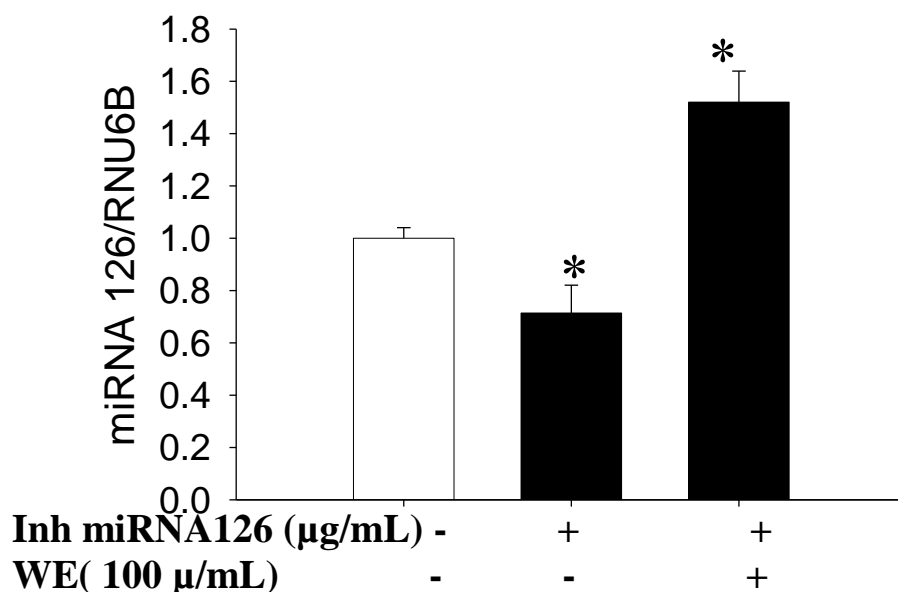
Figure 14 Effect of miRNA-126 Inhibitor (100 nM) with and without wine extract. Cells were treated with solvent (DMSO) or different concentration of wine extract (25-100 µg GAE/mL) for 30 min before LPS challenge (1 µg /mL) for 16 hrs and relative micro-RNA levels were determinated by qRT-PCR as described in materials and methods. Each experiment was performed at least three times, and results are expressed as means ± SEM. Statistically significant difference from LPS-induced cells,* indicates significantly changes at P< 0.05).

Moreover the gene expression of ICAM, VCAM, PECAM and NF-KB was increased up to 3.25, 1.49, 2.91 and 1.75-fold respectively by the inhibitor of miR-126. The wine extract reversed this effect and decreased mRNA levels down to 1.04, 1.02, 1.01, and 1.045-fold respectively of miRNA-126 knockout cells (Figure 15).
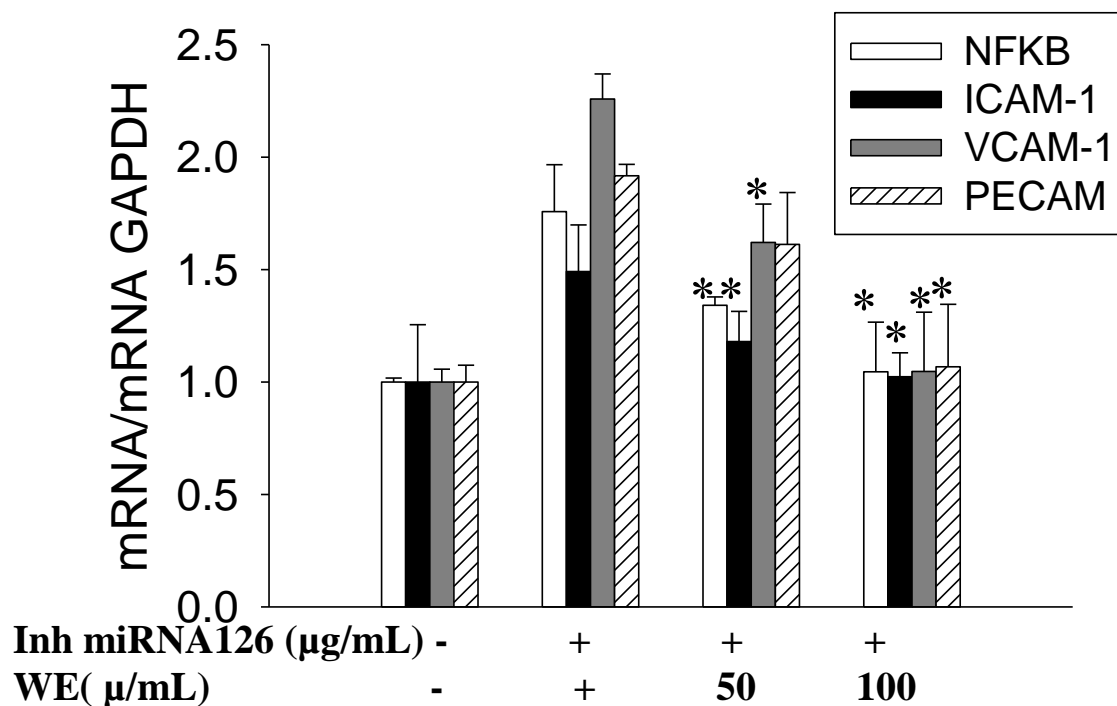
Figure 15 Effect of wine extract on gene expression of ICAM, VCAM, PECAM and NF-kB after cells were transfected with miRNA-126 inhibitor. Each experiment was performed at least three times, and results are expressed as means ± SEM. Statistically significant difference from LPS-induced cells,* indicates significantly changes at P< 0.05).

Overall, these results suggest that the anti-inflammatory effects exerted by wine extract are at least in part mediated thought the induction of miRNA-126, which significantly decreases VCAM-1 levels (Figure 16). Likewise, by transfecting CCD-18Co cells with miRNA-126 mimic, we confirmed the role of miRNA-126 in decreasing VCAM-1 protein levels (Figure 17).
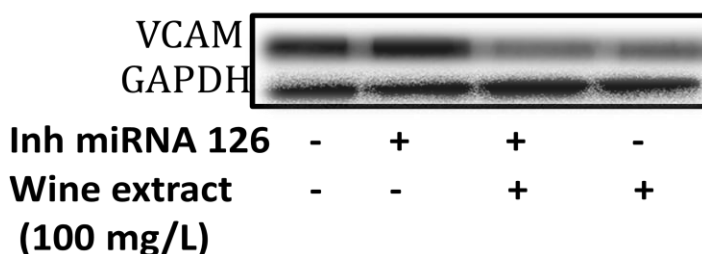
Figure 16 VCAM protein expression after cells were transfected with miRNA-126 inhibitor. Protein levels were assessed in cells transfected with antisense miRNA-126 inhibitor, cell lysates were analyzed by Western blot as described materials and methods. Each experiment was performed at least three times, and results are expressed as means ± SEM. Statistically significant difference from LPS-induced cells,* indicates significantly changes at P< 0.05).
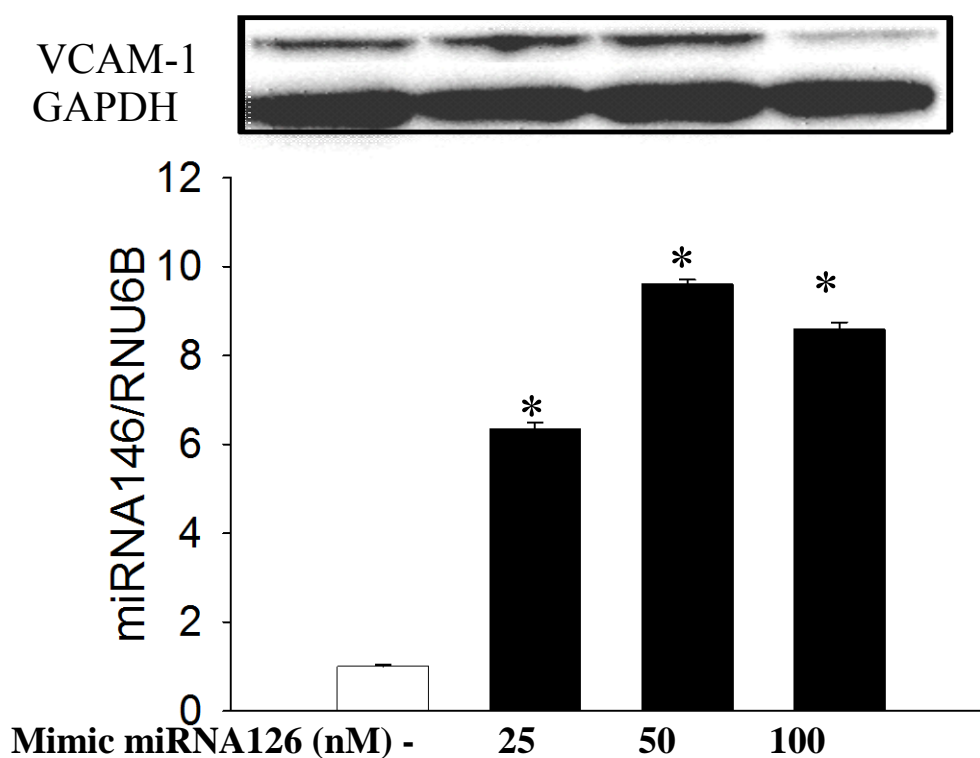
Figure 17 Effect of VCAM protein and gene expression after cells were transfected with miRNA-126 mimic. Protein levels were assessed in cells transfected with antisense miRNA-126 inhibitor, cell lysates were analyzed by Western blot as described materials and methods. Each experiment was performed at least three times, and results are expressed as means ± SEM. Statistically significant difference from LPS-induced cells,* indicates significantly changes at P< 0.05).

Conclusion

This is the first time that the anti-inflammatory effects of red wine polyphenols from Black Spanish grapes were investigated in colonic human fibroblast cells. Wine extract induced a concentration-dependent decrease of ROS (25-100 μg GAE/mL) without decreasing the growth of CCD-18Co colonic fibroblast cells. The protection against production of ROS was accompanied by decreased gene expression and reduced activation of NF-kB transcription factor and NF-kB-dependent pro-inflammatory cytokines and cell adhesion molecules. Induction of miR-126 by wine extract was determined to be the underlying molecular mechanism by which wine extract decreased VCAM-1 and inflammation. This is relevant to IBD, in which the balance of pro-inflammatory and anti-inflammatory molecules is disrupted and the inflammatory protective events fail to turn off once the pathogenic agent is cleared resulting in excessive cytokine release and disruption of intestinal mucosa (158).

CHAPTER IV

DETERMINATION OF 2-ISOPROPYL-3-METHOXYPYRAZINE in GREEN JUNE BEETLE (COLEOPTERA: SCARABAEIDAE) BY SOLID-PHASE-MICROEXTRACTION GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Summary

There is currently no scientific literature describing the presence of volatile compounds in GJB that could affect the quality of Texas wines, yet the presence of green june beetles (GJB) in some vineyards in Texas and their aroma strongly resembling methoxypyrazines led to the hypothesis that GJB when present in harvested wine grapes, may contribute to increasing methoxypyrazines in wines. Volatile 2-alkyl-3-methoxypyrazines were determined in GJB by GC-MS using a solid phase micro-extraction (SPME) headspace device. 2-isopropyl-3-methoxypyrazine (IPMP) was identified in GJB at 4.3 ± 0.2 ng /L. Relative amounts of IPMP based on body mass showed that GJB contain IPMP (24.21 ng/gr beetle). This amount seems sufficient to increase the levels of IPMP above the level of perception of 4 ppt in approximately 1 gallon of wine.

This study demonstrated that GJB contain MP, which can negatively affect the quality of Texas wines.

Introduction

A major challenge to wine quality worldwide are elevated methoxypyrazine concentration that can interfere with the fruity/floral aroma in winee flavor (41). The most commonly found methoxypyrazines in wine are 3-Isobutyl-2-methoxypyrazine (IBMP), 3-sec-butyl-2-methoxypyrazine (SBMP), and 3-isopropyl-2-methoxypyrazine (IPMP). The latter are predominantly present at elevated levels in grapes grown incooler climates and under ripe fruit of low quality (36, 37, 159).

Isobutyl-2-methoxypyrazine (IBMP) are also know to be secreted by ladybugs (members of *coccinellidae* family) as a defensive pheromone (160). The multicolored Asian Ladybeetle (*Harmonia axyridis*) (commonly called the Multicolored Asian Lady Beetle, MALB), was deliberately introduced from its native Western Asia into North America, first in 1918, but more successfully in 1980s to control aphids on pecan trees, pine trees, ornamental shrubs, cotton, wheat, tobacco, roses, etc (161). If ladybugs are present around ripe clusters during grape harvest it is likely that these will be incorporated into the must (162). MALB are isfound in many winemaking regions of the world, including Italy, France, Spain, Greece, South Africa, Argentina, Brazil, Canada and United States (46). Due to their contribution of IPMP to grape must they have a negative impact on the winery industry (37).

GJB have been identified in grape growing regions of Texas around grape harvest time (163). The GJB is native to the Southeaster region of the United States from Kansas to Connecticut and south to Texas and northern Florida (164). The beetles injure fruits of many kinds, including grapes, peaches, raspberry, blackberry, apple, pear, plum,

prune, apricot, and nectarine, and are attracted to ripe fruits (Figure 18) (163).

Figure 18 Green June beetle feeding on a cluster of grapes (9)

Materials and Methods

Analysis of 2-Alkyl-3-Methoxypyrazines Compounds Released by Green June Beetles

Green June Beetles (GJB) were collected in Northern Texas in August 2010 and entymologically identified by Dr. Bart Drees from Texas A&M University Deparment of Entomology. GJB were maintained in a 20 mL glass bottle at 4 °C prior to experiments. Average weights of GJB were determined by separately weighing 8 GJB using an electronic balance. One GJB was macerated into 1000 mL of model wine and equilibrated for 24 hrs at 21 °C before SPME-GC injection. Approximately 9mLof the analyte was added to a 10mL volumetric flask, spiked with 10 µL of deuterated MPs (40 µgL−1 each of IPMPand IBMP, diluted in methanol) and 400 µL of 2 mol/LNaOH. The

solution was adjusted to 40 ng/L concentration of deuterated MPs at pH of 6.6. Samples were prepared, extracted and analyzed in triplicate.

Sample Extraction

A 20 mL glass cylinder was placed on a heating plate and clamped in place. The DVB/Carboxen™/PDMS StableFlex™ SPME fiber was inserted into the sample vial and the MPs and their deuterated analogues were adsorbed onto the 2 cm, 23 gauge fiber. The fiber stayed inserted into the headspace of the sample vial for 30 min, with close attention paid to ensure the fiber did not come in contact with the liquid.

Instrumental Analysis

Samples were analyzed using a solid phase micro-extraction (SPME) headspace device. Analysis was conducted using a ThermoElectron Trace GC Ultra (Waltham, MA) equipped with a TriPlusAutosampler and a DSQII mass spectrometer. The autosampler was fitted with a DVB/Carboxen™/PDMS StableFlex™ SPME fiber (Supelco, Bellefonte, PA). Samples (10 mL) were incubated for 30 min at 70˚C and allowed to adsorb from the headspace onto the fiber for 30 min. The fiber was desorbed onto a DB-5 column (30 m x 0.53 mm x 5μm film thickness, J&W Scientific, Agilent Technologies, Santa Clara, CA). The injector was held at 250 °C with no purge for 5 min, then was purged at 50 mL/min for an additional 5 min. The oven was held at 70 °C for 5 min and then was increased 3 ◦ C/min up to 110 °C held for 1 min at 110 °C, and then was increased 25 °C/ mi up to 230 ◦ C. Helium was used as the carrier gas at

constant pressure (10.36 psi) with a nominal initial flow of 1.2 mL/min. The MSD interface was held at 250 °C while the temperature of the ion source was at 200 °C. Identification was achieved using selected ion monitoring (SIM). For IBMP selected mass channels were m/z 109 and 124 and m/z 112 and 127 for [^{2}H$_3$]-IBMP. Ions 124 and 127 were used for quantification, while ions 109 and 112 were used as qualifier ions. For IPMP, selected mass channels were m/z 137 and 152 and m/z 140 and 155 for [^{2}H$_3$]-IPMP. Ions 137 and 140 were used for quantificationwhile ions 152 and 155 were used as qualifier ions. All samples were analyzed in duplicate or in triplicate.

Standards and Solutions

Reference standards used for identification and quantification of MPs, were purchased from Sigma–Aldrich and included IPMP (97%, IPMP) and IBMP (97%, IBMP). The isotopically labeled internal standards [^{2}H$_3$]-IBMP and [^{2}H$_3$]-IPMP, were purchased from CDN isotopes (Quebec, Canada).

Standard Curve

A standard solution containing IBMP and IPMP with their correspondingdeuterated MPs was prepared from each individual standard and subsequently diluted with methanol within a dark colored flask and sealed with parafilm. All the solutions were stored in dark at 4 ◦ C until use. For the standard curve a model wine was prepared containing 12% (v/v) ethanol and 4 gr/L of tartaric acid, adjusted to pH 6.6 with NaOH. Approximately 9 mL of model wine was added to a volumetric flask spiked with IBMP

and IPMP to give MPs concentrations in the range of 2.5–50 ng/ L. Then10 uL of deuterated MPs were added to the volumetric flask to have a final concentration of 40 ng/L [^{2}H$_3$]-IBMP and [^{2}H$_3$]-IPMP respectively and topped to the mark with model wine solution. Each solution containing the MPs and the deuterated MPs were added to 20 mL glass cylinder bottles containing 3 gr of NaCl and closed with a septum cap.

Results and Discussion

Identification of 2-Alkyl-3-Methoxypyrazines Released by Green June Bettles

Its been demonstrated that wine fermentation in the presence of an *H. axyridis* results in elevated IPMP levels with concentration of approximately 50 ng/L (165). However, there is no previous evidence of methoxypyrazine in the Green June Beetle (GJB). IPMP was detected in GJB at concentrations of 4.3 ± 0.2 ng /L.

At an average weight of 177.60 mg/beetle, one GJB contains 24.21ng/gr of IPMP which is higher than the condentration previously found in *Harmonia axyridis* (8.06 ng/gr) (166). The chromatogram and mass spectrum for IPMP found in GJB with the deuterated IPMP is shown in Figure 19.

The retention time obtained for IPMP was 7.95 min while [^{2}H$_3$]-IPMP had a retention time of 8.19 min. IPMP was confirmed by matching the retention time of the unknown compound with the retention time of the IPMP standard. IBMP was not found in GJB.

Previous studies have shown that lady beetles secrete pyrazines that act as aggregation pheromones (57, 167, 168). This Dead beetles may provide elevated levels of IPMP because MPs may be produced by the gut flora of GJB (169).
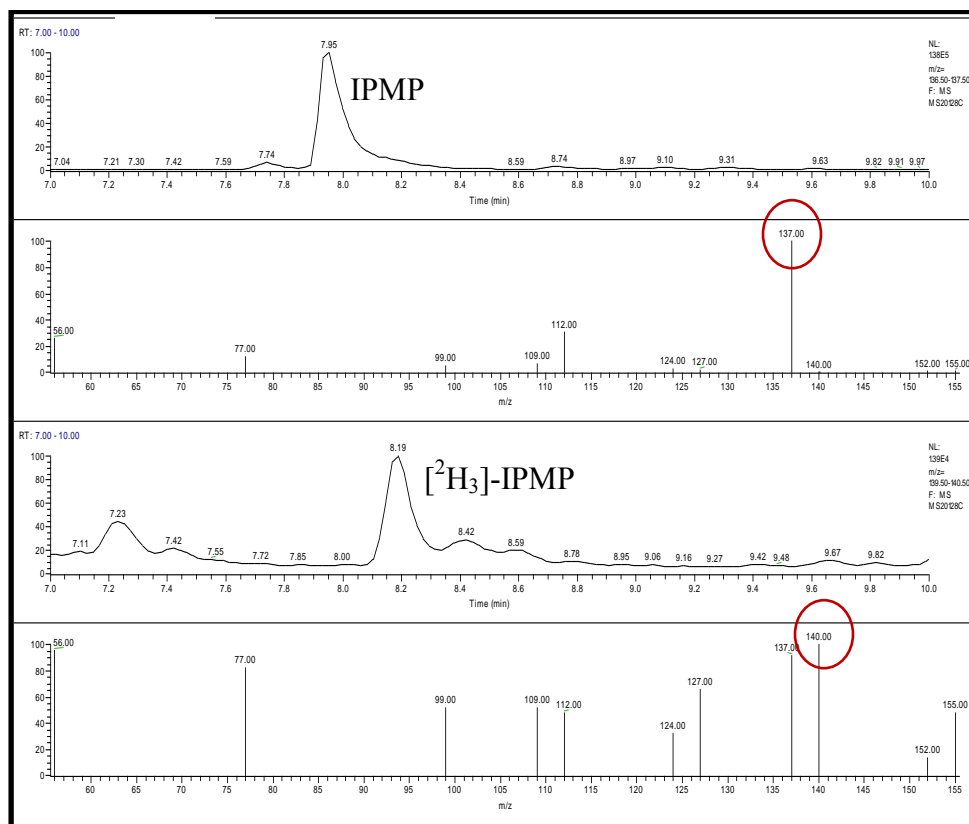
Figure 19 Model wine after addition of 1 green june beetle/mL. (a) Mass chromatogram of IPMP (b) Mass spectrum (SIM) of IPMP (ion 137). (c) Mass chromatogram of [²H₃]PMP. (d) Mass spectrum (SIM) of [²H₃]PMP (ion 140).

Methoxypyrazines are primary and secondary metabolites of micro-organisms such as bacteria (170), fungi (171), *Candida* yeast (172), *Pseudomonas* species (173) as well as *Serratia*, members of the *Enterobacteriaceae* (*48*). Candida yeast was reported to be present in the gut flora of GJB (169). Therefore, the microflora of GJB may play a role in the production of IPMP.

Conclusion

This study demonstrated of the presence of IPMP in Green June Beetles. The undesired incorporation of this insect into grape must of specifically red grapes could negatively impact the sensory properties of wines by adding green and vegetative aromas to wines.

CHAPTER V

EFFECT OF MICROOXYGENATION ON LEVELS OF 2-ALKYL-3-METHOXYPYRAZINES IN TEXAS WINES

Summary

3-alkyl-2-methoxypyrazines (MPs) volatile-plant and insect-derived aroma compounds which may negatively impact grape quality. The most commonly found in wine are, 3-Isobutyl-2-methoxypyrazine (IBMP) and 3-isopropyl-2-methoxypyrazine (IPMP). The objective of this study was to investigate the effects of micro-oxygentation (MOX) and other accelerated aging techniques in the potential reduction of MPs in red wine. Treatments were applied for 96 days in red wines storaged in a 55 gallons stainless steel drum (Control), a stainless steel drum with the addition of inner staves (SSD+oak), an American oak barrel medium toast (Oak Barrel), and micro-oxygenation treatment wine blend along with 30% oak portion in the inner stave (MOX). Changes in MPs concentration were monitored with headspace solid-phase microextraction and gas chromatography. IBMP and IPMP were detected in the control wine at 11.24 ± 0.49 ng/L and 5.60 ± 0.22 ng/L respectively. Results indicated that there was not a significant difference in IBMP and IPMP levels after MOX treatment compared to the control. Data imply that micro-oxygenation may not reduce actual concentrations of MPs. The perceived reduction of herbaceous character in red wines through MOX previously reported may be due to a masking effect of MOX or oak-derived compounds and a reduction of volatiles thiols.

Introduction

A problem associated with wine quality worldwide and specifically in Texas are high concentration of 3-Alkyl-2-methoxypyrazines (MPs) that add a green, vegetative aroma to wines and can mask over fruity/floral aroma-notes in wines. The olfactory threshold at which these compounds can be perceived is extraordinarily low, namely 1 ng/L in water while it may be slightly higher in wines (159).

MPs are widely distributed in plants, and can reach total concentrations in excess of 1000 pg/g in the vegetative tissue and unripe fruits, including bell pepper, potatoes, asparagus, and peas. MPs are responsible for the vegetative, herbaceous, capsium-like aromas (38). They can contribute positively to certain varietal wine aroma profiles. MPs are appreciated to a certain extent in Cabernet sauvignon, Sauvignon blanc, Cabernet franc and Merlot noir among others (39, 40). MPs may be considered a negative sensory attribute, as their aroma may not be suitably balanced by other aroma compounds in the wine. The most commonly found in wine are, 3-Isobutyl-2-methoxypyrazine (IBMP), 3-sec-butyl-2-methoxypyrazine (SBMP), and 3-isopropyl-2-methoxypyrazine (IPMP) as shown in Figure 20 (40, 41).

isopropyl methoxypyrazine
(IPMP)

isobutyl methoxypyrazine
(IBMP)

Sec-butyl methoxypyrazine
(SBMP)
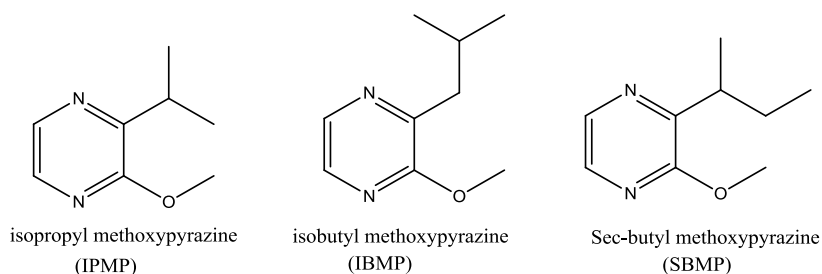
Figure 20 Grape-derived methoxypyrazines

MPs concentrations in grapes are influenced by grape variety, fruit maturity, season, climate, and solar exposure of the fruit. Moreover these compounds are associated in higher concentrations with wines from cooler climates as well as under-ripe and low quality fruit.

Recently, lady beetles were identified as an exogenoussource of elevated MPs in wine that has been named "ladybug taint" (LBT). LBT is a wine defects resulting from the undesired incorporation of lady beetles (Coleoptera: Coccinellidae), particularly *Harmonia axyridis* (commonly called the Multicolored Asian Lady Beetle, MALB), into the fermentation process. MALB can be found in vineyards in large numbers around the time of commercial grape harvest (45, 46) and are found in many winemaking regions of the world, including Italy, France, Spain, Greece, South Africa, Argentina, Brazil, Canada and United States (46).

Winemakers have used several techniques in an effort to enhance the quality of wines. In recent years micro-oxygenationhas has proven most effective at meeting the needs of winemakers. During the winemaking process, oxygen is required and plays an important role in wine maturation. At early stages oxygen aids in the fermentation, and later on helps to stabilize color and develop aromas during aging (22). Despite these beneficial effects of oxygen, an excess may cause wine oxidation having negative effects such as phenol oxidation, higher astringency, and adverse microbial activity (93).

Micro-oxygenation (MOX) is a winemaking technique that describes introducing small amounts of oxygen into wines at different stages of the winemaking process. The rate at which oxygen is supplied to the wine is equal or lower than the rate at which it is consumed in

order to avoid oxygen accumulation. Typically dosage rates are relatively small, ranging from 2-90 mg of O2/L of wine/month, this value depends on the type of wine, the stage of maturation, and preference of the wine maker (95).

MOX is mainly used in red wines, as such can be applied during any stage of the winemaking process. However, oxygen is usually added during the alcoholic fermentation and before beginning the malolactic fermentation (35). Furthermore, oak chips are commonly used in combination with the micro-oxygenation in order to imitate aging in oak barrels. This technique is called accelerated aging and has been widely applied in recent years in several countries such as France, Italy, Australia, New Zealand, The United States, Spain and Chile. (35).

Several studies have reported positives effects of MOX such as improve palatability, enhance color stability and intensity (94, 174, 175). This is due among other factors, to the fact that oxygen takes part in condensation and polymerization reactions between tannins and anthocyanins. This reactions produces new pigments which can stabilize wine color and reduce astringency (176). Furthermore MOX improves sensorial characteristic to the wine by enhancing the development of fruity flavors and integrates the aroma of the wood.

More importantly, MOX decreased herbaceous characteristics such as leafy and green pepper aromas in sensorial analysis. However the effect of micro-oxygenation in the reduction of methoxypyrazines concentration measured by analytical techniques such as SPME-GC-MS has not been well investigated (177). The objective of this study was to examine the effect of micro-oxygenation treatment and accelerated aging techniques on methoxypyrazines concentration as quantified by SPME-GC-MS in a red wine blend.

Materials and Methods

Winemaking

A red wine blend consisted of Cabernet Franc 45%, Malbec 45%, and Merlot 10% was prepared following traditional winemaking practices by Llano Estacado Winery (Lubbock, TX). Malolactic fermentation was allowed to completion before MOX.

Experimental Design for Micro-Oxygenation Wines

The experimental design consisted of four treatments as follows: a 55 gallons stainless steel drum (Control), a 55 gallon stainless steel drum with the addition of inner staves, (SSD+oak), a standard 59 gallons two years old American oak barrel medium toast (Demptos, Napa, CA) (Oak Barrel), and 3142 gallons tank with 30% oak portion in the inner stave (Innerstave, Sonoma, CA) (MOX), which was used previously one time. Winery laboratory staff was in charge of sampling during the experiment that lasted approximately three months. Wine samples were taken in triplicate in a 750 mL bottle, immediately flush with nitrogen, and stored at 4°C until need them for methoxypyrazines analysis.

Micro-oxygenation Procedure

A MOX procedure developed by Llano Estacado Winery (Lubbock, TX) was used during the experiment. In brief, sulfur dioxide was adjusted to 0.5 molecular before start wine MOX. The initial oxygen level was set at 10 mg/L/month using a micro-

oxygenation unit model SAEN 400x/5 (PARSEC Srl, American Tartaric Products Inc. Windsor, CA).

The wine was monitored on a weekly basis for pH, SO_2 (free, molecular, and total), volatile acidity and sensory; and depending on the degradation of free SO_2 and sensory, oxygen concentration was kept or adjusted as follows: if SO_2 drops the first time below 0.3 molecular, then is adjusted again to 0.5 and the oxygen level is maintained to 10mg/L/month; when the SO_2 drops for the second time below 0.3 molecular, the oxygen level is reduced to 5 mg/L/month and the SO_2 is adjusted to 0.5 once again; the third time that SO_2 goes below 0.3 the oxygen level is reduced to 2 mg/L/month and molecular is adjusted once again to 0.5; finally when the SO2 reaches 0.3 or below, the micro-oxygenation is stopped.

Instrumental Analysis

The samples were analyzed using a solid phase micro-extraction (SPME) headspace device. Analysis was conducted using a ThermoElectron Trace GC Ultra (Waltham, MA) equipped with a TriPlusAutosampler and a DSQII mass spectrometer. The autosampler was fitted with a DVB/Carboxen™/PDMS StableFlex™ SPME fiber (Supelco, Bellefonte, PA). Samples (10 mL) were incubated for 30 min at 70 ˚C and allowed to adsorb from the headspace onto the fiber for 30 min. The fiber was desorbed onto a DB-5 column (30 m x 0.53 mm x 5μm film thickness, J&W Scientific, Agilent Technologies, Santa Clara, CA). The injector was held at 250 °C with no purge for 5 min, then was purged at 50 mL/min for an additional 5 min. The oven was held at 70 °C

for 5 min and then was increased 3 ◦ C/min up to 110 °C held for 1 min at 110 °C, and then wasincreased 25 °C / mi up to 230 ◦ C. Helium was used as the carrier gas at constant pressure (10.36 psi) with a nominal initial flow of 1.2 mL/min . The MSD interface will be held at 250 ◦ C while the temperature of the ion source will be at 200 °C. Identificationwas achieved using selected ion monitoring (SIM). For IBMP selected mass channels were m/z 109 and 124 and m/z 112 and 127 for [^{2}H$_3$]-IBMP. Ions 124 and 127 were used for quantification, while ions 109 and 112 were used as qualifier ions. For IPMP, selected mass channels were m/z 137 and 152 and m/z 140 and 155 for [^{2}H$_3$]-IPMP. Ions 137 and 140 were used for quantification while ions 152 and 155 were used as qualifier ions. All samples were analysed in duplicate or in triplicate. Refer to Appendix A to see the validation of the method.

Standards and Solutions

Reference standards were used for identification and quantification of MPs, were purchased from Sigma–Aldrich and included IPMP (97%, IPMP) and IBMP (97%, IBMP). The isotopically labelled internal standards [^{2}H$_3$]-IBMP and [^{2}H$_3$]-IPMP were purchased from CDN isotopes (Quebec, Canada).

Standard Curve

A standard solution containing IBMP and IPMP with their corresponding deuterated MPs was prepared from each individual standard and subsequently diluted with

methanol in a dark colored flask and sealed with parafilm. All solutions were stored in dark at 4 ◦ C until use.

For the standard curve, a model wine was prepared containing 12% (v/v) ethanol and 4 gr/L of tartaric acid, adjusted to pH 6.6 with NaOH. Approximately 9 mL of model wine was added to a volumetric flask spiked with IBMP and IPMP to give MPs concentrations in the range of 2.5–50 ng/ L. Then 10 uL of deuterated MPs was added to the volumetric flask to have a final concentration of 40 ng/L of [^{2}H$_3$]-IBMP and [^{2}H$_3$]-IPMP respectively and topped to the mark with model wine solution. Each solution containing the MPs and the deuterated MPs were added to 20 mL glass cylinder bottles containing 3 gr of NaCl and closed with a septum cap.

Wine Analysis

Approximately 9 mL of wine were added to 10 mL volumetric flask, followed by 10 µL of deuterated MPs (40 ng/L each of IPMP and IBMP, diluted in methanol) and 400 µL of 2 mol/L NaOH. The flask was then topped to the 10 mL mark with wine to achieve a 40 ng/L concentration of deuterated MPs with a pH of 6.6. The 10 mL solution was added to a 25 mL volumetric flask and topped to the mark with deionised water (dilution 2.5 times). Two 10 mL aliquots were then removed from the flask and added to two 20 mL glass cylinder bottles. The pH was measured and was adjusted to 6.6 to improve the adsorption of the SPME. Then 3 gr of NaCl was added to the glass cylinders bottles and closed with a septum cap.

Sample Extraction

The 20 mL glass cylinder was placed on a heating plate and clamped in place. The DVB/Carboxen™/PDMS StableFlex™ SPME fiber was inserted into the sample vial and the MPs and their deuterated analogues were adsorbed onto the 2 cm, 23 gauge fiber. The fiber stayed inserted into the headspace of the sample vial for 30 min without contacting the sample.

Statistical Analysis

Statistical analysis was performed using JMP ® statistical software version 5.0 (SAS Institute Inc. Cay NC., USA). All data for each analyte underwent Analysis of Variance (ANOVA). Fisher's Protected Least Significant Difference $(LSD)_{0.05}$ was used as the mean separation test.

Results and Discussion

During the last decade the use of SMPE-GC-MS has received substantial attention for the quantification of methoxypyrazines because its requires minimal sample preparation and is very effective in concentrating the analytes (178). However, the use of SPME with the stable isotope labeled internal standard is necessary since the analytes are present in the ng/L to pg/L range. The majority of scientific studies focus on IBMP determination but the recent identification and description of ladybug taint has highlighted the importance to measured IPMP in wine as well. The use of SPME

isolation methods and the deuterium labeled analogues [^{2}H$_3$]-IBMP and [^{2}H$_3$]-IPMP as internal standards is necessary to measures low levels of MPs in Texas wines.

Method Validation

A standard curve was created using the wine model described in material and methods. Standards of IBMP and IPMP were added in a concentration range of 2.5 to 50 ng/L. Three replications of each of seven standard concentration (2.5, 5, 10, 20, 30, 40, 50 ng/L) were extracted and analyzed. The IBMP peek area (m/z 124) in relation to the [^{2}H$_3$]-IBMP internal standard peek area (m/z 127) was linearly correlated with the IBMP standards over the range examined (R2= 0.994) The IPMP peek area (m/z 137) in relation to the [^{2}H$_3$]-IPMP internal standard peek area (m/z 140) was linearly correlated with the IPMP standards over the range examined (R2= 0.994). After every 20 samples, quality control standards were analyzed to verify the method. Relative standards deviations of replicate samples were 7.1%, 4.5% and 6.5% at 2.5, 20 and 50 ng/L. The lower detection limit was <1.5 ng/L for wine and juice respectively.

Wine Sample Analysis

IBMP and IPMP were confirmed by matching the retention time of the unknown compound with the retention time of the IBMP and IPMP standard.

The chromatogram for IBMP and IPMP found in the control wine with the deuterated IBMP and IPMP are shown in Figure 21. The retention time obtained for IBMP and IPMP was 11.67 and 8.41 min while [^{2}H$_3$]-IBMP and [^{2}H$_3$]-IPMP gave a retention time

of 12.2 and 8.19 min respectively. IBMP and IPMP were quantified in wines at 0, 13, 24, 52, 66, 94, 96 days for each 4 treatments. IBMP and IPMP were detected in the control wine at 11.24 ± 0.49 ng/L and 5.60 ± 0.22 ng/L respectively. In the case of IBMP similar concentrations were reported in cabernet franc wines (178, 179). Likewise IPMP was detected at similar concentrations in red wines (165). To analyze the effects of oak on MPs levels, oak pieces made from staves used in the MOX were added into a stainless steel drum, keeping the oak/wine proportion similar as in the MOX tank. Results show that after 96 days IBMP and IPMP concentrations were relatively stable and were not statistically significant affected by the interaction of oak pieces.
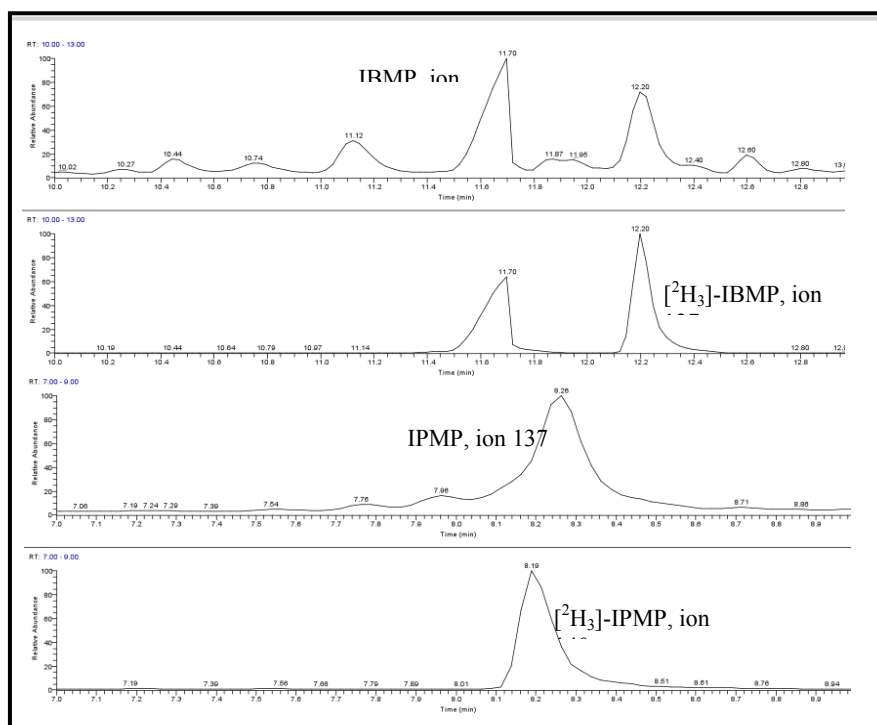
Figure 21 Gas chromatograms of red wine (control). Mass chromatogram (SIM) of IBMP (ion 124), [²H₃]-IBMP (ion 127), IBPMP (ion 137) and [²H₃]-IPMP (ion 140).

Similar results were previously reported with model wines storage from 10 months with oak chips (180). Furthermore, the wine stored in an American oak barrel showed similar levels of IBMP and IPMP over time as the control wine (Table 1 and Table 2).

Table 1 Levels of IBMP in red wines[1]

Time (days)	Control	SSD+Oak[2]	Oak Barrel
0	11.243 ± 0.49a	11.243 ± 0.49a	11.243±0.49a
13	11.876 ± 0.09a	11.744 ± 0.67a	11.720±0.21a
24	11.235± 0.22a	11.227 ± 0.36a	11.49±0.04a
52	11.097 ± 0.37a	11.118 ± 0.96a	12.408±0.34a
66	11.754 ± 0.14a	11.063 ± 0.04a	11.002±0.66a
94	11.863 ± 0.16a	11.107 ± 0.48a	12.156±0.51a
96	11.086 ± 0.13a	11.122 ± 0.67a	12.385±0.28a

[1]Quantified as ng/L of IBMP. [2]SSD+Oak: Stainless steel drum with oak pieces. Data represent average values ± standard error from triplicate measurements; values with different letters between columns represent a significant difference (Tukey-Kramer HSD, $p < 0.05$). Values with an asterisk (*) mean significant difference when compared to control (Tukey-Kramer HSD, $p < 0.05$).

Table 2 Levels of IPMP in red wines[1]

Time (days)	Control	SSD+Oak[2]	Oak Barrel
0	5.604±0.22a	5.604±0.22a	5.604±0.22a
13	5.236±0.12a	5.292±0.69a	5.293±0.23a
24	5.094±0.06a	5.628±0.13a	5.424±0.41a
52	5.982±0.21a	5.920±0.33a	5.568±0.44a
66	5.982±0.25a	5.578±0.75a	5.769±0.65a
94	5.728±0.43a	5.491±0.16a	5.830±0.15a
96	6.239±0.29a	5.133±0.37a	5.668±0.114a

[1]Quantified as ng/L of IBMP. [2]SSD+Oak: Stainless steel drum with oak pieces. Data represent average values ± standard error from triplicate measurements; values with different letters between columns represent a significant difference (Tukey-Kramer HSD, $p < 0.05$). Values with an asterisk (*) mean significant difference when compared to control (Tukey-Kramer HSD, $p < 0.05$).

Sensorial analyses have reported that red wines treated with oak chips, could significantly decrease the intensity of "green leafy" attribute, as well as increase the "vanilla" and "oak blend" attributes that potentially masked the MPs aroma and flavor attributes (177, 181).

The aroma of oak is due to the complex compounds occurring in the wood and the reaction with wine components during aging (182). Previous studies investigated the adsorption of aroma compounds by oak in a model wine. Eight aroma compounds, such as terpene alcohols, and ethyl esters, were added to a model wine containing 12.6% ethanol. Most added aroma compounds were be adsorbed by the oak (183). However results demonstrated that oak does not have a strong affinity for 3-alkyl-methoxypyrazines.

The effect of micro-oxygenation in 3-alkyl-methoxypyrazines is not well understood. According with previous studies, it is well known that micro-oxygenation can reduce herbaceous aroma and flavor in wines with micro-oxygenation treatment when determined by sensorial studies (177). Figure 22 and Figure 23 show the effect of MPs levels in wines treated with MOX. Results indicated that there was no significant difference in the IBMP and IPMP levels after 96 days MOX treatment and the control.

From these analytical data, it is not possible to assert that micro-oxygenation reduces the levels of MPs in red wines. From a sensorial point of view, the micro-oxygenation treatment increased the fruity favors, integrated the aroma of the wood, and reduced the reductive, green and herbaceous aroma (184).
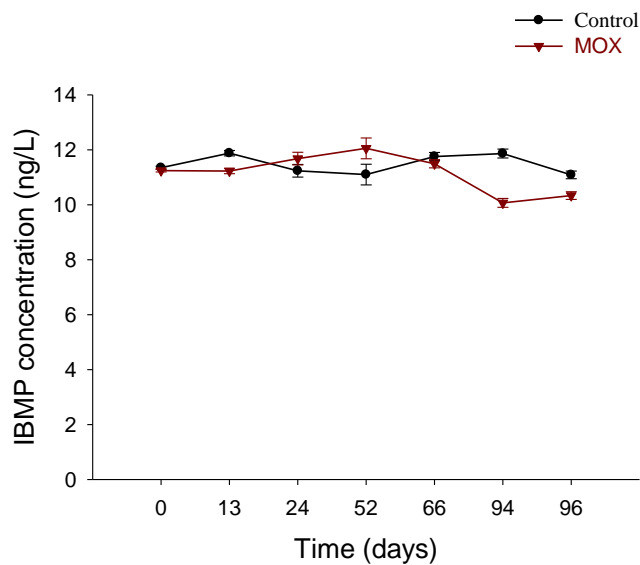
Figure 22 Effect of MOX on IBMP levels through time
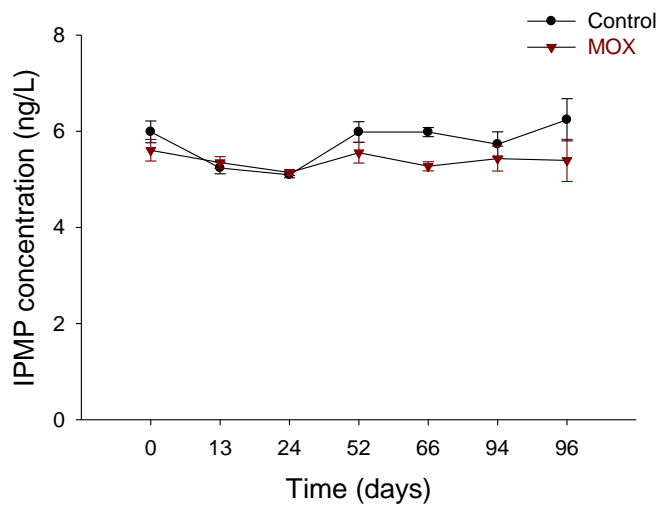
Figure 23 Effect of MOX on IPMP levels through time

These compounds help to reinforce the herbal or vegetative sensory perception in wines. Thus, the reduction of some thiols that can enhance the green aroma by MOX treatment in red wines may result in partially muting of the vegetal character of treated wine.

Conclusion

One of the purposes of micro-oxygenation is to reduce herbaceous character. Our results did not support the hypothesis that MOX lowers the concentration of these compounds in wine and thus reduces herbaceous character. The reduction of herbaceous character in red wines with MOX treatments may be due to a masking effect with oaked-aromas and reduction of volatiles thiols.

CHAPTER VI

EFFECT OF THREE COMMERCIALLY AVAILABLE WINE YEAST STRAINS ON 2-ALKYL-3-METHOXYPYRAZINES

Summary

Due to environmental conditions such as late freeze and hail, Texas wine and grape producers face considerable challenges to their continued growth and success. Quality of Texas wines has been affected by an elevated concentration of undesirable volatile compounds like the 3-Alkyl-2-methoxypyrazines (MPs) which can mask the fruity/floral aroma in wine, causing green and vegetative flavors.

MPs represent an important and potent class of grape and insect-derived odor–active compounds associated with wine quality.

Forty nanograms per litre each of 3-isobutyl-2-methoxopyrazine (IBMP) and 3-isopropyl-2-methoxypyrazine (IPMP) were added to Black Spanish must to investigate the effect of three commercially available yeast strains on MPs by SPME-GC-MS.

Wines fermented with K1 and D80 reduced IBMP levels down to 21.86±1.34 ng/L and 20.52± 2.81ng/L, respectively. Wines fermented with BM45 increased IBMP levels up to 32.31±1.54 in comparison with the control (26.31±1.31ng/L). The effects of different yeast strains in IPMP concentrations were non-significantly different from the control juice.

In addition we evaluated MPs levels of wines fermented during 20 days with a chemical defined grape juice medium containing a yeast available nitrogen of 200 mg

N/L. Data suggest that wines fermented with BM45 and D80 yeast strains reduced IBMP but K1 yeast did not show any effect in comparison with the control.

The effects of different yeast strains in IPMP concentrations were non-significantly different from the control in all the fermentations treatments. Moreover, we evaluated MPs levels in yeast mannoproteins and demonstrated evidence for interactions between mannoproteins secreted by the D80 yeast strain and IBMP.

Overall the data suggests that different yeast strains strongly associate with changes in IBMPs during the winemaking process due to sorptive interactions with yeast mannoproteins.

Introduction

A real problem associated with the winemaking quality is the high concentration of undesirable volatile compounds like 3-Alkyl-2-methoxypyrazines (MPs), which can mask the fruity/floral aroma in wine, providing a green and vegetative flavor. The most commonly found in wine are 3-Isobutyl-2-methoxypyrazine (IBMP), 3-sec-butyl-2-methoxypyrazine (SBMP), and 3-isopropyl-2-methoxypyrazine (IPMP). These compounds are associated with wines from cooler climates and as well from under ripe, low quality fruit. Recently lady beetles were identified as a second source of elevated MPs in wine forming what is known as the "ladybug taint" (LBT). LBT is a wine defect resulting from the undesired incorporation of lady beetles (*Coleoptera: Coccinellidae*), particularly *Harmonia axyridis* (commonly called the Multicolored Asian Lady Beetle,

MALB), into the fermentation process. It is responsible for millions of dollars of lost revenue from downgraded or discarded wine in the United States.

Strategies for reducing methoxypyrazine concentrations are necessary to improve wine quality. This may be achieved through the reduction of "green" flavor and aroma characteristics caused by suboptimal grape ripeness or MALB-affected vintages.

Yeast choice may provide one such mitigating approach, as certain strains have been shown to positively influence volatile wine compounds across a range of chemical species (53, 68, 185). Moreover, Treloar and Howell (186) investigated for the first time, the capacity of yeast to affect MP concentration in wine. They examined strain effects on IBMPin Cabernet Sauvignon and Cabernet Franc wines and noted differences of up to 37% (1.53 ng/L).

Lalvin BM-45, and Lalvin ICV-D80 produced wines with the lowest IBMP concentrations. However the study did not report on the analytical method used, which makes it difficult to assess the quality of the data and the validity of the conclusions reached. This is particularly important given the known analytical challenges of isolating and quantifying trace compounds within a wine matrix. Moreover, sensory analyses have demonstrated that the use of different yeast strains decreases the vegetative aroma in wine (53, 59, 187).

Saccharomyces cerevisiae yeast requires a relatively high level of nutrients to complete the fermentation of grape must, typically producing 12-15% v/v ethanol. Yeasts assimilate a variety of nitrogen compounds, predominantly primary amino acids, ammonium ions, and small peptides. Yeast assimilable nitrogen (YAN) has been

identified as a key nutrient that is often suboptimal in many grape musts in many wine regions worldwide (77-81). Insufficient YAN is often associated with lower biomass yield, which in turn slows fermentation rate, increasing the risk of sluggish or stuck fermentation, production of undesirable thiols (eg. Hydrogen sulfide), and higher alcohols and as well, lowering the production of esters and long chain volatile fatty acids (77, 80).

Moreover previous studies have demonstrated that yeast can reduce the concentrations of wine derived aromatic compounds through metabolic processes (188) and sorption on the cell wall (63). The aim of this project was to determine the effect of commercial *Saccharomyces* yeast strains on IBMP and IPMP levels in Black Spanish wine by SPME-GC-MS. Additionally, we were interested in monitoring yeast growth during fermentation using a grape juice medium with medium nitrogen demands to investigate if the lack of nitrogen nutrients would force yeast to metabolize MPs as a source of nitrogen.

Materials and Methods

Black Spanish Winemaking and Treatments

Black Spanish (*Vitis aestivalis*) must was kindly providedby Messina Hof Winery (College Station, TX). A concentration of 40 ng/L of IBMP and IPMP respectively was added to the must. In preparation for inoculation, 8 x1 L aliquots of MPs spiked-juice were then separated into autoclaved Erlenmeyer flasks. The Black Spanish must was then inoculated at 300 mg/L with three commercially available yeast strains. Lalvin

BM45 (BM45), LalvinV1116 (K1), and Lalvin ICV-D80 (D80) (Lallemand Inc., Santa Rosa, California, USA). The commercial freeze-dried yeast preparations were added to distilled 40°C water and allowed to rehydrate for 20 min with intermittent stirring. After 20 min, the starter culture volume was doubled by adding must and 20 min later was added to black Spanish must in the volumetric flask.

These strains were chosen to represent the best performing yeast with respect to their reported effects on IBMP concentration in wine (186).

A control treatment was processed as above without the addition of MPs. All treatments were performed in triplicate, and fermentations were conducted in a temperature-controlled water bath (Fisher Scientific Pittsburgh, PA) at a constant temperature of 18°C.

Growth Conditions for the Grape Juice Model by Saccharomyces cerevisiae Wine Yeast

The wine yeast was inoculated in a grape juice medium (GJM) previously reported by several authors (78, 80). The sugar content was 225 g/L of sugars (112.5 g/L of glucose and 112.5 g/L of fructose). To obtain a YAN level of 200 mg N/L, the following mixture of amino acids was used (values in milligrams per liter): alanine 74.4, arginine 98.5, asparagine 14.9, aspartic acid 24.9, cysteine 1.4, glutamine 111.9, glutamic acid 75.3, glycine 4.7, histidine 19.6, isoleucine 11.0, leucine 11.2, lysine 5.2, methionine 3.7, ornithine 1.1, phenylalanine 20.1, serine 50.8, threonine 48.6, tryptophan 10.9, tyrosine

18.7 and valine 18.6. The YAN content was then increased from 117 mg/L up to 200 mg/L by adding 317 mg of NH_4Cl.

Monitor Yeast Growth

Yeast starter cultures were made in 50 mL of GJM, incubated aerobically at 21°C. Starter cultures were used to inoculate 100 mL GJM at 1.8×10^5 CFU/mL. A typical winemaking process is conducted in a non-sterile environment involving more than just one microorganism. In this case, to study the effect of commercially available yeasts in the MPs levels, one single strain of yeasts was used experiment (D80).

Fermentations were carried out in triplicates in Erlenmeyer flasks (250 mL) plugged with non-absorbent cotton (Fisher Scientific Pittsburgh, PA) and incubated at 21 °C. Samples were spiked with IBMP and IPMP at 40 ng/L respectively. Samples were taken every 12 hrs throughout the 10 days trial. A new flask was used to analyze each sampling event. The controls without the addition of the yeasts were analyzed in accord with the same schedule. Yeast growth was monitored by successive dilutions. Differential enumeration of wine yeasts was carried out by plating them onto selective yeast and mold petrifilms (3M, Minneapolis, Minn., U.S.A.) in triplicates. One mL of the appropriate dilution was plated according to the manufacturer's instructions. The yeast and mold petrifilms were incubated at 25 °C, and colonies were counted within 72-96 hours after incubation.

Chemical Analysis

Wine malic acid, alpha-amino nitrogen and ammonia concentrations were determined using the Megazyme enzymatic kits (Napa California, USA) according to the manufacturer's instructions.

Standards and Solutions

Reference standards used for identification and quantification of MPs were purchased from Sigma–Aldrich (St. Louis, MO, USA) and included IPMP (97%, IPMP) and IBMP (97%, IBMP). The isotopically labeled internal standards $[^2H_3]$-IBMP and $[^2H_3]$-IPMP were purchased from CDN isotopes (Quebec, Canada).

Standard Curves, Reproducibility and Detection Limits

A standard solution containing IBMP and IPMP with their corresponding deuterated MPs was prepared from each individual standard and subsequently diluted with methanol within a dark colored flask and sealed with parafilm. All the solutions were stored in the dark at 4^oC until use.

For standard curve, a model wine was prepared containing 12% (v/v) ethanol and 4 g/L of tartaric acid adjusted to pH 6.6 with NaOH. To measure the MPs levels in GJM, a standard curve was created using the GJM described above. Seven standard concentrations of IBMP and IPMP (2.5, 5, 10, 20, 30, 40, 50 ng/L) were added to 10 mL volumetric flasks. Then 10 uL of deuterated MPs was added to the volumetric flasks to have a final concentration of 40 ng/L $[^2H_3]$-IBMP and $[^2H_3]$-IPMP, respectively, and

topped to the mark with model wine or GJM. Each solution containing the MPs and the deuterated MPs was added to 20 mL glass cylinder bottles containing 3 grams of NaCl (Sigma-Aldrich, St. Louis, MO, USA) and closed with a septum cap.

The repeatability was calculated at different concentration levels for each MPs: 2.5, 20 and 50 ng/L (n=5). Data was analysed and compared using means and relative standard deviation. The method detection limit was calculated using the standard deviation multiply by the t-value at 99.5% of confidentiality and 7 degrees of freedom (3.4994).

Analysis of Black Spanish Wines

Approximately 9 mL of wine was added to a 10 mL volumetric flask followed by 10 µL of deuterated MPs (40 ng/L each of IPMP and IBMP, diluted in methanol) and 400 µL of 2 mol/L NaOH. The flask was then topped to the 10 mL mark with wine to achieve a 40 ng/L concentration of deuterated MPs with a pH of 6.6. The 10 mL solution was added to a 25 mL volumetric flask and topped to the mark with deionized water (dilution 2.5 times). Two 10 mL aliquots were then removed from the flask and added to two 20 mL glass cylinder bottles. The pH was measured and was adjusted to 6.6 to improve the adsorption of the SPME. Then 3 grams of NaCl were added to the glass cylinder bottles and closed with a septum cap.

Grape Juice Medium Analysis

Approximately 9 mL of the GJM sample was added to a 10 mL volumetric flask followed by 10 µL of deuterated MPs (40 ng/L each of IPMP and IBMP, diluted in methanol). The flask was then topped to the 10 mL mark with the GJM sample to achieve a 40 ng/L concentration of deuterated MPs. Then the solution was added to a 20 mL glass cylinder bottle. NaCl (3 g) was added to the glass cylinders and a septum was placed on the top.

Sample Extraction

The 20 mL glass cylinder was placed on a heating plate and clamped in place. The DVB/Carboxen™/PDMS StableFlex™ SPME fiber was inserted into the sample vial and the MPs and their deuterated analogues were adsorbed onto the 2 cm, 23 gauge fiber. The sample has heated to 70°C. The fiber stayed inserted into the headspace of the sample vial for 30min with close attention paid to ensure the fiber did not come into contact with the liquid.

Instrumental Analysis

MPs were quantified by SPME-GC-MS according to the method reported by (189). Samples were analyzed using a solid phase micro-extraction (SPME) headspace device. Analysis was conducted using a ThermoElectron Trace GC Ultra (Waltham, MA) equipped with a TriPlusAutosampler and a DSQII mass spectrometer. The autosampler was fitted with a DVB/Carboxen™/PDMS StableFlex™ SPME fiber (Supelco,

Bellefonte, PA). Samples (10 mL) were incubated for 30 min at 70 ˚C and allowed to adsorb from the headspace onto the fiber for 30 min. The fiber was desorbed onto a DB-5 column (30 m x 0.53 mm x 5µm film thickness, J&W Scientific, Agilent Technologies, Santa Clara, CA). The injector was held at 250º C with no purge for 5 min, followed by a purge at 50 mL/min for an additional 5 min. The oven was held at 70ºC for 5 min and temperatures then were increased 3 ºC/min up to 115ºC, 1◦ C/min up to 120ºC, and finally 10 º C up to 230ºC before holding for 10 min. Helium was used as the carrier gas at constant pressure (10.36 psi) with a nominal initial flow of 1.5 mL/min. The MSD interface was held at 250 ºC while the temperature of the ion source was at 200ºC. Identification was achieved using selected ion monitoring (SIM). Selected mass channels were m/z 109 and 124 for IBMP and m/z 112 and 127 for $[^2H_3]$-IBMP. Ions 124 and 127 were used for quantification, while ions 109 and 112 were used as qualifier ions. For IPMP, selected mass channels were m/z 137 and 152 and m/z 140 and 155 for $[^2H_3]$-IPMP. Ions 137 and 140 were used for quantification while ions 152 and 155 were used as qualifier ions. All samples were analysed in duplicate or in triplicate.

Statistical Analysis

All statistical data was performed using JMP ® statistical software version 5.0 (SAS Institute Inc. Cay NC., USA). All data for each analyte underwent analysis of variance (ANOVA). Fisher's protected least significant difference $(LSD)_{0.05}$ was used as the mean separation test.

Results and Discussion

To quantify the levels of MPs in Black Spanish red wines, a standard curve was created using a model wine described in materials and methods. At least three replications of each of the seven standard concentrations were run.

The IBMP peak area (m/z 124) in relation to the [2H3]-IBMP internal standard peak area (m/z 127) was linearly correlated with the IBMP standards over the range examined (R^2= 0.994 for wine and R^2=0.991 for GJM). The IPMP peak area (m/z 137) in relation to the [2H3]-IPMP internal standard peak area (m/z 140) was linearly correlated with the IPMP standards over the range examined (R^2= 0.994 for wine and R^2-0.993 for GJM). After approximately every 20 samples, standards were analyzed to verify the method. Relative standard deviations of replicate samples were 7.1%, 4.5% and 6.5% at 2.5, 20 and 50 ng/L. The detection limit was <1.5 ng/L for wine and juice respectively.

A deuterated analogue of IBMP and IPMP was chosen as an internal standard in order to quantify trace levels of these MPs in samples. The IBMP and IPMP react nearly identically to their respective deuterated isotopes during isolation and measurement. For this reason the ratios of the IBMP with $[^2H_3]$-IBMP and IPMP with $[^2H_3]$-IPMP remain constant, despite potential variations in sampling efficiency and GC-MS response (*167*).

Standard addition tests were run by adding 40 ng/L to the Black Spanish wines. Recovery of the spikes averaged 95.32 and 97.03 %for IBMP and IPMP, respectively (Table 3). Recovery of MPs is comparable with previous studies (41, 178).

Black Spanish red wine from the 2009 vintage year contains a concentration of 26.32±0.51 ng/L of IBMP and 3.81±0.81 ng/L of IPMP. IBMP concentration was

detected at almost 10 times the concentration of IPMP. This result was in agreement with previous reports of these MPs reported in *Vitis vinifera* grape wines (162, 190, 191). However, this is the first time that MPs levels are being reported in *Vitis aestivalis* grape wines (Black Spanish).

Table 3. Recovery Tests for IBMP and IPMP Spiked into Black Spanish Wine[a].

Sample	Initial concentration (ng/L)	Spike concentration (ng/L)	Measured after spike (ng/L)	% Recovery of spike
IBMP	26.32±1.31	40	63.22± 0.32	95.32
IPMP	3.81±1.69	40	42.51± 0.45	97.03

The data are mean ± standard deviation obtained from three independent fermentations.

The IBMP and IPMP concentrations of the Black Spanish wines made with the three different strains varied widely, depending on the yeast strains used for fermentation (Figure 24). The MB45 strain resulted in the highest amount of MPs followed by K1 and D80. Results show that wines fermented with K1 and D80 reduced IBMP levels down to 21.86±1.34 ng/L and 20.52± 2.81ng/L, respectively. Wines fermented with BM45 increased IBMP levels up to 32.31±1.54 in comparison with the control (26.31±1.31ng/L). In the case of IPMP levels, wines fermented with K1, D80, and BM45 showed a concentration of IPMP of 3.95±1.62, 3.75±1.58 and 4.30±0.62, respectively. The effects of different yeast strains in IPMP concentrations were non-significantly different from the control juice.

An increase in IBMP was not expected. There is no literature which suggests that wine yeasts may produce MPs and that this may vary with strain. MPs are known as primary and secondary metabolites of micro-organisms and can be produced by bacteria (170), fungi (171), *Candida* yeasts (172), *Pseudomonas* species (173) as well as *Cedeceadavisae* and *Serratia*, members of the *Enterobacteriaceae* (48).
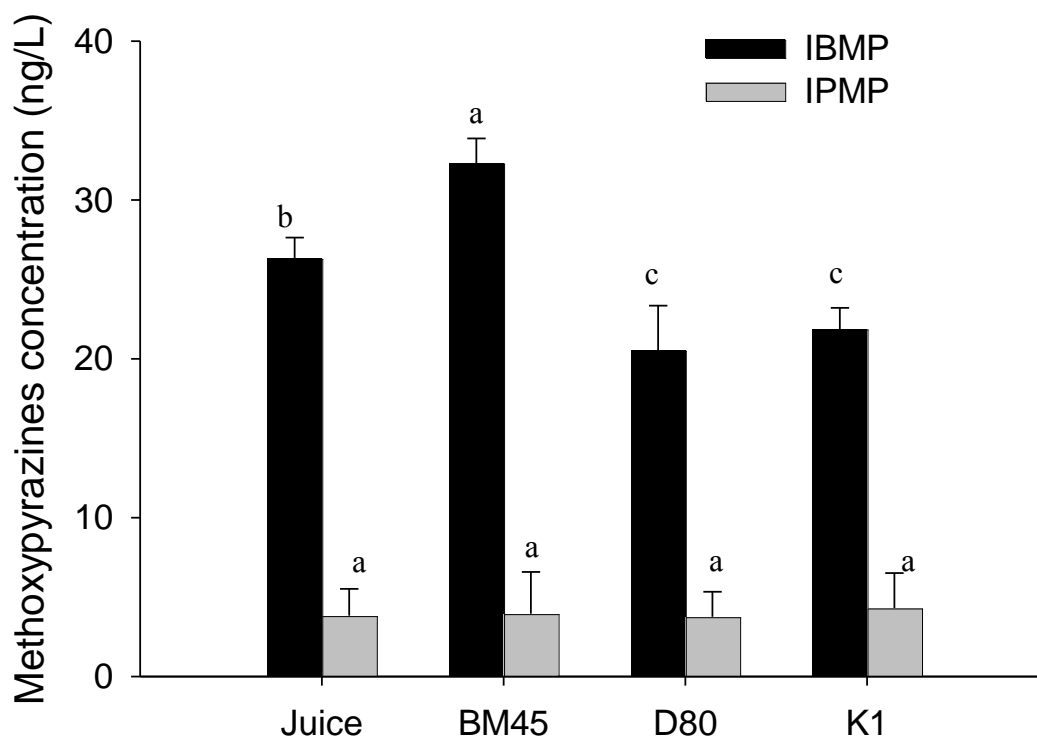
Figure 24 Methoxypyrazines concentrations in Black Spanish wines made with different commercial yeast strains (BM45, D80 and K1). Results indicate the average of three fermentation replicates. (a,b) means values with different letters are significantly different.

Biosynthesis of MPs by *Saccharomyces cerevisiae* has not been previously reported. However it has been demonstrated that amino acids valine and leucine are precursors to IPMP and IBMP respectively, because of similarities in the alkyl side chains (47). It has been proposed that the respective amino acid gains a second nitrogen through an unknown amidation reaction and them undergoes a condensation reaction with 1,2-dicarbonyl compound such as glyoxal to produce 3-alkyl-2-hydroxypyrazine (HP) (47).

Previous studies suggested the biosynthetic pathway of IPMP by *Pseudomonas perolens* (192). The metabolic pathway proposed involves condensation of valine and glycine to yield 3-isopropyl-2, 5-diketopiperazine leading to 3-isopropyl-2- (1H)-pyrazinone, which undergoes O-methylation to 2-methoxy-3- isopropylpyrazine (193).

Recent studies have demonstrated that the final step in the pathway involving the methylation of HP to MP is possible due to an enzymatic reaction with S-adenosyl-L-methionine-dependent O-methyltransferase (OMT) purified and characterized from *Vitis vinifera* grapes (49).

Functional enzyme assays showed that this enzyme is multifunctional and has the ability to methylate each of the respective HP precursors of MPs (49, 50).

If *Vitis aestivalis* grapes contained a similar OMT enzyme to that found in *Vitis vinifera* grapes and furthermore if *S. cerevisiae* contained this second IPMP biosynthesis pathway found in *Pseudomonas perolens* (192), BM45 may have led to the increased IBMP observed in the fermentation.

It is known that nitrogen availability can also affect many aspects of yeast metabolism, including the formation of volatile and non-volatile compounds (77, 78).

The implication of nitrogen supplements on fermentation is clear from literature (77, 78, 80, 194). There are no studies reporting the metabolic response of *S. cerevisiae* with a high nitrogen demand in conjunction with interaction with MPs levels in a standardized grape juice medium. Therefore we monitored yeast growth during 10 fermentation days using a grape juice medium with 200 mg/L of YAN to investigate if the lack of nitrogen nutrients would force yeast to metabolize MPs as a source of nitrogen.
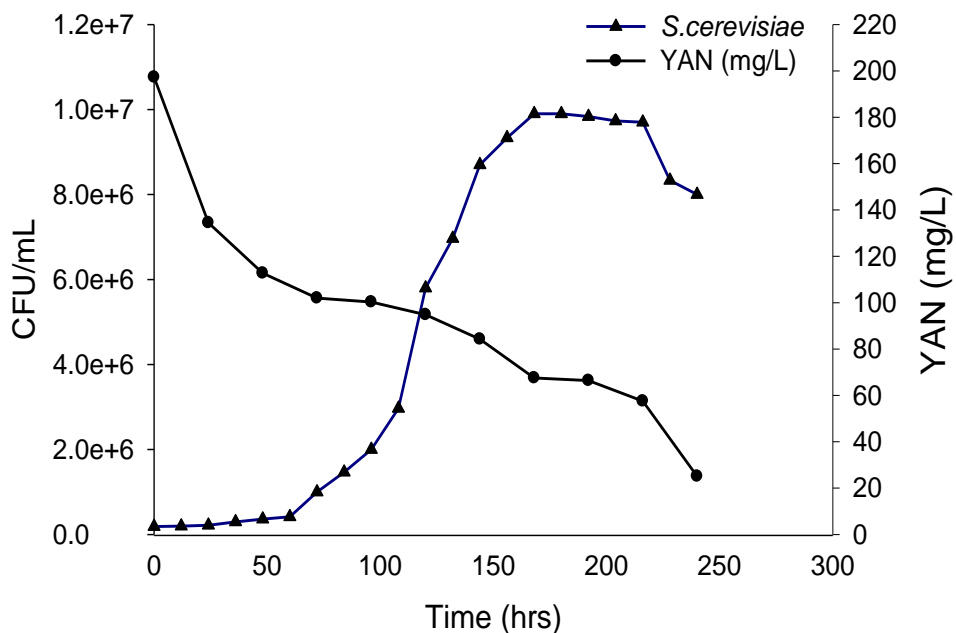
Figure 25 Average yeast cell population (CFU/ml) growth during fermentation and YAN consumption in grape juice medium inoculated with D80. Results indicate the average of three fermentation replicates.

Monitoring yeast growth during fermentation is important to investigate if these are associated with changes in MPs concentrations. Figure 25 shows the change in yeast cell growth during fermentation.

The adaptation time of D80 lasted 3 days, followed by the growth phase. Eight days after the fermentation was started, D80 achieved its highest population levels, 9.8×10^6 CFU/mL. YAN is expressed as the sum of the α-amino acid nitrogen plus ammoniacal nitrogen. Consumption of YAN is show in figure 25. D80 yeasts consumed up to 89% of YAN by the end of the fermentation.

Levels of Methoxypyrazines in GJM Wines

GJM was spiked with IBMP and IPMP at a rate of 40ng/L each. Levels of MPs was measured every twelve hours during 10 days of fermentation. Figure 26 shows the evolution of IBMP and IPMP during D80 yeast strain fermentation. IBMP was significantly reduced down to 24.10 ± 1.07 ng/L, while no change in IPMP (35.39 ± 2.16 ng/L) was observed in comparison with the final concentration of GJM in the control samples (36.42 ± 2.56). The reduction of IBMP was observed at the end of the fermentation (10 days) after the majority of the consumption of YAN had taken place.

In addition, we evaluated MPs levels of wines fermented during 20 days with a chemical defined grape juice medium (GJM) containing a YAN concentration of 200 mg N/L. Levels of MPs were measured every 0, 5, 10, 15 and 20 hours during 20 days.
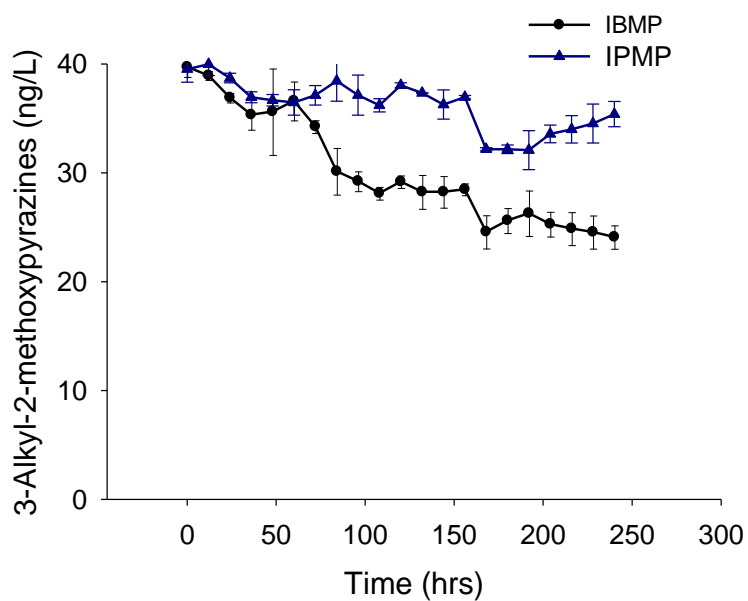
Figure 26 IBMP and IPMP concentrations from GJM spiked with 40 ng/L. Results indicate the average of three fermentation replicates.
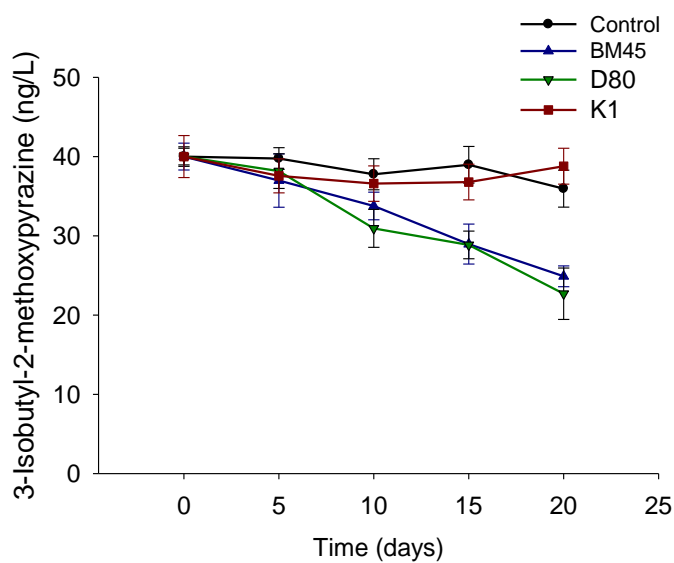
Figure 27. -Effect on IBMP levels during fermentation with three commercially available yeast strains (BM45, D80 and K1)

Fermenting wine with BM45 and D80 yeast strains exhibited the greatest IBMP reduction according to our data. Figure 28 shows the evolution of IBMP during fermentation. Wines fermented with BM45 and D80 yeast reduced IBMP down to 24.89 ± 1.24 and 22.69 ± 3.21 in comparison with the control.

Conversely, wines fermented with K1, D80, and BM45 showed a concentration of IPMP of 36.78±2.26, 34.69±3.58 and 35.31±1.89, respectively. The effects of different yeast strains in IPMP concentrations were non-significantly different from the control.
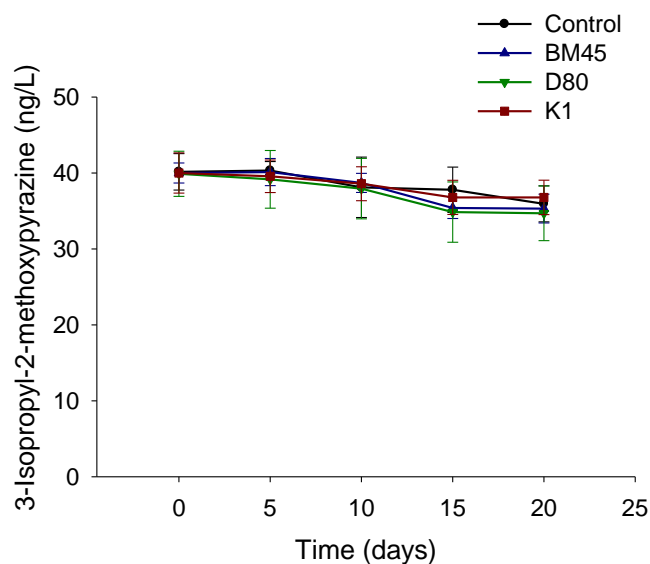
Figure 28-Effect on IPMP levels during fermentation with three commercially available yeast strains (BM45, D80 and K1)

There are many parameters associated with the reduction of IBMP levels during GJM fermentation. It is known that the ability of some yeast strains to reduce volatile aroma compounds may actually be due to sorptive processes involving the yeast cell wall (67).

In addition, we analyzed MPs levels in the yeast mannoproteins at the end of the fermentation. Yeast cells and medium were separated by centrifugation (6000g, 20 min) at the end of the fermentation (10 days). Pellets containing secreted mannoproteins were analyzed for MPs by SPME-GC-MS.

Results show that MPs were retained in the yeast cell wall. Quantifiation of IBMP in yeast mannoproteins was not possible due to areas of the qualifier and quantifier ions were below the method detection limit <1.5 ng/L. The matrix of mannoprotein interfered with intensity signal of these ions. On the contrary, no interaction was shown with IPMP under the same condition. This may allude to the role of the conformational and compositional structure of these macromolecules in the interaction with aroma compounds.

This is the first study that demonstrates evidence for interactions between mannoproteins secreted by the D80 yeast strain and IBMP as show in figure 8. The chromatogram and mass spectrum for IBMP found in yeast mannoproteins with the deuterated IBMP isotope is shown in Figure 29. The retention time obtained for IBMP was 11.47 min while $[^2H_3]$-IPMP had a retention time of 12.2 min.

IBMP was confirmed by matching the retention time of the unknown compound with the retention time of the IBMP standard. However, traces of MPs quantification were not achievable in this study due to the fact that concentration of IBMP was below the detection limit. A selectivity fractionation from the complex matrix of the whole mannoprotein extract needs to be considered.

It is known that wine mannoproteins have the capacity to bind to different wine compounds. This interaction depends on the physical and chemical nature of the volatile compounds. It is known that hydrophobic compounds have a greater degree of interaction with wine mannoproteins (63, 67, 195).
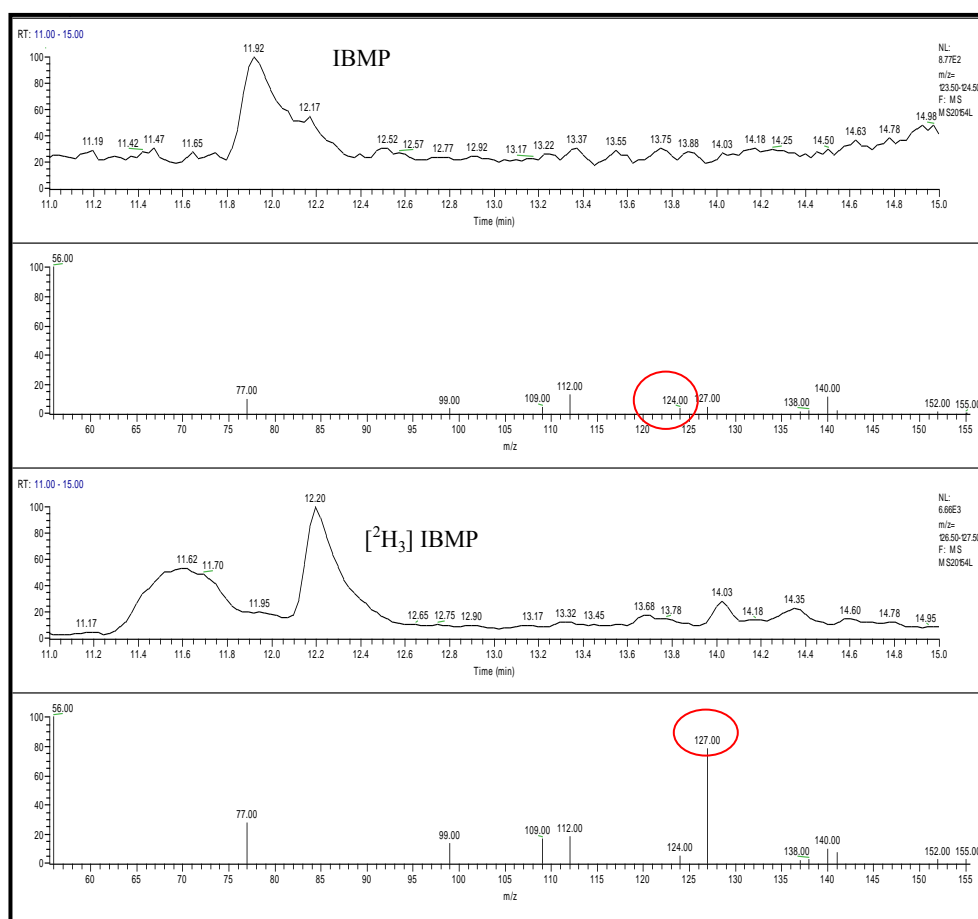
Figure 29 - Mass chromatogram of IBMP and mass spectrum (SIM) of IBM [^{2}H$_3$]IBMP ion 127 in yeast mannoproteins extract.

However It is important to mention that yeast walls from different yeast strains could show a different ability to bind volatile compounds as the composition of yeast cell walls would vary depending on the species and strain of the yeasts (196). Other authors have also observed that the saturation of the sorbent substrate (yeast walls) is the limiting factor in the sorption process of volatile compounds (66). Consequently it has been demonstrated that the retention of volatile compounds by different mannoproteins depends on the accessibility of the binding site (63).

Conclusion

The IBMP and IPMP concentrations of the Black Spanish wines made with the three different strains varied widely depending on the yeast strain used for fermentation. The MB45 strain resulted in the highest amount of MPs followed by K1 and D80. The effects of different yeast strains in IPMP concentrations were not significantly different from the control juice. Furthermore, when MPs were evaluated in a grape juice model, IBMP was decreased by D80 and BM45; without any change in K1 yeast. In the case of IPMP, no significant changes were observed for all yeast strains evaluated.

In addition, yeast mannoproteins were evaluated as a potential mechanism to bind MPs, results showed its presence in D80 yeast strain.

Overall the data suggests that different yeast strains strongly associate with changes in IBMP during the winemaking process due to hydrophobic interactions with yeast mannoproteins. Further study is required to elucidate changes in other volatiles compounds and should include collection of sensory data.

CHAPTER VII

GENERAL CONCLUSIONS

The presented studies focused on research needs centering around quality aspects and health benefits of red wine with increased relevance to the grape and wine industry in the State of Texas, which has been continuously growing within the last decade.

The chemopreventive effect of red wine polyphenols from Black Spanish (*Vitis aestivalis)* were studied in colonic human fibroblast cells and results showed that wine extract decreased gene expression and activation of NF-kB transcription factor and target pro-inflammatory cytokines and cell adhesion molecules. In addition, induction of miR-126 by wine extract was found to be one of the underlying molecular mechanism by which wine extract decreased VCAM-1 and inflammation in colon cells.

In addition, the Green June Beetle was investigated as a potential source of IPMP in wines made from grapes grown in Texas, which was demonstrated by SPME-GC-MS. The undesirable incorporation of GJB into the grape must during harvest, could negatively impact quality of wines by adding green and vegetative aroma.

Moreover, levels of methoxypyrazines were monitored in industry-scale accelerated aging techniques including micro-oxygenation. Results showed that MPs were not significantly decreased over the 96 days of experiment duration in all treatment groups (control, wine with oak pieces, oak barrel, and micro-oxygenation). Previous reports indicating the decrease of MPs by MOX are likely due to masking of the taste and aroma of MPs by MOX or oak-derived compounds.

In contrast, IBMP and IPMP concentrations in Black Spanish wines fermented with three different yeast strain (BM45, D80, and K1) varied widely between strains. Wine fermented withMB45 yeast exhibited an increase in concentration of IBMP, while wine prepared with K1 and D80 showed lower concentrations when compare to the control. Moreover, IPMP, did not show significant differences between yeast strains when compare to the control. Furthermore, when MPs were evaluated in a grape juice model fermented with different yeast strains, IBMP was decreased by D80 and BM45; without any change caused by K1 yeast. In the case of IPMP, no significant changes were observed for all yeast strains. In addition, yeast mannoproteins appeared to bind MPs, a mechanism which has possible application in the wine industry.

In summary, the presented studies provide valuable information concerning potential health benefits and the reduction of methoxypyrazines in red wines, with relevance to the grape and wine industry in Texas.

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APPENDIX A

METHOD VALIDATION AND LINEARITY OF 2-ALKYL-3-METHOXYPYRAZINES STANDARD CURVES IN WINE AND GRAPE JUICE

The linearity of IBMP and IPMP curves in the wine model and the grape juice medium were investigated to assure the validation of the method.

Material and Methods

Standards and Solutions

Reference standards used for identification and quantification of MPs were purchased from Sigma–Aldrich (St. Louis, MO, USA) and included IPMP (97%, IPMP) and IBMP (97%, IBMP). The isotopically labeled internal standards $[^2H_3]$-IBMP and $[^2H_3]$-IPMP were purchased from CDN isotopes (Quebec, Canada).

Standard Curves, Reproducibility and Detection Limits

A standard solution containing IBMP and IPMP with their corresponding deuterated MPs was prepared from each individual standard and subsequently diluted with methanol within a dark colored flask and sealed with parafilm. All the solutions were stored in the dark at 4^oC until use.

For standard curve a model wine was prepared containing 12% (v/v) ethanol and 4 g/L of tartaric acid adjusted to pH 6.6 with NaOH. To measure the MPs levels in GJM, a standard curve was created using the GJM described chapter VI. Seven standard

concentrations of IBMP and IPMP (2.5, 5, 10, 20, 30, 40, 50 ng/L) were added to a 10 mL volumetric flask. Then 10 uL of deuterated MPs was added to the volumetric flask to have a final concentration of 40 ng/L $[^2H_3]$-IBMP and $[^2H_3]$-IPMP, respectively, and topped to the mark with model wine or GJM. Each solution containing the MPs and the deuterated MPs was added to 20 mL glass cylinder bottles containing 3 grams of NaCl (Sigma-Aldrich, St. Louis, MO, USA) and closed with a septum cap.

The repeatability was calculated at different concentration levels for each MPs: 2.5, 20 and 50 ng/L (n=5). Data were analysed and compared using means and relative standard deviation. The method detection limit was calculated using the standard deviation multiply by the t-value at 99.5% of confidentiality and 7 degrees of freedom (3.4994).

Sample Extraction

The 20 mL glass cylinder was placed on a heating plate and clamped in place. The DVB/Carboxen™/PDMS StableFlex™ SPME fiber was inserted into the sample vial and the MPs and their deuterated analogues were adsorbed onto the 2 cm, 23 gauge fiber. The sample has heated to 70°C. The fiber stayed inserted into the headspace of the sample vial for 30min with close attention paid to ensure the fiber did not come into contact with the liquid.

Instrumental Analysis

MPs were quantified by SPME-GC-MS according to the method reported by (*189*). Analysis was conducted using a ThermoElectron Trace GC Ultra (Waltham, MA) equipped with a TriPlus Autosampler and a DSQII mass spectrometer. The autosampler was fitted with a DVB/Carboxen™/PDMS StableFlex™ SPME fiber (Supelco, Bellefonte, PA). Samples (10 mL) were incubated for 30 min at 70˚C and allowed to adsorb from the headspace onto the fiber for 30 min. The fiber was desorbed onto a DB-5 column (30 m x 0.53 mm x 5μm film thickness, J&W Scientific, Agilent Technologies, Santa Clara, CA). The injector was held at 250 ◦ C with no purge for 5 min, then was purged at 50 mL/min for an additional 5 min. The oven was held at 70 ◦C for 5 min and temperatures then were increased 3 ◦ C/min up to 115 °C, 1◦ C/min up to 120 °C, and finally 10 °C up to 230 ◦ C before holding for 10 min. Helium was used as the carrier gas at constant pressure (10.36 psi) with a nominal initial flow of 1.5 mL/min. The MSD interface was held at 250 °C while the temperature of the ion source was at 200 ◦C. Identification was achieved using selected ion monitoring (SIM). Selected mass channels were m/z 109 and 124 for IBMP and m/z 112 and 127 for [^{2}H$_3$]-IBMP. Ions 124 and 127 were used for quantification, while ions 109 and 112 were used as qualifier ions. For IPMP, selected mass channels were m/z 137 and 152 and m/z 140 and 155 for [^{2}H$_3$]-IPMP. Ions 137 and 140 were used for quantification while ions 152 and 155 were used as qualifier ions.
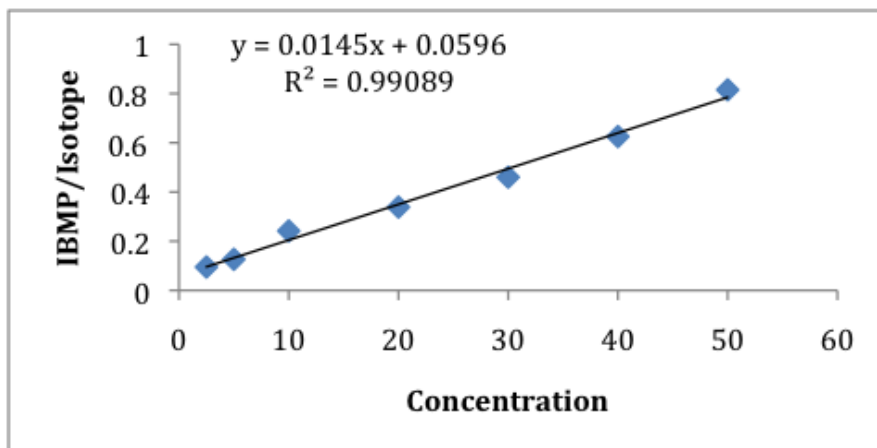
Results and Discussions

To quantify the levels of MPs in Black Spanish red wines. A standard curve was created using a model wine described in materials and methods. At least three replications of each seven standard concentrations were run. The IBMP peak area (m/z 124) in relation to the $[^2H_3]$-IBMP internal standard peak area (m/z 127) was linearly correlated with the IBMP standards over the range examined (R^2= 0.990 for wine and R^2=0.994 for GJM)(Figure 30).

The IPMP peak area (m/z 137) in relation to the $[^2H_3]$-IPMP internal standard peak area (m/z 140) was linearly correlated with the IPMP standards over the range examined (R^2= 0.994 for wine and R^2-0.993 for GJM) (Figure 31). After approximately every 20 samples, standards were analyzed to verify the method. Relative standards deviations of replicate samples were 7.1%, 4.5% and 6.5% at 2.5, 20 and 50 ng/L. The detection limit was <1.5 ng/L for wine and juice respectively.

A deuterated analogue of IBMP and IPMP was chosen as an internal standard in order to quantify trace levels of these MPs in samples. The IBMP and IPMP react nearly identically to their respective deuterated isotopes during isolation and measurement. For this reason the ratios of the IBMP with $[^2H_3]$-IBMP and IPMP with $[^2H_3]$-IPMP remain constant, despite potential variations in sampling efficiency and GC-MS response.
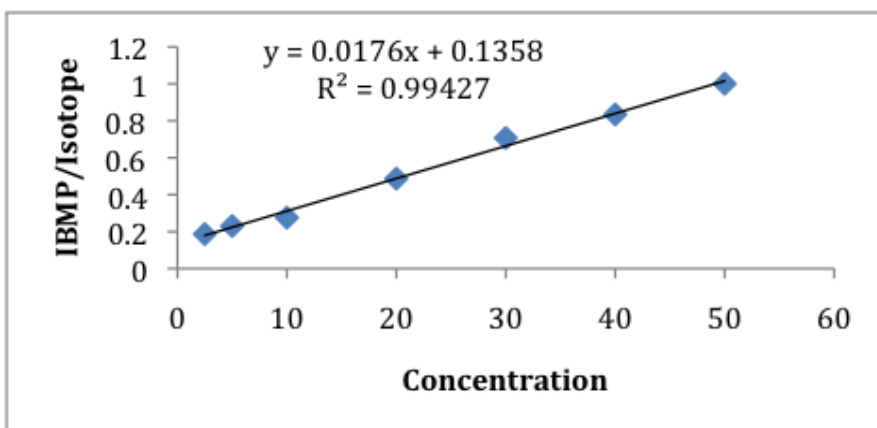
(a)

(b)

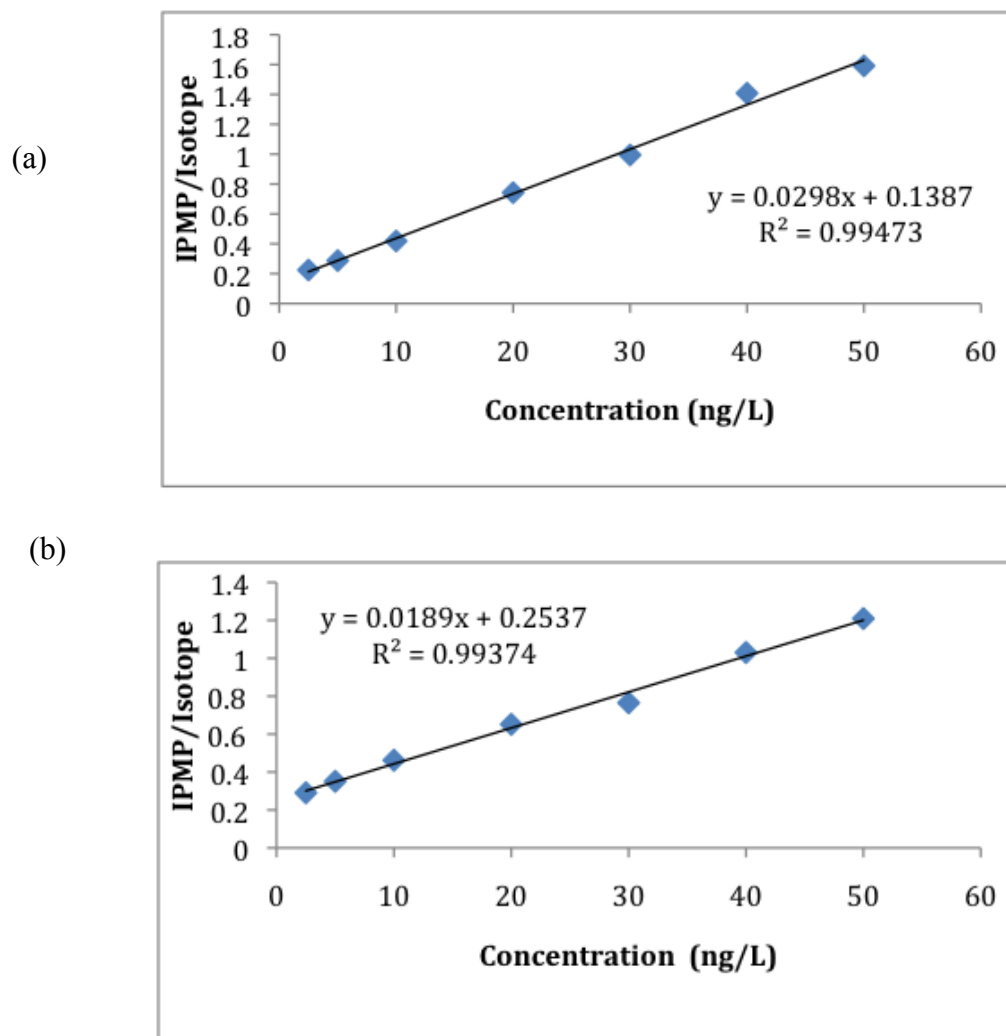**Figure 30** IBMP in relation to the $[^2H_3]$-IBMP internal standard for (a) model wine, (b) grape juice medium.

(a)

(b)

Figure 31. IPMP in relation to the $[^2H_3]$-IPMP internal standard for (a) model wine, (b)grape juice medium.

VITA

Gabriela del Carmen Angel Morales received her Bachelor of Science degree in biochemical engineering from Instituto Tecnologico de Tuxtla Gutierrez in 2003. She entered at Instituto Tecnologico y de Estudios Superiores de Monterrey Campus Monterrey and received her Master of Science degree in biothecnology in May 2005. She finally received her Doctor of Philosophy degree in food science and technology, from Texas A&M University in August 2011.

While at Texas A&M, Gabriela was active in a number of organizations and held the positions of Product Development Team Leader, and Graduate Student Council representative of the Food Science Graduate Student Association.

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