REDUCTION OF 2-ISOBUTYL-3-METHOXYPYRAZINE and 2-ISOPROPYL-3-METHOXYPYRAZINE

A Thesis

by

CLINT BYRON DAILY

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

MASTER OF SCIENCE

Approved by:

Chair of Committee,	Susanne Mertens-Talcott
Committee Members,	Stephen T. Talcott
	Thomas J. McDonald
Intercollegiate Faculty Chair,	Alejandro Castillo

December 2012

Major Subject: Food Science and Technology

ABSTRACT

One of the largest problems in the Texas wine industry is a sensory flaw due to methoxypyrazines (MP). A precise method or material for the remediation of excessive levels of MP in finished wine has not been reported. Wine makers, enologist, and research scientist have been experimenting for years to find a material or method that will selectively reduce MP concentrations in wine or bind them into solution reducing aromatic volatility. MP when present in wines in excessively high concentrations occasion flawed wines; even to the extreme of making them unmarketable to be discarded as waste. Winemakers have developed various solutions to this problem allowing some salvageability. Most winemakers review MP changes subjectively, using before and after sensory tests. If sensory perception for the aromatic profiles of MPs in wines does not indicate the presence of MP quantitative analysis may be used to confirm this observation.

The overall objective of this project was a quantitative investigation of the binding capacity of various materials for 3-alkyl-2-methoxypyrazines in model wine.

Several materials currently used as fining agents have previously been reported to reduce the sensory perception of MP in wine. A material screening was conducted for alumina oxide, diatomaceous earth, copper sulfate, activated carbon, Isinglass, Bocksin, bentonite, toasted oak, untoasted oak, Amberlite XAD-4, FXP H0320, fibresol-2,

aluminum foil, and polyvinylopolypyrrolidone (PVPP) using GC-MS SPEME on a model wine systems spiked with MP. The various fining agents and alternative materials chosen were found to have reduced MP concentrations in the model wine. For the first study quantitative assessments were recorded before and after treatment.

The second objective of this work was an investigation of the time it takes for the reduction in MP levels to occur. The time involved in fining wines is variable depending on the type of wine, the winemaking method, the fining material used, the condition of the wine, and the winemaker's decision. The goal of these time trials is to establish the amount of time it takes for fining agents to bind MPs; in order to provide information to aid winemakers in implementing the selected materials into their wine making method.

ACKNOWLEDGEMENTS

I would like to thank to Dr. Susanne Talcott, my advisor, for giving me the opportunity to work in her lab and for guiding me through the M.S. trip. Also for believing in me and for the opportunities and experiences she made possible during my studies.

I am also very grateful to Dr. Steve Talcott, for demonstrating by example how enjoyable it is to do research in food science and enology. Also to Dr. McDonald and Dr. Cobb for their support and time throughout this trip, they made graduation possible.

I also thank my lab mates and friends Chris, Emily, Nieve, Hunter, Christina, Kimmy, Libby, Shirley, Manuela, for all the good and bad times we spent together. Thanks to Mike Sipowicz for sharing his experiences in the wine industry, and for making this trip fun.

A special thanks to my family for all their support, I am grateful for you in my life, you were the only reason I made it through the "return to academics" experience.

TABLE OF CONTENTS

Page

CHAPTER Page

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

TABLE Page

NOMENCLATURE

Methoxypyrazine	(MP)
Alkyl-methoxypyrazine	(MP)
2-isobutyl-3-methoxypyrazine	(IBMP)
2-isopropyl-3-methoxypyrazine	(IPMP)
Sec-butyl-methoxypyrazine	(SBMP)
Hydroxypyrazine	(HP)
2-hydroxy-3-isobutylpyrazine	(IBHP)
2-hydroxy-3-isopropylpyrazine	(IPHP)
O-methyltransferase	(OMT)
Solid phase extraction	(SPE)
Liquid-liquid extraction	(LLE)
Solid phase microextraction	(SPME)
High performance liquid chromatography	(HPLC)
Gas chromatography	(GC)
Gas chromatography-mass spectrometry	(GC-MS)
Headspace-solid phase microextraction	(HS-SPME)
Gas chromatography flame photometric detection	(GC-FPD)
Headspace gas chromatography-mass spectrometry	(HS-GC/MS)
Carbowax	(CW)

Polyacrylates	(PA)
Polydimethylsiloxanes	(PDMS)
Microoxygenation	(MOX)
Mass selective detector	(MSD)
Environmental protection agency	(EPA)
National oceanic and atmospheric administration	(NOAA)
Untoasted oak	(UO)
Toasted Oak	(TO)
Aluminum foil	(AF)
Polyethylene terephthalate	(PETE)
Diatomaceous earth	(DE)
Alumina basic	(AL)
Copper Sulfate (aqueous solution)	(Cu)
FXP H0320 (Soy Protein)	(FXP)
Polyvinylopolypyrrolidone	(PVPP)

CHAPTER I

INTRODUCTION

Wine making is an ancient process dating back 4000-6000 years B.C.E. In Egypt residue of fermented grapes was found in containers with cork stoppers. Wine making took a major transition in 1866 with Louis Pasteur's work on wine spoilage. He was the first to isolate bacteria from wine which in turn led to his famous work "Etudes sur le vin," translated "a study in wine" (1). The global wine industry was last reported to have produced 26,759,900 liters of wine in 2009 as depicted in Table 1, statistics provided by the Wine Institute (2). The United States is fourth in the world for wine production by volume (Table 1).

TABLE 1 World Wine Production (liters/volume) 2006-2009 (2)

World Wine Production 2006-2009 Top Ten Wine Producing Countries and Percent Change Since 2009/2006 (Liters 000)					
Country (1)	**2006**	**2007**	**2008**	**2009**	**% of Total Liters**
World Total	**28,729,000**	**27,128,800**	**27,173,900**	**26,759,900**	**100.00%**
France	5, 302, 500	4,654,700	4,280,600	4,700,000	17.56%
Italy	5,460,000	4,918,900	5,047,000	4,650,000	17.38%
Spain	4,367,900	4,207,00	4,190,900	3,800,000	14.20%
United States	2,438,300	2,510,800	2,431,500	2,777,200	10.38%
Argentina	1,539,600	1,504,600	1,470,000	1,210,000	4.52%
Australia	1,325,000	955,000	1,237,000	1,171,000	4.38%
Chile	844,800	828,000	869,000	987,000	3.69%
Germany	899,500	1,036,300	999,100	928,000	3.47%
South Africa	939,800	851,600	763,300	780,700	2.92%
Portugal	754,200	604,900	562,000	600,000	2.24%

The Texas wine industry has seen tremendous growth over the last decade. While wine production was once struggling Texas is now the fifth largest winemaking state in the US. Wine is a complex matrix consisting of a myriad of compounds that when brought together in various combinations and concentrations can be beneficial or detrimental to the wine as a final product. In the myriad of compounds contained in wine, the compound group of methoxypyrazines (MP) has been the subject of several investigations worldwide.

Methoxypyrazines

Pyrazines are a class of volatile odorants found in most plants throughout the plant kingdom. As potent aromatic compounds pyrazines are found to produce desirable aromas in some foods and beverages. However, pyrazines are considered a sensory defect in others such as certain fresh fruits and wines. Many pyrazines yield pleasant aromas associated with roasted meats, roasted peanuts, cocoa, coffee, and cereal grains (3). Providing complex aromas and flavors; pyrazines are often used as a food additive to enhance sensory characteristics by attributing a mosaic of flavor and aroma layers triggering various sensory receptors. MPs are a subclass of pyrazine compounds that are powerful odorants having a sensory profile most often described as herbaceous. The herbaceous aromas are correlated with vegetables such as asparagus, green bell peppers, peas, and potatoes (4). Intrinsic to some grape varieties are higher concentration levels of MPs compared to other varieties. If MP levels are in balance with other aromatic volatiles, tannin

structure, residual sugar, and acidity, MPs become a desirable descriptive character for some wines such as Sauvignon Blanc, Semillon, and Cabernet Sauvignon (5). Essential for Sauvignon Blanc are the green capsicum aromas provided by MP, without these green notes the wine is found to be of inferior quality (4). In 2004, adulteration of Sauvignon Blanc was discovered in South Africa. Wine makers were adding MP to wine, increasing the unique aroma in their Sauvignon Blanc that previously had lower than desired MP concentrations, thereby increasing the perceived value of the wine (6).

In contrast, elevated concentrations of MPs overpower desirable aromatic volatiles leading to strong, unpleasant, herbaceous aromas. When this occurs, MPs are considered a flavor defect. MP detection through the human ortho-nasal passage occurs at very low concentrations and is reported to have been detected by sensory analysts at concentration levels as low as 0.32 ng/L in water (7). The extremely low concentrations of MPs detectable by olfactory bulb indicate a very low sensory threshold. This low threshold and distinct contributing odor has made this class of compound the target of research. Particularly 2-Isobutyl-3-methoxypyrazine & 2-isopropyl-3-methoxypyrazine as they are commonly found in water supplies, food, and plant based beverages such as wine are considered are considered undesirable in higher concentrations. MP concentrations in Texas wines are often higher than desired.

The objective of this study was to analyze various materials for their binding capacity of MPs in wine. The materials were analyzed in a model wine system for binding capacity. The relationship of MP binding materials will be examined for traits and characteristics.

Future research will further investigate these materials based on the findings in this study where the affinity of materials to desired volatiles and other wine components will be assessed as well as the feasibility of its use in wine production. The goal of this study is to lay the groundwork to develop an, affordable, accessible, material or method, to bind MPs and improve, the overall quality of the wine. Linked to this goal is the intention to provide more information for the Texas wine industry to increase the production of high quality wines.

CHAPTER II

LITERATURE REVIEW

Pyrazines in Wine and Food

Pyrazines occur in a wide variety of wines and foods (Table 2) imparting an essential element to the flavor composition of these products. Scientists and flavor chemist have therefore set out to isolate pyrazines from various foods. In the 1960's scientist began reporting on isolation of pyrazines from foods. MPs were first reported to be isolated from green bell peppers in 1969 (8). Of the several identified pyrazine compounds the most abundantly occurring are 2-alkyl-3-methoxypyrazines (9).

In wine it is 2-alkyl-3, & 3-akyl-2-methoxypyrazines that are garnering attention for research due to their mostly undesirable contribution to wine aroma and flavor. The aromatics of 2-alkyl-3-methoxypyrazines are pungent, specifically from 2-isopropyl-3-methoxypyrazine and 2-isobutyl-3-methoxypyrazine (3).

TABLE 2 Pyrazines in Food and Wine Products

Pyrazines in Food and Wine Products					
Foods and Beverages			**Wines and Spirits**		
Raw Products	**Cooked Products**	**Oil and Fat**	**Red**	**White**	**Spirits**
Coffee beans	Bread	Peanut	Merlot	Sauvignon Blanc	Whiskey
Whey Powder	Roast Coffee Beans and Coffee products	Sesame	Syrah	Chardonnay	Dark Rum
Legumes	Roast Meat	Soy Bean	Tempranillo	Gewürztraminer	
Potatoes	Fried Meat	Galbanum	Cabernet Franc	Cabernet Blanc	
Nuts	Pressure Cooked Meats	Olive	Pinot Noir		
Green Beans	Legumes and Products	Avocado	Baco Noir		
Cruciferous Vegetables	Potatoes & products	Walnut	Marechel Foch		
Sugar Beets	Molasses	Hazelnut			
Asparagus	Beef Broth	Pine Nut			
Tomatoes	Chicken Broth	Beef			
Mushrooms	Pork Broth	Pork			
Avocados	Fish Broth	Fish			
Leafy Vegetables	Offal Products	Poultry			
Peppers					

Pyrazine Structure

Within the pyrazine compound class there are several derivatives (Table 3) that have been isolated and synthesized for the food and fragrance industry. The basic chemical structure of pyrazine compounds is heterocyclic with nitrogen in the 1 and 4-positions (Figure 1). The nitrogen in the 1,4 positions create an inductive effect resulting in electron deficiency of the carbon atoms (Figure 1). This results in pyrazines being resistant to electrophylic substitution. Moreover, pyrazines are able to form stable anions in that the electrons on the nitrogen molecules are rarely delocalized (8). The primary resulting anions found in foods and wine are the methyl, ethyl, methoxy, secbutyl, isobutyl, and isopropyl (10).

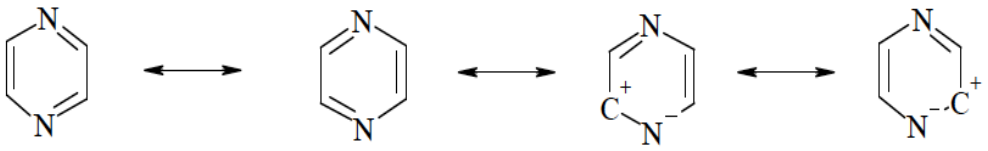

FIGURE 1. Carbon Resonance of Pyrazine.

TABLE 3 Pyrazines and Derivatives in Food, Wine and Spirits

Pyrazines and Derivatives In Food, Wine and Spirits		
Pyrazine	**2-methylpyrazine**	**2-ethylpyrazine**
2,5-dimethylpyrazine	2,6-dimethylpyrazine	2-ethyl-3-methylpyrazine
2-ethyl-S-methylpyrazine	2,3-dimethylpyrazine	2-ethyl-6-methylpyrazine
2,3,S-trimethylpyrazine	2,3 –diethylpyrazine	2-ethyl-3,S-dimethylpyrazine

TABLE 3 Continued

Pyrazine	**2-methylpyrazine**	**2-ethylpyrazine**
2,3,5,6-tetramethylpyrazine	2,3-diethyl-S-methylpyrazine	2-butyl-3-methylpyrazine
2-isobutyl-3-methylpyrazine	2-propyl-3-methylpyrazine	2-propyl-3, S [and 3,6,1-dimethylpyrazine
2-butyl-3,5 (and 3,6)-dimethylpyrazine	2-isobutyl-3,5 [and 3,61-dimethylpyrazine	2-n-propylpyrazine
2-isopropy l pyrazine	2-t-butylpyrazine	2-isobutylpyrazine
2 -vinylpyrazine2-methoxy-3-methylpyrazine	2-methoxy-3-ethylpyrazine	2-methoxypyrazine
2-methoxy-3 -n -propyl pyrazine	2-methoxy-3-isopropylpyrazine	2-ethoxy-3-ethylpyrazine
2-ethoxy-3-isopropylpyrazine	2-methoxy-3-isobutylpyrazine	2-methoxy-3 -sec-buty1 pyrazine
2-n-propoxy-6-methylpyrazine	2-n-propoxy-3-5 -methylpyrazine	2-isopropoxy-3-5 -methylpyrazine
2-methylthio-3-methylpyrazine	Mercapto-methylpyrazines	Pyrazinylmethyl sulfide
2-methylthiopyrazine	2-mercaptomethylpyrazine	Pyrazine-ethanethiol
Pyrazine methanethiol	2-methylthio-3-ethylpyrazine	2-methylthio-3-isopropylpyrazine
2-methyl-3,5 or 6-quinoxaline	5H-5-methyl-6, 7 –dihydrocyclo pentapyrazine	5,6,7,8-tetrahydroquinoxaline
Cyclohexapyrazine	Tetrahydroquinoxaline	2-methylquinoxaline
5-methylquinoxaline	6-methylquinoxaline	5&6-methylquinoxaline
Furfurylthiopyrazine	2-acetylpyrazine	2-acetyl-3-methylpyrazine
2-acetyl-3-ethylpyrazine	2-aminopyrazine	2-amino-6-chloropyrazine
3-aminopyrazine-2-carboxylic acid	2-hydroxypyrazine	2-chloropyrazine
2-cyanopyrazine	2-chloro-3-methylpyrazine	2-chloro-3,(5),(6)-methylpyrazine

TABLE 3 Continued

Pyrazine	**2-methylpyrazine**	**2-ethylpyrazine**
2-chlor-3-ethylpyrazine	2,3-dichloropyrazine	2,6-dichloropyrazine
2,5-distyrylpyrazine	Pyrazinamide	Pyrazine 2-t-butyl-carboxamide
Pyrazine 2-carboxylic acid	Pyrazine 2-carboxylic acid	Pyrazine 2-carboxylic acid
Pyrazine 2,3-dicarboxylic acid	2-methyl-5-pyrazinoic acid	Pyrazine dipotassium tetracarboxylate

The pyrazines used for the investigations in this study are 2-alkyl-3-methoxypyrazines (Figure 2.). 2-isobutyl-3-methoxypyrazine is a pyrazine with isobutyl in the 2 position and a methoxy group in the 3 position (Figure 3, Table 4). A summarization of the chemical and aromatic profiles of studied methoxypyrazines was described by Peter J. Hartman (11).

TABLE 4 Pyrazine Compounds.

Pyrazine Compounds									
Name	**CAS**	**MW**	**Formula**	**Bp°C**	**Mp°C**	**Odor Threshold (ppb in water)**	**Odor**	**Density**	**RI**
Pyrazine	290-37-9	80.09	C4H4N2	115.5	54	.65			
2-isobutyl-3-mehtoxypyrazine	24683-00-9	166.220	C9H14N2O	120-126		0.002	green bell pepper	0.990	1.4922
2-isopropyl-3-methoxypyrazine	25773-40-4	152.200	C8H12N2O	94-100	61.5	0.002	bell Pepper, Asparagus, earthy	0.996	1.4940

The 2-isopropyl-3-methoxypyrazine is a pyrazine with an isopropyl group in the 2 position along with a methoxy group in the 3 position (Figure 4).

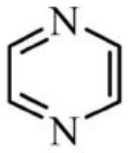

FIGURE 2. Pyrazine.

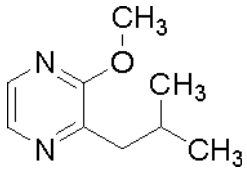

FIGURE 3. 2-isobutyl-3-methoxypyrazine.

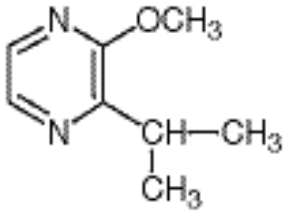

FIGURE 4. 2-isopropyl-3-methoxypyrazine.

It has been observed that 2,3 or 3,2 alkyl-methoxypyrazines are often referred to as the same molecule and used interchangeably (12). To clarify; positions 2,3 vs. 3,2 for 2-alkyl-3-methoxypyrazine & 3-alkyl-2-methoxypyrazine represent isomeric examples of the same molecule differentiating between alkyl/methoxy groups in alternate positions. One of the first articles to illicitly differentiate 2,3 vs. 3,2 positioning was by Cudjoe Erasmus, 2004 (12). Literature reviewed shows that the isomeric differences between the 2,3 & 3,2 positioning provides no distinction of the two isomers through sensory profiling. The two isomers of Alkyl-MPs MP are both referred to as having aromas of

green bell pepper, asparagus, grassy, green, and herbaceous (3, 13). Examples of the molecular differences of these two molecules are shown below (Figure 5).

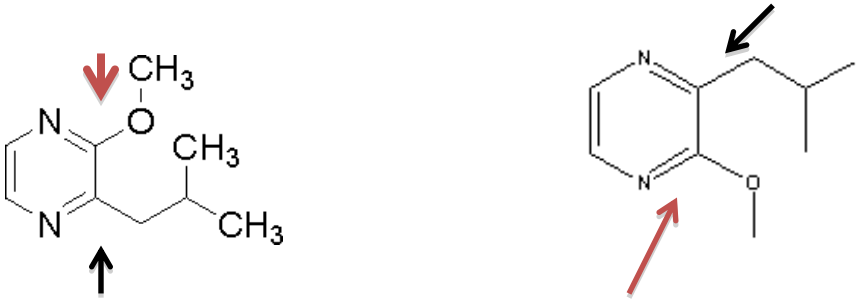

FIGURE 5. Comparison of 2-isobutyl-3-methoxypyrazine Isomers.

Methoxypyrazines in Grapes and Wines

Alky-methoxypyrazines (MPs) are found in several grape varieties particularly that of Sauvignon Blanc and Cabernet Sauvignon. From bud to veraison, throughout maturation, MPs remain in these two varieties at high concentrations, yielding signature "green" notes. Typically MP concentrations are high pre-veraison and decrease throughout the ripening process with sudden drops prior to full maturation then stabilizing throughout the remainder of maturation (13). Grape varieties that commonly do not have high levels of MPs after maturation can end up with high concentrations due to a variety of mechanisms. Late winter freezes (late March into May) occur often in Texas (Table 5). A correlation between weather conditions and MP concentrations has been observed. Grapes from cooler climates that ripen slower coupled with under-ripe grapes result in higher MP concentrations (14).

TABLE 5 First and Last Freeze Date Averages and Extremes in Texas Wine Regions (14)

First and Last Freeze Date Averages and Extremes in Texas Wine Regions						
Location	**First Freeze** (1950-2011) Avg.	**Last Freeze** (1950-2011) Avg.	**Earliest Freeze on Record**		**Latest Freeze on Record**	
Rocksprings	NOV 19	MAR 18	OCT 20,	1976	APR 17,	1947
Del Rio	DEC 1	FEB 22	OCT 27,	1913	MAR 31,	1987
Carrizo Springs	NOV 29	FEB 21	OCT 30,	1980	MAR 30,	1903
Eagle Pass	DEC 4	FEB 18	OCT 17,	1903	APR 5,	1920
Llano	NOV 29	MAR 23	OCT 13,	1977	APR 18,	1921
Fredericksburg	NOV 11	MAR 22	OCT 8,	1952	APR 17,	1947
Lubbock	OCT 31	APR 10	OCT 7,	1952	MAY 8,	1938
Amarillo	OCT 24	APR 13	SEP 21,	1983	May 7,	1915
Blanco	NOV 9	MAR 22	OCT 8,	1952	APR 19,	1921
Johnson City	NOV 13	MAR 22	OCT 19,	1989	APR 18,	1999
Boerne	NOV 11	MAR 23	OCT 8,	1952	APR 22,	1931
Austin Mabry	DEC 2	MAR 23	OCT 26,	1924	APR 9,	1914
Austin Bergstrom	NOV 27	MAR 4	OCT 25,	2005	APR 17,	1999
San Marcos	NOV 22	MAR 5	OCT25,	1955	APR 16,	1961
New Braunfels	NOV 24	MAR 8	OCT 20,	1989	APR 14,	1980
San Antonio	NOV 25	MAR 2	OCT 30,	1917	APR 3,	1987
Hallettsville	NOV 25	FEB 27	OCT 8,	1952	APR 8,	2009
Smithville	NOV 19	MAR 9	OCT 21,	1989	APR 14,	2008
Yoakum	NOV 30	FEB 28	OCT 30,	1993	APR 3	1987

One method that is used by vineyard managers in order to reduce MP levels is canopy pruning. Specific pruning methods are implemented to reduce the vegetative growth of the canopy coupled with leaf removal around the clusters to provide full sun exposure.

In 2011, J.J. Scheiner reported on Leaf removal at 50% and 100% foliage removal. The author states that dramatic effects on the final MP concentration in grapes occur due to leaf removal (15). Other influences on MP concentration in grapes are climate, soil moisture, crop load on the vine, and uneven ripening. Recently a trial was conducted that challenges previous beliefs that MP concentrations are influenced dramatically by grape ripeness/maturity and terroir. Four sequential years, in three separate vineyards were used to monitor MP evolution. MP development starts as fruit develops, increases then declines during veraison. The study reports that climate was the major contributing factor to MP concentrations not necessarily fruit maturation. Plants synthesize MPs as secondary products of amino acid metabolism. The reported biosynthetic pathway involves formation of an amide from an amino acid, then formation of a pyrazine which goes through methylation (16). The complete biosynthetic pathway leading to the formation of MPs is still unknown; however, a number of pathways have been proposed.

All proposals 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 (16). 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 (16). 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 (17).

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 (Figure 6) (16).

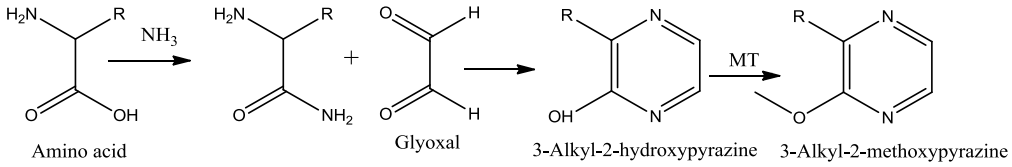

FIGURE 6. Proposed Biogenesis Pathway of Methoxypyrazines.

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. (18). 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 (Figure 7), (18, 19).

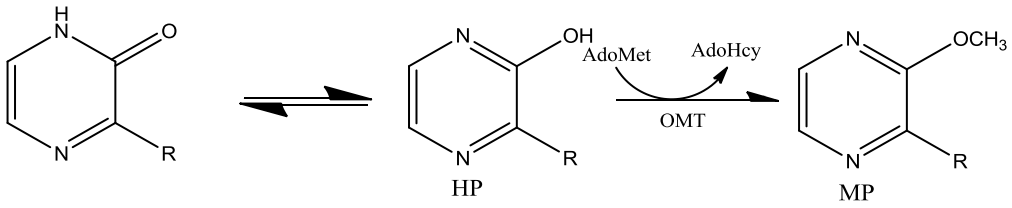

FIGURE 7. Enzymatic 0-methylation Of HP in Grapes.

Sequencing of the N-terminus of the purified methyltransferase enzyme enabled the identification of a grape cDNA that encodes this enzyme (19). While this gene is yet to be functionally characterized, a number of results imply that this gene is involved in the pathway of MP 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 (15), 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.

Methoxypyrazines in Model Wine

Analysis of Methoxypyrazines

Over the last few years, several analytical methods have been implemented in the analysis of methoxypyrazines in foods and wines. Varying methods were solid-phase extraction (SPE), liquid-liquid extraction (LLE) and the most recent, solid phase micro extraction (SPME). High performance liquid chromatography (HPLC) was used by Heymann, et al. in an attempt to quantify MPs in chenin blanc wine (20). For the sample preparations; steam distillation followed by concentration on C_{18} cartridges was used, but resulted in a poor recovery (53 ± 7%) with a high detection level of MP at (1.2 µg/L). As recently as 1999 analysis of MPs using a similar approach only with gas chromatography (GC) was performed and yielded much higher recovery concentrations (21). Due to better recovery concentrations using GC, and the difficulty in which MP are quantified, experimentation of hyphenated analytical systems such as GC-MS began. Other hyphenated analytical systems that have been used are headspace-solid phase microextraction (HS/SPME), gas chromatography flame photometric detection (GC/FPD), and headspace gas chromatography-mass spectrometry (HS-GC/MS) to name a few.

SPME has proven to be the most sensitive, efficient, and cost effective means for MP analysis. SPME allows for one-step sample prep without the need for solvents or columns. SPME is commonly used in the trace analysis of low molecular weight volatile compounds such as MPs. Primarily coupled with GC, but sometimes with HPLC, as

aforementioned, SPME detection varies due to the distribution constant of the compounds partitioned between the SPME fiber (stationary phase) and the sample partition coefficient (Kfs) in the head space. Different SPME fibers are more conducive to binding varying size compounds, the need for the appropriate fiber is necessary. Silica fibers are coated with varying adsorbants such as Carbowax (CW), polyacrylates (PA), and polydimethylsiloxanes (PDMS). Most low molecular weight volatiles require the use of PDMS-Carboxen fibers in the SPME (11).

The occurrence of MPs in such low concentrations coupled with MP volatility; GC/MS has proven currently to be the most effective analytical technique for qualitative and quantitative analysis, proven accurate in detecting MPs in the low ng/L levels 8. Therefore, GC/MS was the analytical technique chosen for use. The method for analyzing MPs in model wines was provided by Belancic 2007 for implementing MP standards and isotopes (22).

For greater accuracy in quantification deuterated labeled IBMP & IPMP isotopes as an internal standard have recently been used in MP analysis. Having MP isotopes coupled with MP standards allows for greater analytical certainty by calibrating the plotted ratio of MP standard concentration to the MP isotope concentration. The experiments later described were performed using deuterated labeled IBMP & IPMP internal standards to provide accurate assessment.

Current Methods for Remediation of MP in Wines

Cellar Practices

Current methods employed for remediation of MP in wines have proven to be inconsistent and often inefficient. Some of the methods currently used to reduce concentrations or alter sensory effects of MP in wines are microoxygenation (MOX), spin cone resonance, active packaging materials, MP reducing yeast strains, malo-lactic fermentation and binding via various materials such as bentonite and oak. Materials and methods with proven efficacy such as activated carbon and thermovinification result in non-selective stripping of desirable polyphenolics and volatiles from the wines in the process of reducing MP. Results of some cellar methods such as bentonite fining, oak additions, and MOX have been suboptimal (22).

MOX: MOX has been found to be beneficial in bringing about better color and stability, greater complexity of organoleptic characteristics, reduction in sulfur off-odors and the acceleration of ageing (22). MOX has not proven to be effective in reducing MP concentrations (23). However, wines treated for MP defects using MOX has shown to be effective in reducing the off-putting "green" aromas. This is speculated by enologist to be due to the synergistic effects of sulfur off-odors with MPs. If the sulfur off-odors are decreased the green characteristics due to MPs decrease as well. Sensory trials need to be conducted to provide more information.

Spinning Cone Columns: The primary use of spin cone columns in wine making is for the removal of excess alcohol due to high sugar levels in grapes upon harvest. Spin cone technology in its most advanced form is a mild, analyte selective, method of steam distillation. The column is made from stainless steel with conical vanes attached alternating on the walls of the column fitted to a central rotation point. The column rotates at high rpm and steam is pumped into the column from below. The rotation allows for a thin layer of liquid to move over the vanes providing high surface area whereby lower molecular weight compounds evaporate. Often the columns are fitted with vacuum to accelerate the process. Temperature, pressure can be adjusted to target specific compounds. The high volatility and low molecular weight results in MP volatilizing with less heat required. Less heat aids in the preservation of desired aromatic compounds. Even though less heat is required; any heat application in wine making alters the varietal characteristics and changes the integrity. These changes that occur using spin cone columns along with the high cost of the units make this technology less than ideal in removing MPs from wines.

Active Packaging: Active packaging is an innovative method used in food, drug, and alcohol industries to preserve product quality, extend shelf life, provide information through indicators about product activity and inhibit microbial growth among other things (24). Active packaging has allowed for the food industry to produce mildly processed foods and wines resulting in fresher looking and tasting products with more vibrant color by reducing the rate of oxidation. By incorporating O_2 scavenging agents

into the packaging such as iron powder, ascorbic acid, photosensitive dyes, and varying enzymes that have been immobilized onto the package. Research was conducted on wines spiked with MP analytes then stored in Tetrapak packaging for 18 months. The results showed a reduction in MP at 45, 32, and 26% (25). While Tetrapak packaging has shown to reduce MP concentrations in wines over long-term periods the drawbacks to using Tetrapak for all wines with elevated MP concentrations are numerous. Tetrapak packaging requires the use of Tetrapak processing facilities. The equipment and packages are owned solely by Tetrapak are expensive compared to bottles and corks and Tetrapak aseptic processors require large volumes to contract the facilities. These reasons make Tetrapak packaging economically unfeasible for the majority of wineries that don't produce enough volume of wine or can afford the packaging and processing costs.

Effects of Yeast Strains on MP: Several studies have been done observing the effects of varying yeast strains on final wine aromas and MP concentrations. Several yeast strains altered the sensory perceptions of the wines reducing the perceived green aromas (26). In 2006, a study was done using the Lalvin BM-45 and Lalvin D80 strains. It was found that these strains reduced MP up to 37% (17). The discovery of the capacity for yeast to lower sensory perception and even actual concentrations of MPs during fermentation is an advance in winemaking. However significant the sensory change and reduction in pyrazine concentrations by yeast may be, the effect still may not be enough to reduce

MP levels below sensory threshold or effectively mask/ alter the potent herbaceous aromas of MPs after fermentation is complete.

It is evident that there currently is no ideal method for the removal of MPs from wines. Therefore, the intention of this research is to further explore methods and materials that may facilitate the reduction of MPs in wines.

CHAPTER III

SAMPLE PREPARATION AND INSTRUMENTAL ANALYSIS

Model Wine

All experiments and standard curves were performed in a MP-free model wine system made to specifications provided by Kotseridis, Y.S. (21). A model wine was chosen, free of all compounds, providing a pure system to quantitatively determine interactions of MP with experimental materials, as demonstrated in Table 6.

TABLE 6 Model Wine

Model Wine	
Components	**Ratio**
Water	100% v/v
Alcohol	12%, v/v
Tartaric Acid	4 g L^{-1}

Model wine was adjusted to pH 6 by using NaOH

Standards and Internal Standards

Reference standard compounds of both MPs (IBMP 99%) & (IPMP-97%) were used in quantification. Standards were obtained from Sigma Alderich. Deuterated labeled isotopes for use as internal standards were procured from CDN isotopes (Quebec, Canada), both [$^2H_3$]-IBMP and [$^2H_3$]-IPMP at 99.9% purity as described by S. Bailey (6) & D.M. Chapman (27). A standard solution containing IBMP and IPMP with

corresponding deuterated MPs were prepared from each individual standard and internal standard isotope. All were diluted with methanol to the concentration of 10,000 ng/L. All the standard & isotope solutions were stored in reagent bottles wrapped in foil, sealed with para-film, in the dark at 4˚ C until use.

Standard Curve

For standard curve model wine was prepared containing 12% (v/v) ethanol and 4 gr/L of tartaric acid, adjusted to pH 6.6 with NaOH. Approximately 9.5 mL of model wine was added to a 10 mL. volumetric flask spiked with IBMP and IPMP to give MPs concentrations in the range of 2.5–50 ng/ L. An internal standard of deuterated MPs were added at a concentration of 40 ng/L of [$^2H_3$]-IBMP and [$^2H_3$]-IPMP to all flasks in the range of 2.5-50 ng/L. The flasks were topped to the mark with model wine solution for a final volume of 10mL. Each solution containing the MPs and the deuterated MPs were added to 20 mL glass GC-vials containing 3 gr of NaCl and closed with a septum cap.

Instrumental Analysis

Analysis was conducted using a ThermoElectron Trace GC Ultra (Waltham, MA) equipped with a TriPlusAutosampler and a DSQII mass spectrometer. The samples were analyzed using a solid phase micro-extraction (SPME) headspace device; 50/30µm DVB/Carboxen™/PDMS StableFlex™ SPME fiber (Supelco, Bellefonte, PA) fitted to the auto-sampler. 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 Rxi-1ms non-polar phase dimethyl polysiloxane Crossbond® column (60 m x 0.25 mm x 1µm film thickness) GC column provided by Restek Innovative Chromatography products, Bellfonte, PA. 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, then increased 3° C/min, up to 110° C and held for 1 min at 110° C, then increased again to 25°C/min 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. Compound 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 [$^2H_3$]-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 analyzed in triplicate and given an allowable error of (± 15%) in accordance with EPA guideline 121.

Sample Preparation

Approximately 12 mL of model wine was added to 16 x 150 mm (25 mL) glass test tubes for each sample. 48 µL of deuterated MPs internal standards were added providing concentration 40 ng/L followed by 60 µL of the MP standards providing concentration 50 ng/L. Theses concentrations were chosen to reflect common MP

conditions in wine post-fermentation and were kept as standard concentrations for all the sample trials as described by Y.S. Kosteridis (25). The MP-laden wine was then treated accordingly with the chosen materials. NaCl (3 g.) was added to glass GC vials followed by the treated model wine trials then closed with a septum cap.

The 20 mL glass cylinder was placed on a heating plate and clamped in place. The 50/30µm DVB/Carboxen™/PDMS StableFlex™ SPME fiber was inserted into the sample vial and the MPs and their deuterated analogues were adsorbed onto the 1 cm, 24 gauge fiber. The fiber stayed inserted into the headspace of the sample vial for 30min, SPME fibers never made contact with solution.

CHAPTER IV

PRELIMINARY RESEARCH

Preliminary research was conducted seeking validation for the hypothesis that MP concentrations can be effectively reduced using adsorbent materials. Other experiments of the like performed by Pickering et al. 2006 were performed during fermentations and not after (22). The goal of performing the experiments in model wine is to simulate post fermentation conditions and concentrate focus on interaction between MP and potential binding materials. The research by Pickering et al was done using various oak chips, bentonite, and activated carbon. It was reported that both oak and carbon when added during fermentation decreased both MP concentrations and sensory perception of "green/herbacious" aromas. Preliminary trials will be conducted in single units over extended/varying time periods (14-17 days) whereby potential binding materials will be added to model wine spiked with MP. The materials used in the preliminary trials are as follows: untoasted oak (UO), aluminum foil (AF), Polyethylene terephthalate (PETE), diatomaceous earth (DE), Alumina basic (AL), and varying combinations of the aforementioned materials.

Elution time for IBMP using the aforementioned column provided peaks in the range of 10-12 min (Figure 8.); however, the IBMP-standard most commonly used would elute between 11: 40-11:55 as did IBMP-isotope (Figure 9).

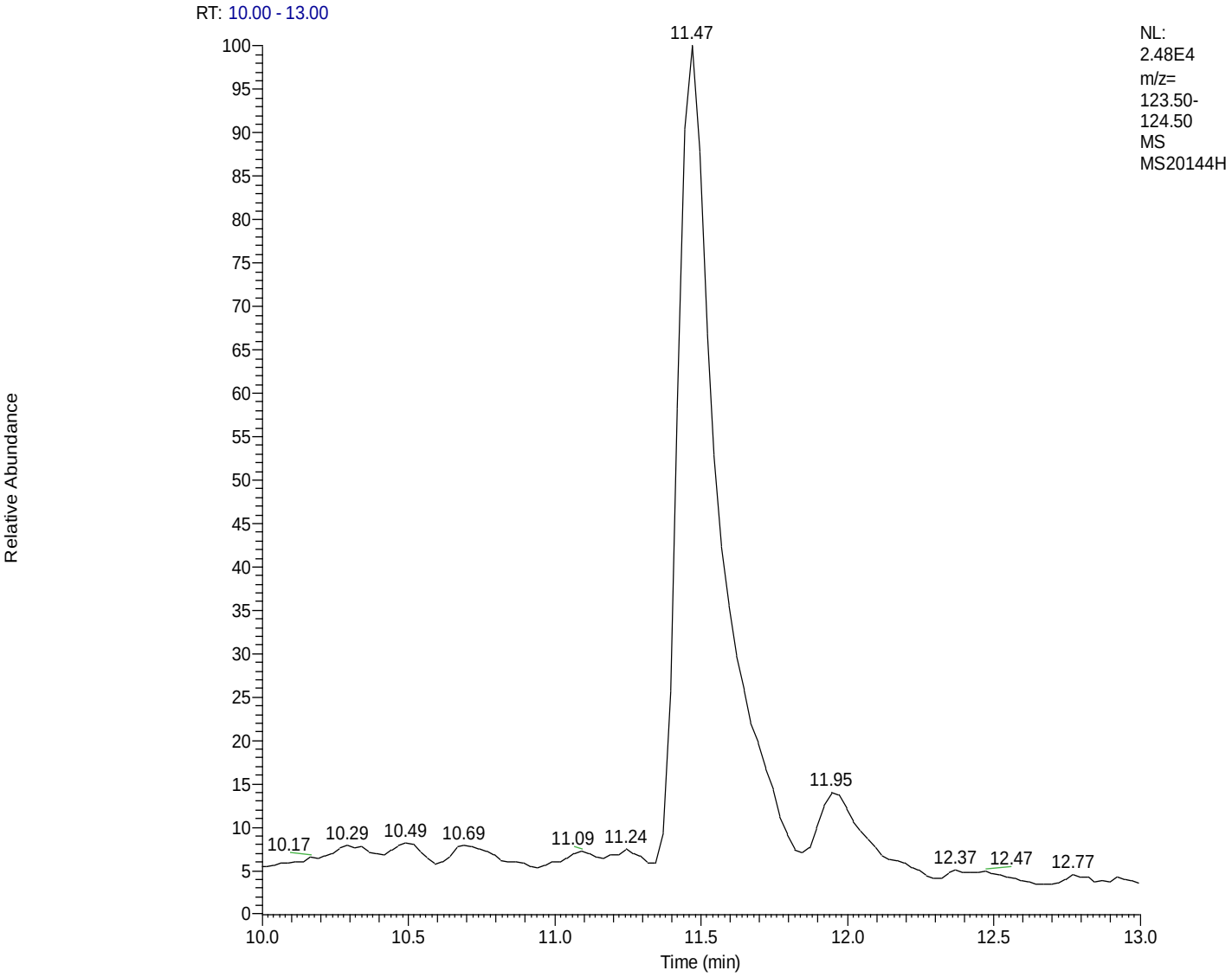

FIGURE 8. IBMP-standard, Retention Time.

Chromatograph for IBMP-standard showing elution from GC column at 11.47.

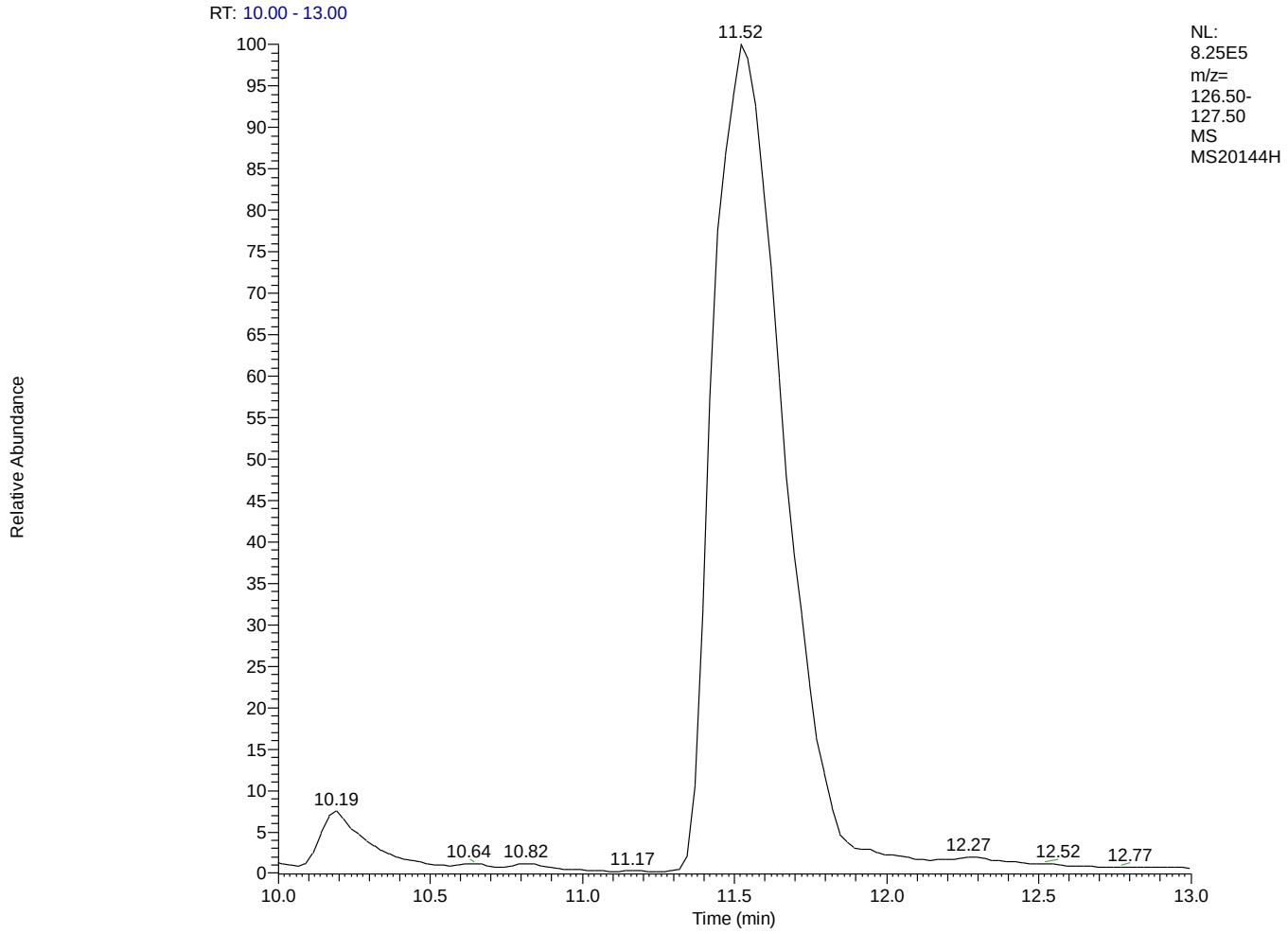

FIGURE 9. IBMP-isotope, Retention Time.

Chromatograph for IBMP-isotope showing elution from GC column at 11.52.

Elution time for IPMP using the same column was in the range of 7-9 min (Figure 10) however IBMP-standard most commonly would elute between 8:15-8:45 where as IPMP-isotope was found to most commonly elute at 8:10-8:30 (Figure 11). All trials were given an allowable error of (± 15%) in accordance with EPA guideline 121 for gas chromatography-mass spectrometry.

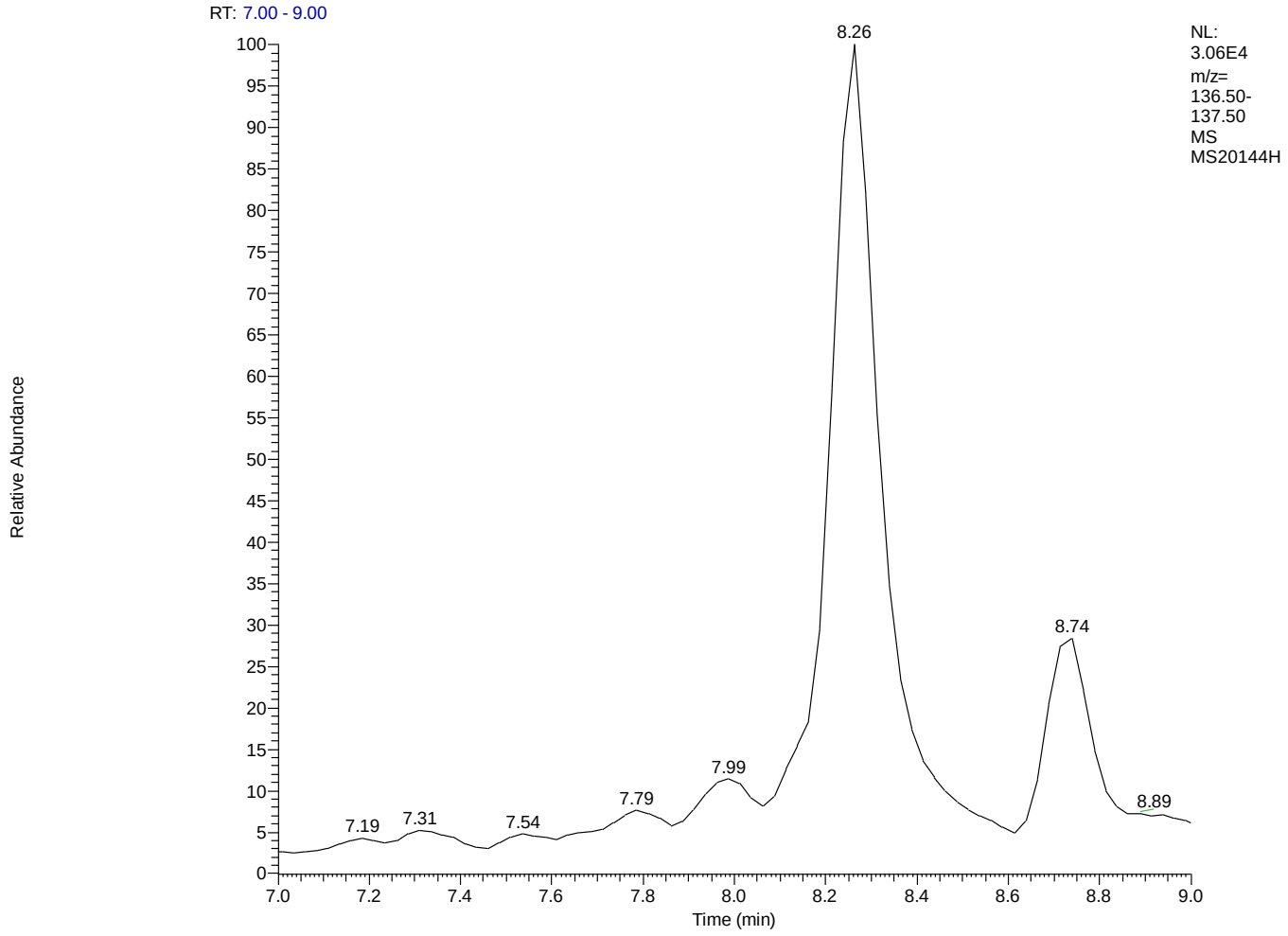

FIGURE 10. IPMP-standard, Retention Time.

Chromatograph for IPMP-standard showing elution from GC column at 8.26.

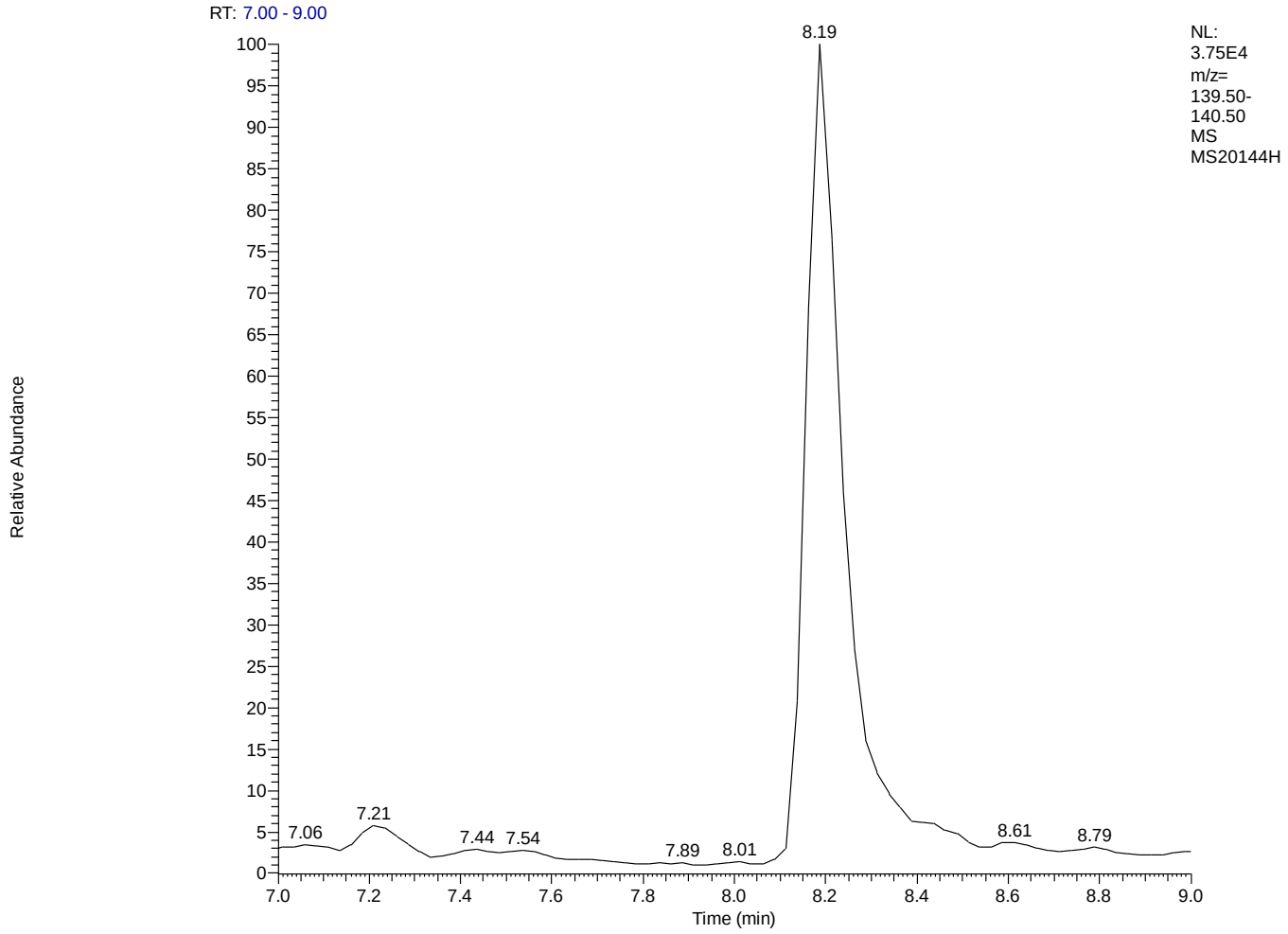

FIGURE 11. IPMP-isotope, Retention Time.

Gas Chromatogram for IPMP-isotope ion showing elution from column at 8.19.

Linearity: A standard curve for both IBMP and IPMP (Figures 12, 13) made with model wine (12% v/v) ethanol and 4 gr./L of tartaric acid, adjusted to pH 6.6 with NaOH was used prior to beginning every trial (all producing $R^2 \geq 0.95$). These preliminary studies confirmed the analytical procedure for both MPs and their isotopes with standard curves as previously described, Y.S. Kotseridis (25).

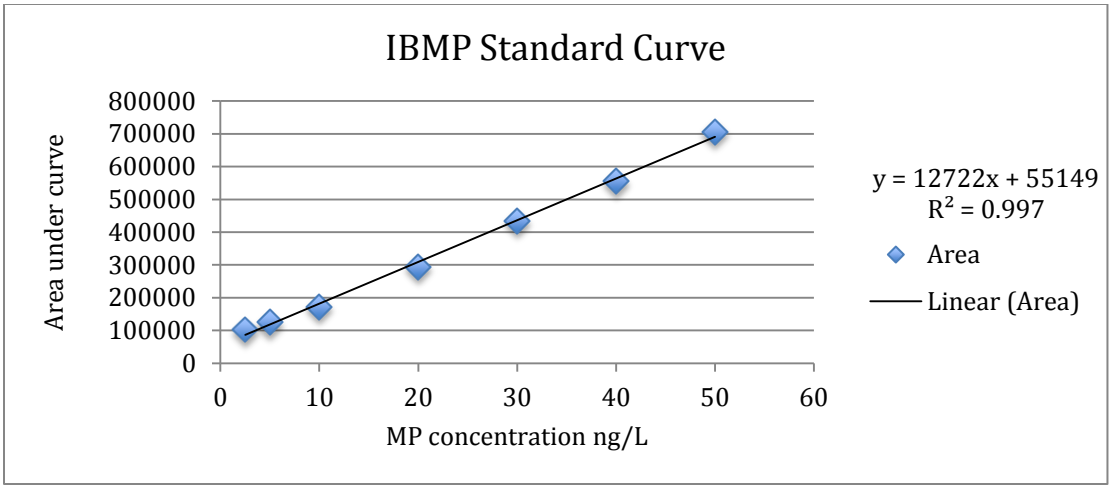

FIGURE 12. IBMP Standard Curve.

A standard curve for IBMP made with model wine (12% v/v) ethanol and 4 gr/L of tartaric acid, adjusted to pH 6.6 with NaOH was used prior to beginning every trial (all producing $R^2 \geq 0.95$).

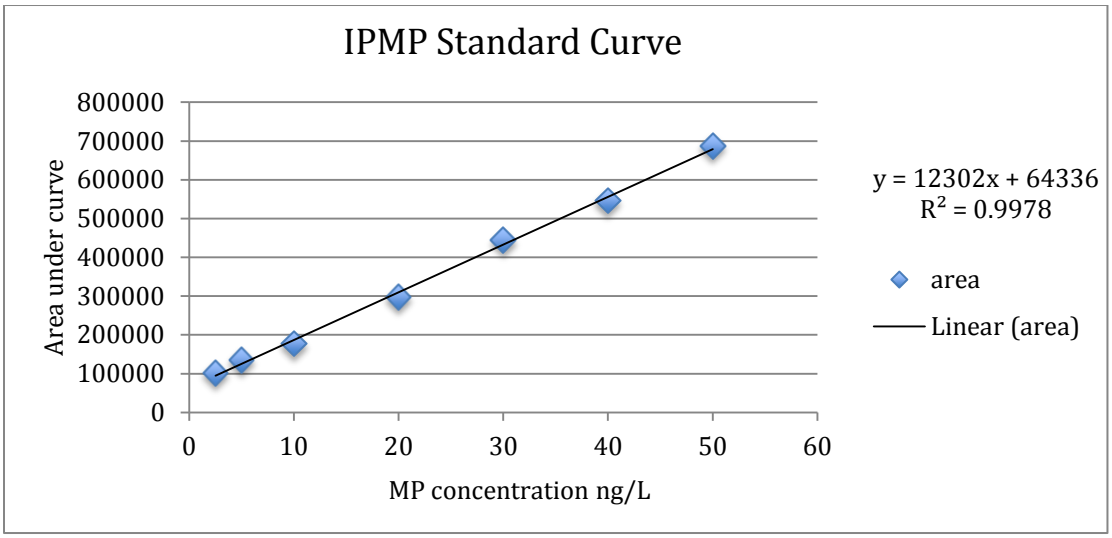

FIGURE 13. IPMP Standard Curve.

A standard curve for IPMP made with model wine (12% v/v) ethanol and 4 gr/L of tartaric acid, adjusted to pH 6.6 with NaOH was used prior to beginning every trial (all producing $R^2 \geq 0.95$).

The files for all described trials were numerically labeled and recorded as such. Analysis trials will be referenced henceforth on in accordance with numerical file names. File numbers and corresponding figures are listed in the table of contents. The first trial was run using PETE and AL as treatments. The SPME fiber used was new; model wine was spiked with both standards, and corresponding isotopes then treatments were applied. Twelve mL of model wine was added to test tubes then spiked with 60µL of IBMP & IPMP standards providing a final concentration of 50µL then 48µL of both IBMP & IPMP isotopes were spike in providing a final concentration of 40 µL. The MP containing model wine was then treated with the prospective binding material.

Trial 142

Trial 142 was performed using two materials AL and PETE. AL was chosen for experimentation based on long established effective use as an adsorbent binder of varieties of compounds, including highly aromatic compounds (28). PETE was chosen as well, based on reports of deceases in MP concentrations in wines stored in PETE lined containers for extended lengths of time 3, 6, 12, 18 months A. Blake (25). For trial 142 the materials were added (2g. AL, 2g. PETE) and allowed to incubate for 20 days (Table 7). After 15 days, 10 mL were pulled off the top simulating the cellar practice known as “pumping over” or “racking.” The wine was then added to 20 mL glass GC-vials with 3g. NaCl and sealed with septum caps. IPMP was shown to reduce 66% with the PETE treated model wine and 90% with the AL treated wine (Figure 14).

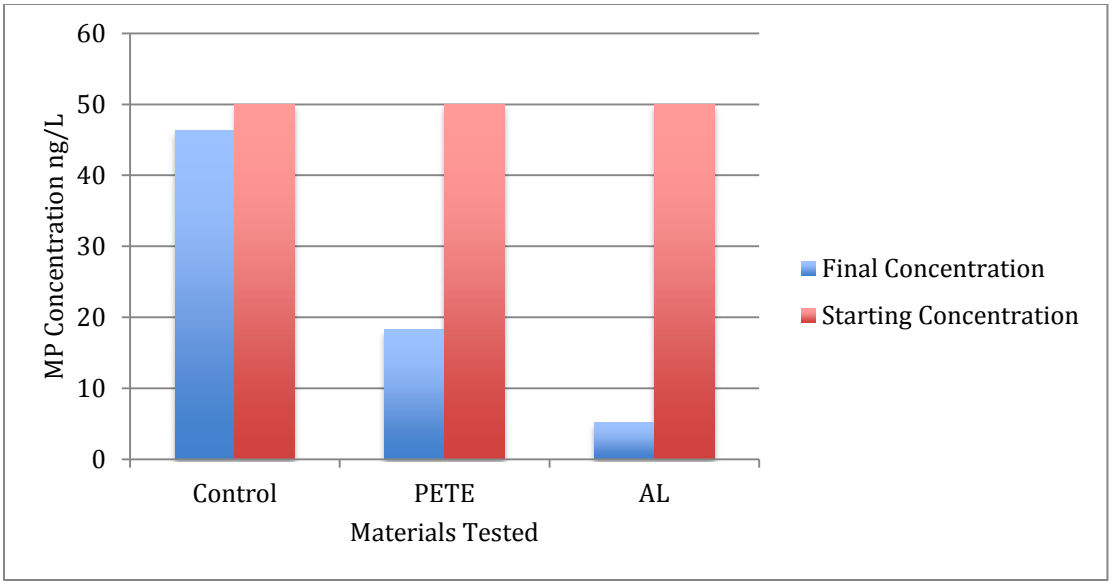

FIGURE 14. Trial 142 IBMP Results.

Preliminary trial for IBMP binding capacity of PETE and AL. Materials were added at 2g/10mL and 5g/10mL for AL & PETE in MP spiked model wine. Materials were soaked for 16 days.

TABLE 7 Trial 142 IBMP Results

Trial 142 IBMP Results		
Material	**Final Concentration**	**Original Concentration**
Control	46.345	50
PETE	18.28064	50
AL	5.111647	50

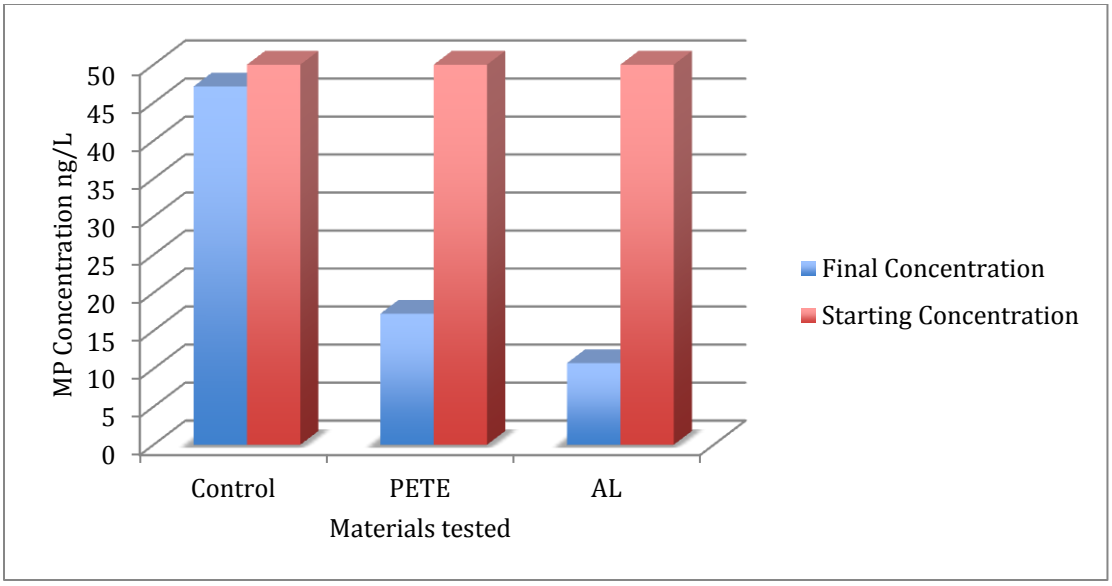

FIGURE 15. Trial 142 IPMP Results.

Preliminary trial for IPMP binding capacity of PETE and AL. Materials were added at 2g/10mL & 5g/10mL for AL & PETE in MP spiked model wine. Materials were soaked for 16 days.

TABLE 8 Trial 142 IPMP Results

Trial 142 IPMP Results		
Material	**Final Concentration**	**Original Concentration**
Control	47.131	50
PETE	17.239	50
AL	10.74	50

The data received from Trial 142 showed changes in MP concentrations in model wine when allowed to soak for extended periods (Table 8). The evidence provided shows the potential of using binging materials for the reduction of MP in wines is plausible. Both

PETE and AL significantly decreased the concentration of IBMP and IPMP by 60 % and 80 %, respectively (Figures14, 15).

Trial 144

The following triall, 144, was performed using the same method; however concentrations of 40μL for both isotope and standard were used. The changes made to isotope concentrations (range 0-40 ng/L) were unintentional and further trials reverted to the range of 0-50 ng/L. The trial utilized four materials in effort to reconfirm MP binding with materials over extended periods of time. AL, Oak, PETE, DE, were used and mixed in various combinations. After witnessing 90% reduction in MP concentrations using AL attention was focused on combining AL with DE simulating an Alumina-bound adsorbent clay. DE in its natural state is found to be 81-91% silica with a significant portion AL and ferric oxide. DE from a natural state is heat treated followed by an acid activation to form bentonite and montmorillomite according to W.T. Tsai (29). DE is commonly used in food processing primarily as a filtering agent and is GRAS certified. Reductions in MP (IBMP & IPMP) concentrations were observed after 15-20 days of treatment (Figures 16, 17).

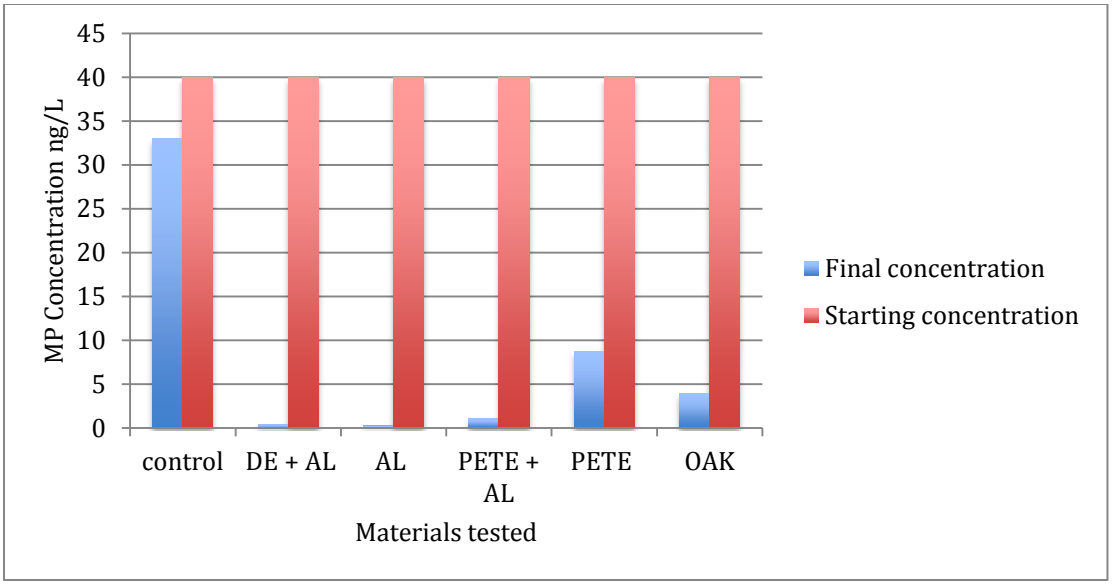

FIGURE 16. Trial 144 IBMP Results.

Trial for IBMP binding capacity of DE+AL, AL, PETE+AL, PETE and Oak powder. Materials were added at 2g/12mL in MP spiked model wine. Materials were soaked for 15-20 days.

TABLE 9 Trial 144 IBMP Results

Trial 144 IBMP Results		
Material	**Original Concentration**	**Final Concentration**
Control	33.023	40
1g. DE+ 1.5g. AL	0.406329	40
AL 2.0g	0.24096	40
PETE 2.0g+AL 1.5g	1.09515	40
PETE 2.0g	8.761595	40
OAK 2.0 g	3.92851	40

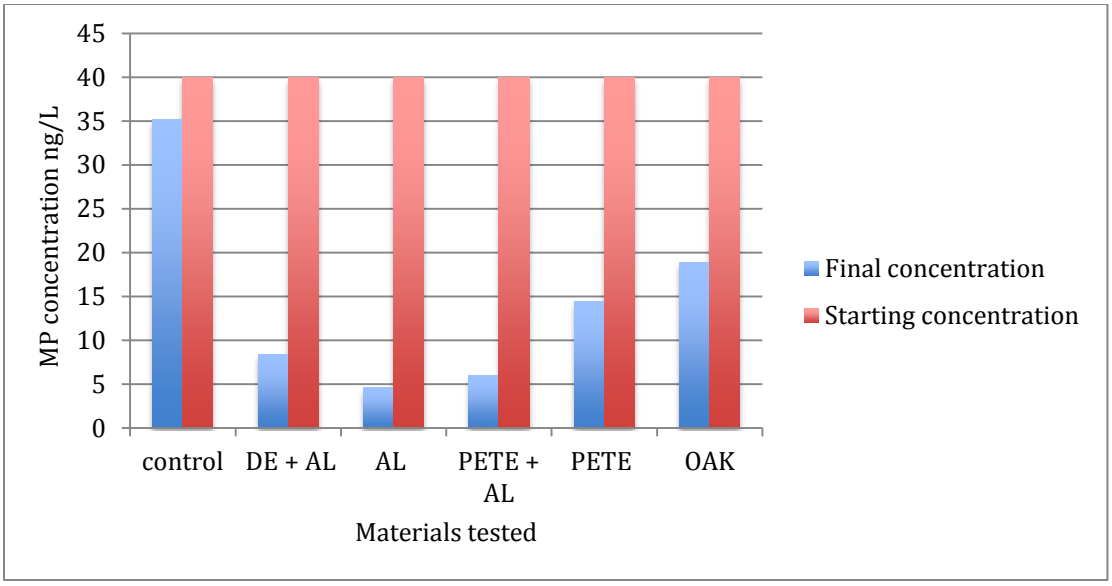

FIGURE 17. Trial 144 IPMP Results.

Trial for IPMP binding capacity of DE+AL, AL, PETE+AL, PETE and Oak powder. Materials were added at 2g/12mL in MP spiked model wine. Materials were soaked for 15-20 days.

TABLE 10 Trial 144 IPMP Results

Trial 144 IPMP Results		
Material	**Final Concentration**	**Original Concentration**
Control	35.159	40
1g. DE+ 1.5g. AL	8.350677	40
AL 2.0g	4.585	40
PETE 2.0g+AL 1.5g	5.95713	40
PETE 2.0g	14.38361	40
OAK 1.0 g	18.84032	40

Reductions were shown to occur with all tested materials after a 15-20 day treatment.

MP concentrations were decreased with all of the applied materials. DE + AL decreased

IBMP by 98.98% and IPMP by 79.12%, respectively, while AL alone induced a higher decrease at 88.54%, PETE + AL decreased 85.11%, PETE alone decreased 64.04%, and oak decreased 52.89% (Tables 9, 10).

Trial 167

Preliminary trial 167 was conducted with three intentions; a) test new materials currently in use for MP reduction, b) establish new elution times for a new, longer, column on the GC, and c) determining the number of samples that can be run on one SPME fiber before its absorbance decreases. The trial implemented the same methodologies, standard curve, trial concentrations and media however the time MPs in model wine were treated changed to 7 days. Materials tested were isinglass, bocksin, and carageenan. Since retention times were presumed to change with the new column, vials of IBMP, IPMP, IBMP-isotope, & IPMP-isotope were used in ultra-high concentrations to locate new retention times. Using ultra-high concentrations of standard and isotope ran with the new longer column showed elution times were delayed. The ultra-high concentrations of the four compounds allowed for easy location of compound elution (Figure 18, 19).

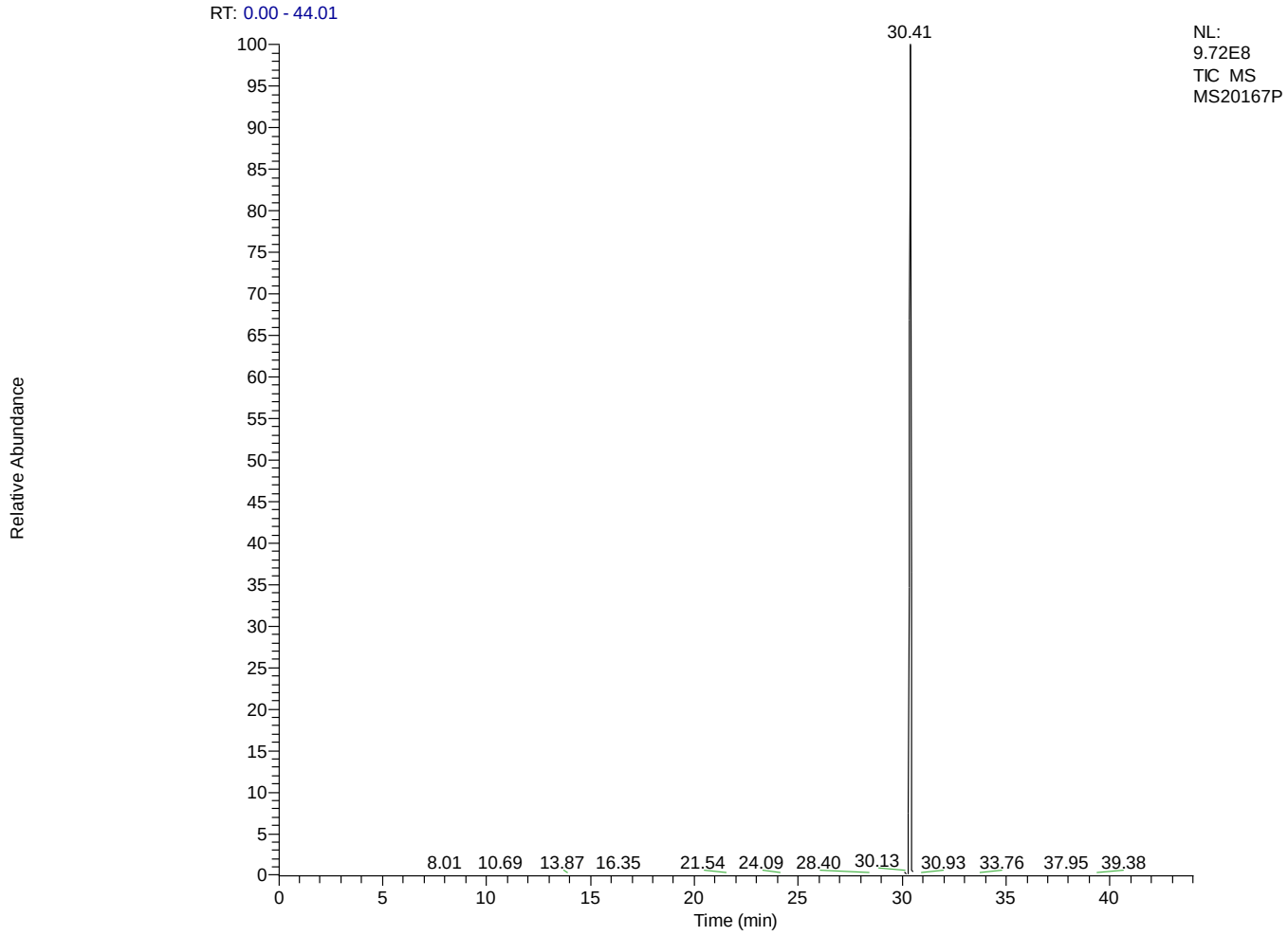

FIGURE 18. IBMP-standard. 200,000 ng/L.

Gas Chromatogram for IBMP-standard ion showing elution from column at 30.41.

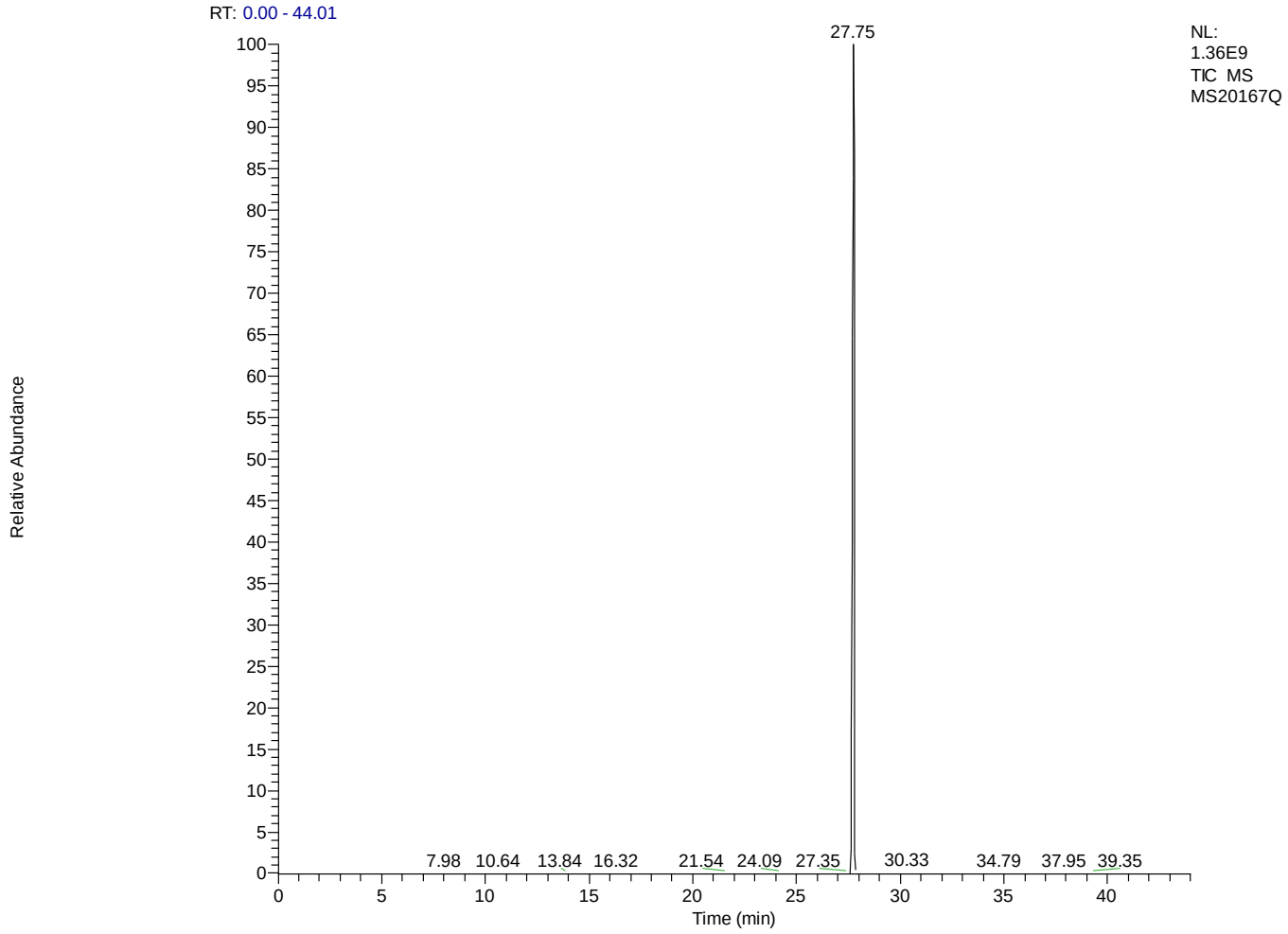

FIGURE 19. IPMP-standard. 200,000 ng/L.

Gas Chromatogram for IPMP-standard ion showing elution from column at 27.75.

Elution time for IBMP using the aforementioned column provided peaks in the range of 30-32 min (Figure 18) however IBMP-standard most commonly would elute between 30:30-31:00 as did IBMP-isotope (Figure 20).

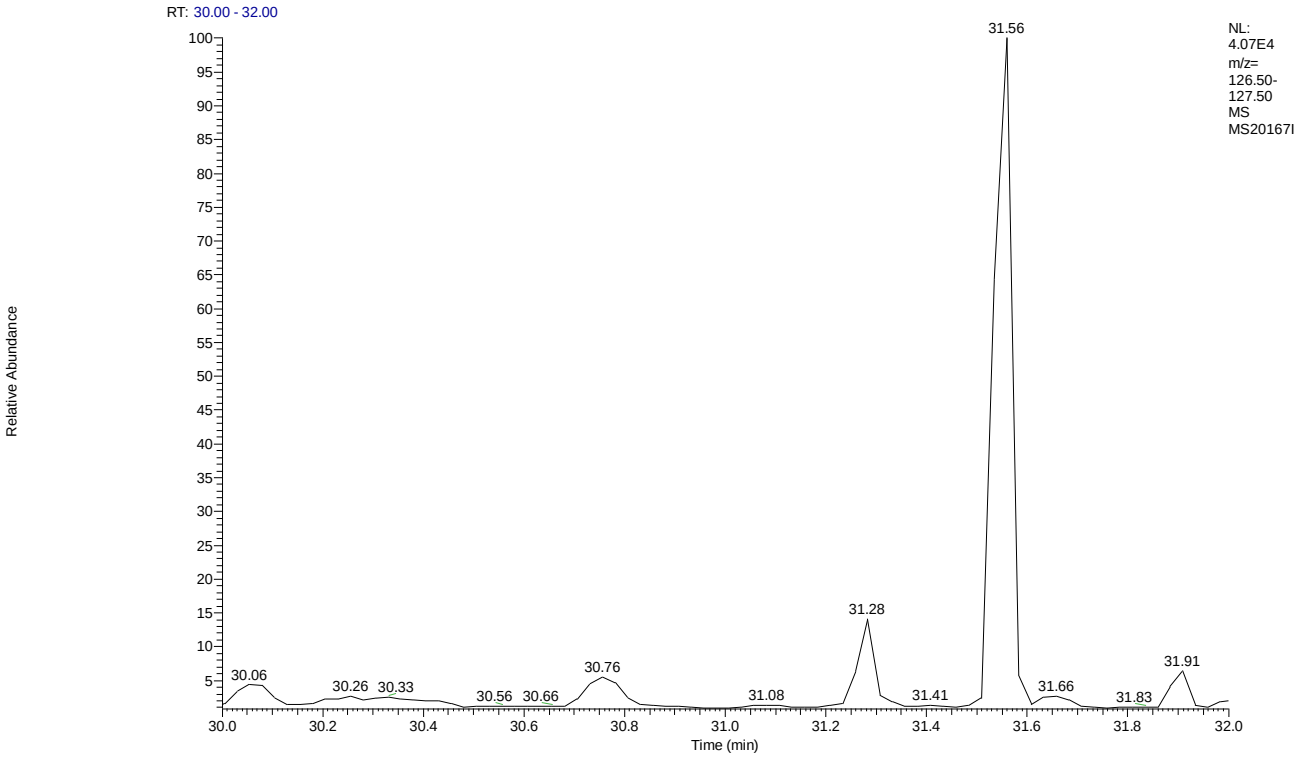

FIGURE 20. IBMP-isotope. 200,000 ng/L.

Gas Chromatogram for IBMP-isotope ion showing elution from column at 31.56.

Elution time for IPMP using the same column was in the range of 27-29 min (Figure 17). IPMP-isotope was found to most commonly elute between 27:30-28:00 for these trials (Figure 21).

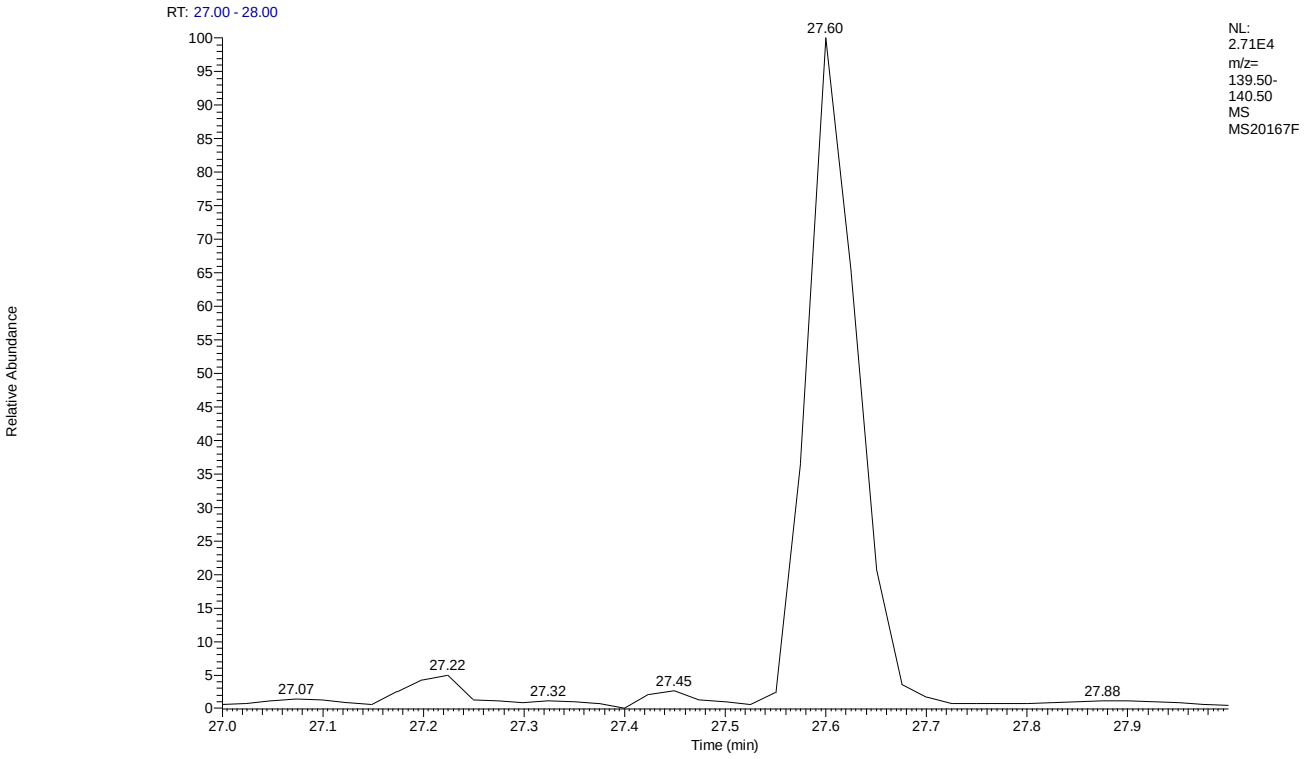

FIGURE 21. IPMP-isotope. 200,000 ng/L.

Gas Chromatogram for IPMP-isotope ion showing elution from column at 27.60.

The elution time identification trials were run qualitatively; however in the same trial sequence quantification trials were run testing material absorbency to MPs. Results for absorbency of MPs to bocksin, isinglass, and carageenan are depicted (Figures 22, 23). All trials were given an allowable error of (± 15%) in accordance with EPA METHOD 8260B. VOLATILE ORGANIC COMPOUNDS by GAS CHROMOTOGRAPHY / MASS SPECTOMETRY (GC/MS).

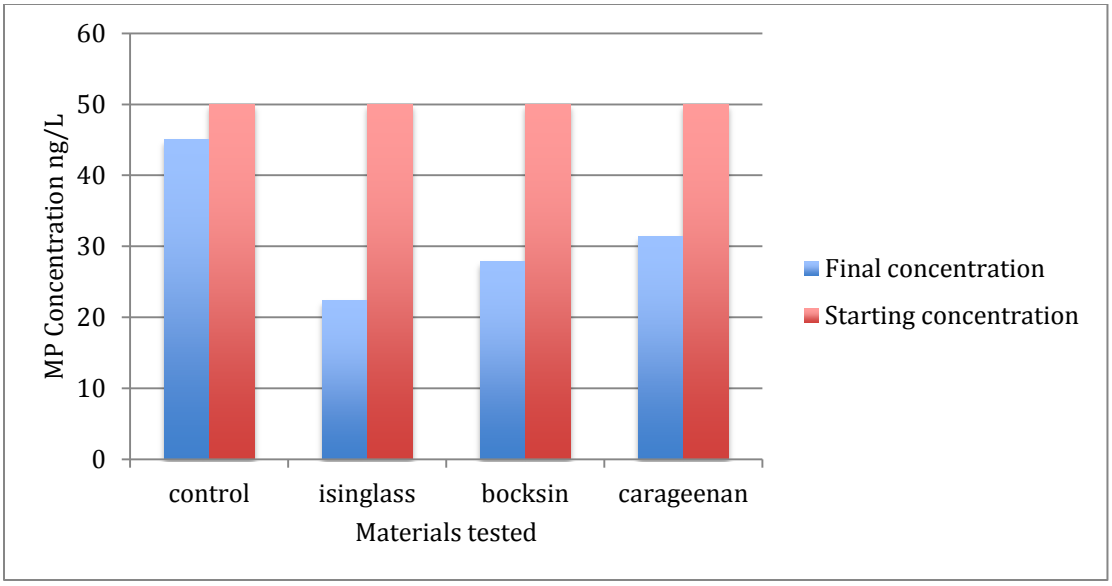

FIGURE 22. Trial 167 IBMP Concentration (7 day soak).

Trial for IBMP binding capacity of Isinglass, AL, Bocksin, and Carageenan. Materials were added at 2g/12mL in MP spiked model wine. Materials were soaked for 7 days.

TABLE 11 Trial 167 IBMP Concentration

Trial 167 IBMP Concentration		
Material	**Final Concentration**	**Original Concentration**
Control	45.032	50
Isinglass	22.383	50
Bocksin	27.832	50
Carageenan	31.348	50

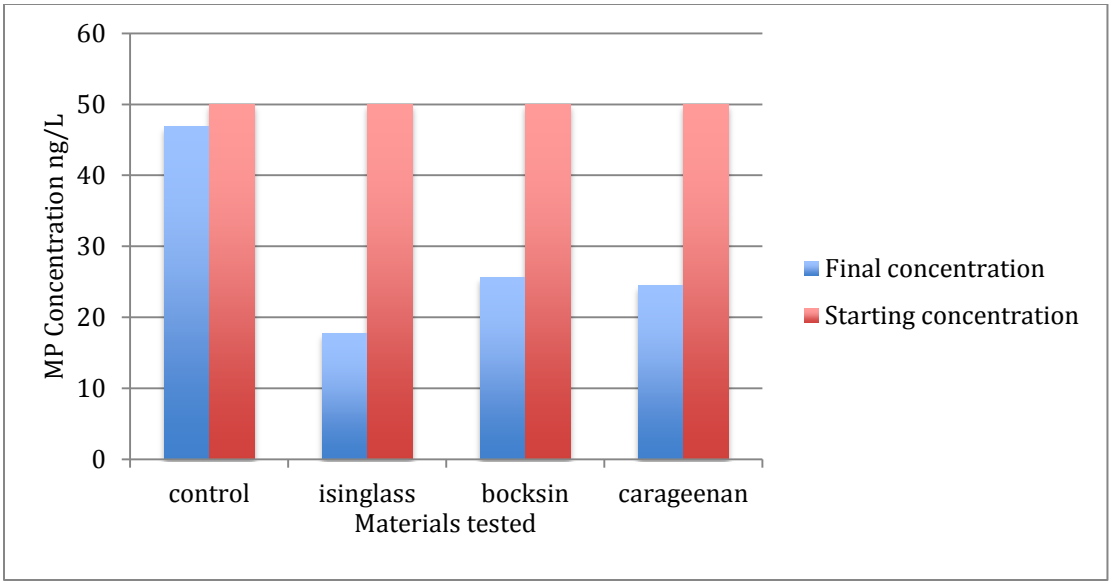

FIGURE 23. Trial 167 IPMP Concentration (7 day soak).

Trial for IPMP binding capacity of Isinglass, AL, Bocksin, and Carageenan. Materials were added at 2g/12mL in MP spiked model wine. Materials were soaked for 7 days.

TABLE 12 Trial 167 IPMP Concentration

Trial 167 IPMP Concentration		
Material	**Final Concentration**	**Original Concentration**
Control	46.861	50
Isinglass	17.679	50
Bocksin	25.659	50
Carageenan	24.4917	50

Results from preliminary trial 167 demonstrate that isinglass caused the highest decrease in IBMP and IPMP by 56 % and 66%, respectively, while carageenan and

bocksin showed a lesser decrease of 50%, respectively, for both MP compounds (Figures 22, 23), (Tables 11, 12).

Results and Conclusions

Preliminary trials observing effects of adding potential binding materials to MP laden model wine provided results indicating possibility of reduction in MP concentrations through adsorbent binding treatments. The trials were not conducted in triplicate however accurate quantification was performed for each sample. All trial results were well within the allotted ± 15% error for GC-MS quantification of volatile aromatic compounds. The reduction in MP concentrations encouraged further trials into MP binding materials.

Overall, these preliminary studies demonstrate that AL, PETE + AL, AL + DE, and Oak appeared to be the most efficient MP-reductants for both IBMP and IPMP.

CHAPTER V

METHOXYPYRAZINES POTENTIAL BINDERS: A SCREENING

The reduction of MPs in wine through binding materials has been investigated previously; however, extensive screening has not been described in current literature. Initial trials providing preliminary data supporting the hypothesis of using additive materials to bind MPs in order to reduce concentrations prompted a screening of materials. A screening of 14 potential binding agents (Table 13) was performed using the same method as the preliminary trials where a new GC-column of the same characteristics was utilized. The longer column delayed elution time of MPs from the previously shown ranges to later ranges. All trials were conducted in triplicate with addition 60µL of IBMP and IPMP standards providing a final concentration of 50µL/12mL wine system & 48µL of both IBMP and IPMP isotopes providing a concentration of 40 µL/12mL wine system.

TABLE 13 Materials Used in MP-Binding Trials and Their Common Uses

Materials Used in MP-Binding Trials and Their Common Uses	
Material	**Common Uses**
1. Alumina Oxide	Used in Ceramics, Porcelain, Glass, Plastic, Heat Resistant Fibers, paper, Petrochemicals, Chromatographic Analysis, Abrasives, Adsorbent
2. Alumina Oxide + Diatomaceous Earth (DE)	(DE) Insecticides, Anti-caking Agent, Dynamite, Fire Resistant Barriers, Adsorbent, Hydroponic Growth Medium
3. Copper Sulfate (Aqueous Solution)	Fining Agent, Removes/Reduces Hydrogen Sulfide
4. Activated Carbon	White Wine Fining Agent
5. Drifine (Isinglass)	White Wine Fining Agent
6. Bocksin (Aqueous Silica)	Reduces/Removes Sulfur Odors
7. Bentonite	Wine Clarifier, Fining Agent
8. Toasted Oak	Wine Barrels
9. Untoasted Oak	Wine Barrels
10. Amberlite XAD-4	Polymeric Adsorbent, Commonly Used for Phytochemicals
11.FXP H0320 (Soy Protein)	Food Additive
12.Fibersol-2 (Resistant Maltodextrin)	Food Additive. Fortification of Dietary Fibers
13. Aluminum Foil	Potential Ion Disruption
14. Polyvinylopolypyrrolidone (PVPP)	Wine Clarifier, Fining Agent, Reduces Bitterness Improves Hue in Reds and Rose' Wines

Trial 176

Materials were selected based upon two criteria; a) use in current cellar practices, b) known binding capacity with follow up on preliminary research. Materials were incubated with model wine for 5 days after which quantitative analysis was performed, data extrapolated and put into charts. Bases on reductions in MP concentrations seen in preliminary trials, material screening was performed to provide greater insight into material binding potential whereby further research will be

conducted. Provided reductions in MP concentrations continue to occur for the screening trials as indicated by preliminary trials, further research will be conducted into the specific behaviors of these materials in true wine systems. Three individual trials were run testing material binding capacity and results were averaged using the mean (Figure 24, Table 14).

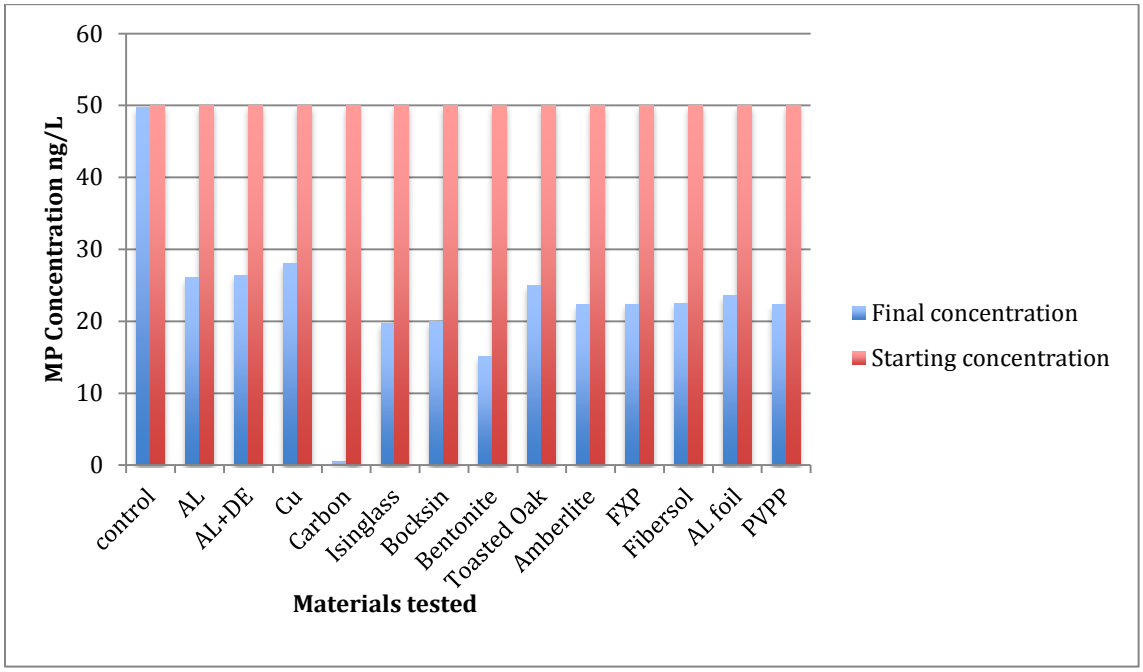

FIGURE 24. Trial 176 IBMP Screening.

Trial for IBMP binding capacity ofAL, AL+DE, CU, Carbon, Isinglass, Bocksin, Bentonite, Toasted Oak, Amberlite, FXP, Fibersol, Aluminum Foil, and PVPP. Materials were added at 1g/12mL in MP treated model wine, at 50 ng/L stadards and 40 ng/L isotope. Materials were soaked for 5 days.

TABLE 14 Trial 176 IBMP Screening

Trial 176 IBMP Screening		
Material	**Final Concentration**	**Original Concentration**
Control	49.71825	50
AL	26.06259	50
AL+DE	26.40779	50
Cu	28.00231	50
Carbon	0.439072	50
Isinglass	19.65077	50
Bocksin	19.90201	50
Bentonite	15.11649	50
TO	24.9943	50
Amberlite	22.3544	50
FXP	22.32076	50
Fibersol	22.46403	50
AL foil	23.54443	50
PVPP	22.33013	50

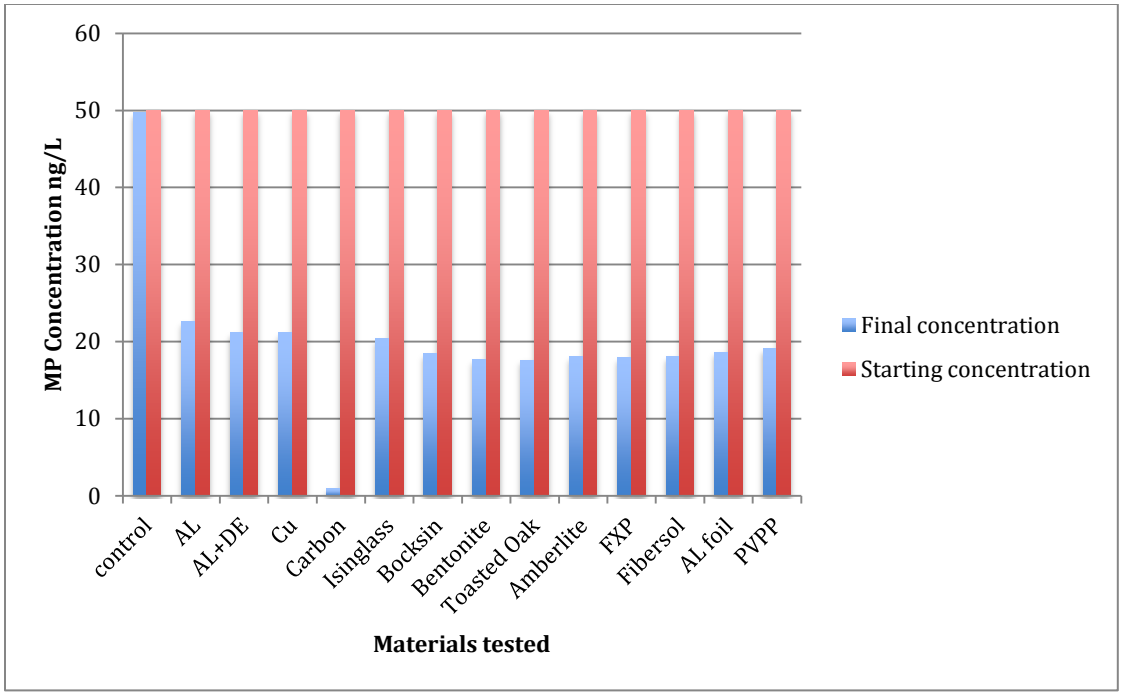

FIGURE 25. Trial 176 IPMP Screening.

Trial for IPMP binding capacity ofAL, AL+DE, CU, Carbon, Isinglass, Bocksin, Bentonite, Toasted Oak, Amberlite, FXP, Fibersol, Aluminum Foil, and PVPP. Materials were added at 1g/12mL in MP treated model wine, at 50 ng/L stadards and 40 ng/L isotope. Materials were soaked for 5 days.

TABLE 15 Screening Trial 176 IPMP

Screening Trial 176 IPMP		
Material	**Final Concentration**	**Original Concentration**
Control	49.71825	50
AL	22.59928	50
AL+DE	21.21295	50
Cu	21.22006	50
Carbon	0.96843	50
Isinglass	20.40332	50
Bocksin	18.42424	50
Bentonite	17.681	50
Toasted Oak	17.57918	50
Amberlite	18.10328	50
FXP	17.96199	50
Fibersol	18.03126	50
AL foil	18.62527	50
PVPP	19.05228	50

Results from trial 176 demonstrate that carbon almost reduced both MPs completely. Most of the other treatments reduced both MPs by around 50% within the 5 day incubation time (Figure 26, Table 15).

CHAPTER VI

METHOXYPYRAZINE TIME TRIALS

Following the material screening, time trials were conducted in effort to better understand adsorption rates and methods by which attenuation of MP concentrations may be achieved. Five materials from the previous screening, showing greatest MP binding, were selected, Amberlite, PVPP, Bocksin, Bentonite, and toasted oak. If varying materials are found to reduce MP concentrations in short time durations then sequential, multiple treatments, will be conducted. The goal is to reduce MP concentrations to below sensory thresholds. Quantitative analysis of the adsorbent materials and time trials will provide more insight into reducing MP concentrations in wines.

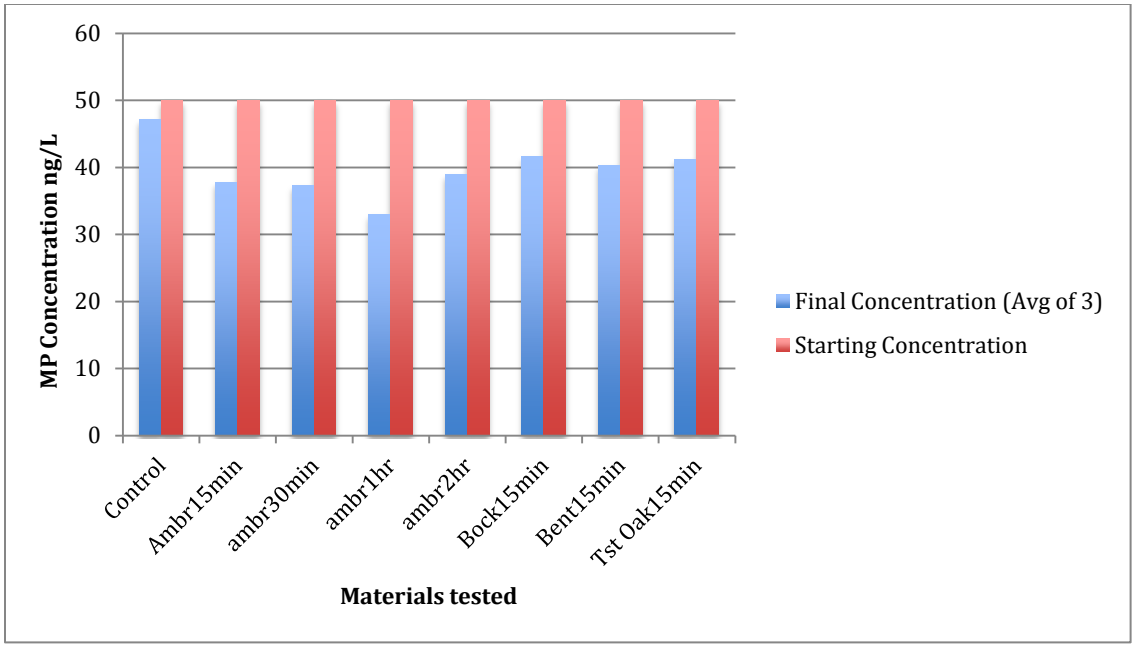

FIGURE 26. Time Trial 181 IBMP.

Time Trial for IBMP binding capacity of Amberlite at 15, 30, 60 & 120 minute soaks. Bocksin, Bentonite and toasted Oak were treated at 15 minute soaks. Materials were added at 1g/12mL in MP treated model wine, at 50 ng/L stadards and 40 ng/L isotope.

TABLE 16 Time Trial 181 IBMP

Time Trial 181 IBMP			
Trial	**Final Concentration (Average of Three)**	**Original Concentration**	**Control Concentration**
Control	47.16	50	47.16
Ambr15min	37.81213	50	47.16
Ambr30min	37.31744	50	47.16
Ambr1hr	32.91254	50	47.16
Ambr2hr	38.97858	50	47.16
Bock15min	41.57794	50	47.16
Bent15min	40.23474	50	47.16
TO 15min	41.25285	50	47.16

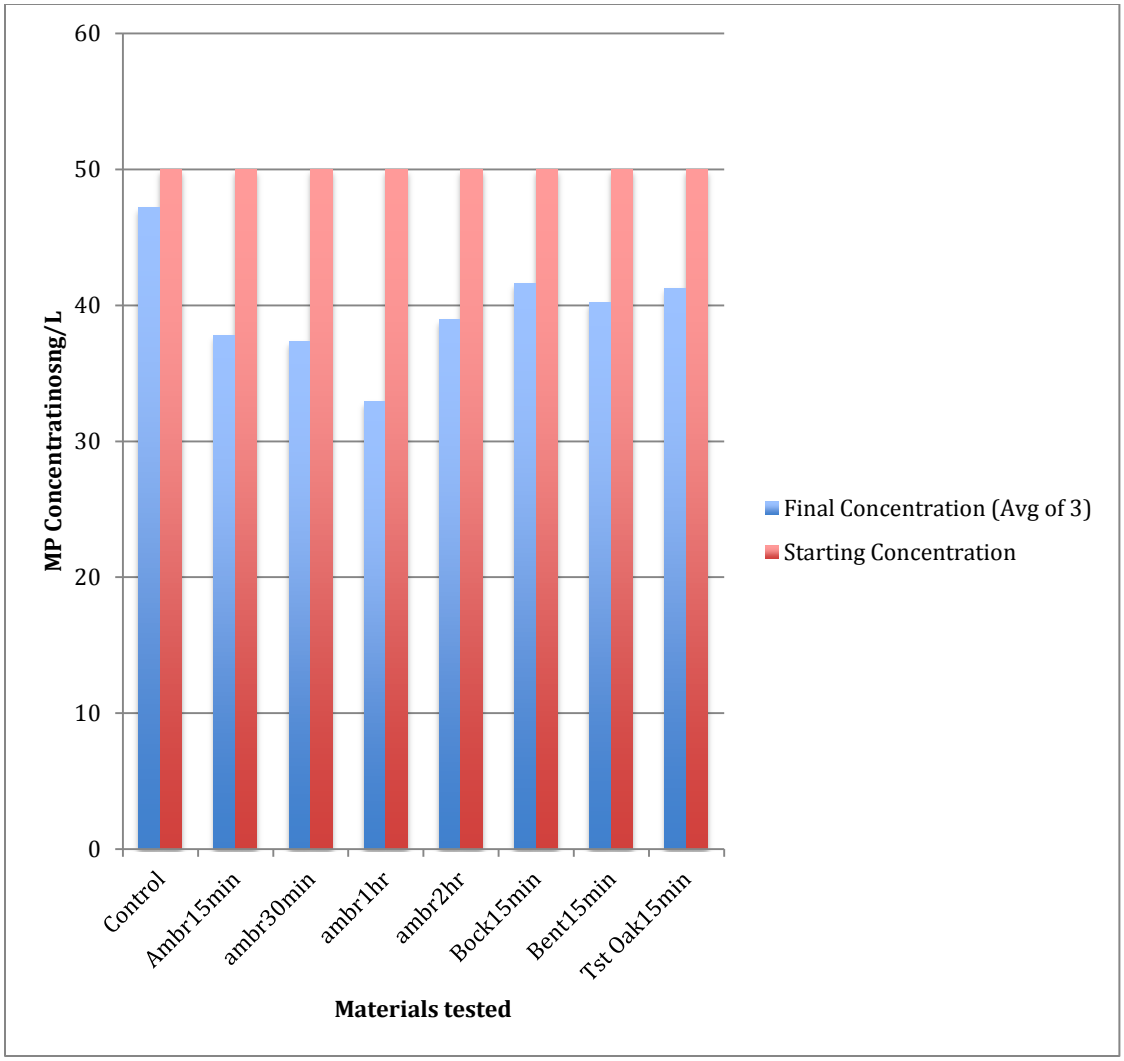

FIGURE 27. Time Trial 181 IPMP.

Time Trial for IPMP binding capacity of Amberlite at 15, 30, 60 & 120 minute soaks. Bocksin, Bentonite and toasted Oak were treated at 15 minute soaks. Materials were added at 1g/12mL in MP treated model wine, at 50 ng/L stadards and 40 ng/L isotope.

TABLE 17 Time Trial 181 IPMP

Time Trial 181 IBMP			
Trial	**Final Concentration (Average of Three)**	**Original Concentration**	**Control Concentration**
Control	48.08878	50	48.08878
Ambr15min	27.28927	50	48.08878
Ambr30min	25.82685	50	48.08878
Ambr1hr	25.72874	50	48.08878
Ambr2hr	26.33546	50	48.08878
Bock15min	30.12604	50	48.08878
Bent15min	30.89177	50	48.08878
TO 15min	31.14998	50	48.08878

Trial 181

Results from time trial 181 demonstrate that within a short term range of 15 min -2h, the reduction of MPs did not change significantly for any of the treatments (Table 16, Figure 26). Amberlight reduced IBMP by up to 17 % after 2h and IPMP by up to 46 % after 1h (Table 17). Bock, Bent and toasted oak reducted MPs to a lesser extent after 15 min to up to 18 % and 38 % for IBMP caused by bocksin and IPMP by bentonite respectively (Figure 27).

Trial 182

In time trial 182, additional treatments (PVPP) were tested within a short-term range of 15 min – 2h and compared to the treatments already tested in trial 181, bocksin, bentonite and toasted oak (Figure 28, Table 18).

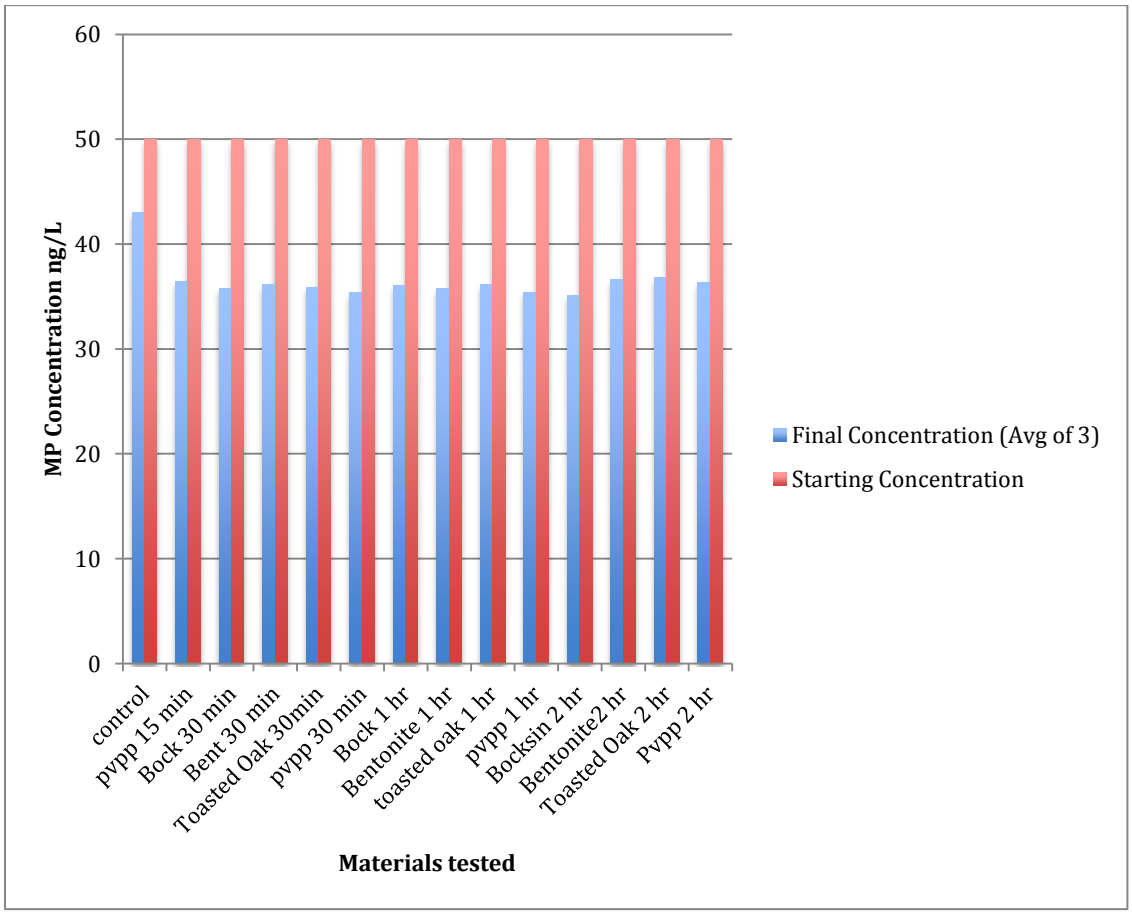

FIGURE 28. Time Trial 182 IBMP.

Time Trial for IBMP binding capacity of PVPP, Bocksin, Bentonite, and Toasted Oak at 15, 30, 60, & 120 minutes. Materials were added at 1g/12mL in MP treated model wine, at 50 ng/L stadards and 40 ng/L isotope.

TABLE 18 Time Trial 182 IBMP

Time Trial 182 IBMP			
Trial	**Final Concentration (Average of Three)**	**Original Concentration**	**Control Concentration**
Control	42.9909	50	42.9909
PVPP 15 min	36.427	50	42.9909
Bocksin 30 min	35.81	50	42.9909
Bent 30 min	36.186	50	42.9909
TO 30min	35.919	50	42.9909
PVPP 30 min	35.374	50	42.9909
Bocksin 1 hr	36.063	50	42.9909
Bentonite 1 hr	35.76	50	42.9909
TO 1 hr	36.145	50	42.9909
PVPP 1 hr	35.444	50	42.9909
Bocksin 2 hr	35.07	50	42.9909
Bentonite2 hr	36.662	50	42.9909
TO 2 hr	36.81	50	42.9909
PVPP 2 hr	36.364	50	42.9909

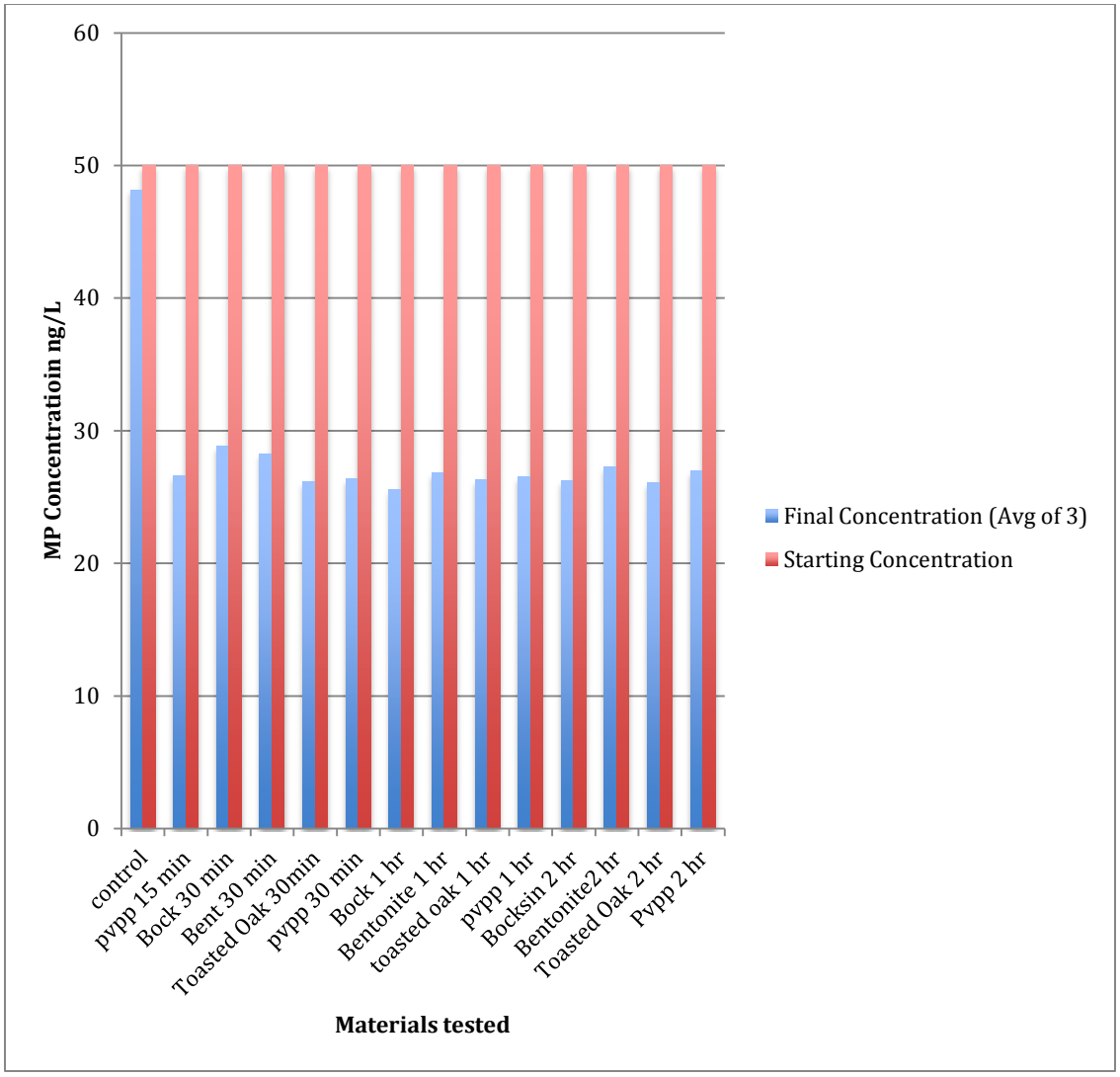

FIGURE 29. Time Trial 182 IPMP.

Time Trial for IBMP binding capacity of PVPP, Bocksin, Bentonite, and Toasted Oak at 15, 30, 60, & 120 minutes. Materials were added at 1g/12mL in MP treated model wine, at 50 ng/L stadards and 40 ng/L isotope.

TABLE 19 Time Trial 182 IPMP

Time Trial 182 IPMP			
Trial	**Final Concentration (Average of Three)**	**Original Concentration**	**Control Concentration**
Control	48.16814	50	48.16814
PVPP 15 min	26.615	50	48.16814
Bock 30 min	28.851	50	48.16814
Bent 30 min	28.278	50	48.16814
TO 30min	26.173	50	48.16814
PVPP 30 min	26.398	50	48.16814
Bock 1 hr	25.547	50	48.16814
Bentonite 1 hr	26.85	50	48.16814
TO 1 hr	26.279	50	48.16814
PVPP 1 hr	26.536	50	48.16814
Bocksin 2 hr	26.231	50	48.16814
Bentonite2 hr	27.262	50	48.16814
TO 2 hr	26.058	50	48.16814
PVPP 2 hr	26.968	50	48.16814

This second short-term time trial confirmed that there was not significant reduction in MPs with increasing incubation time for all treatments. PVPP showed similar MP-reducing capacity to bocksin (Figure 29, Table 19).

Trial 186

Since the short-term incubation of materials with the MP-spiked model wine did not show significant improvement of MP-reduction over time, a longer term trial was performed where materials were incubated for 8-24h (Figure 30, Table 20).

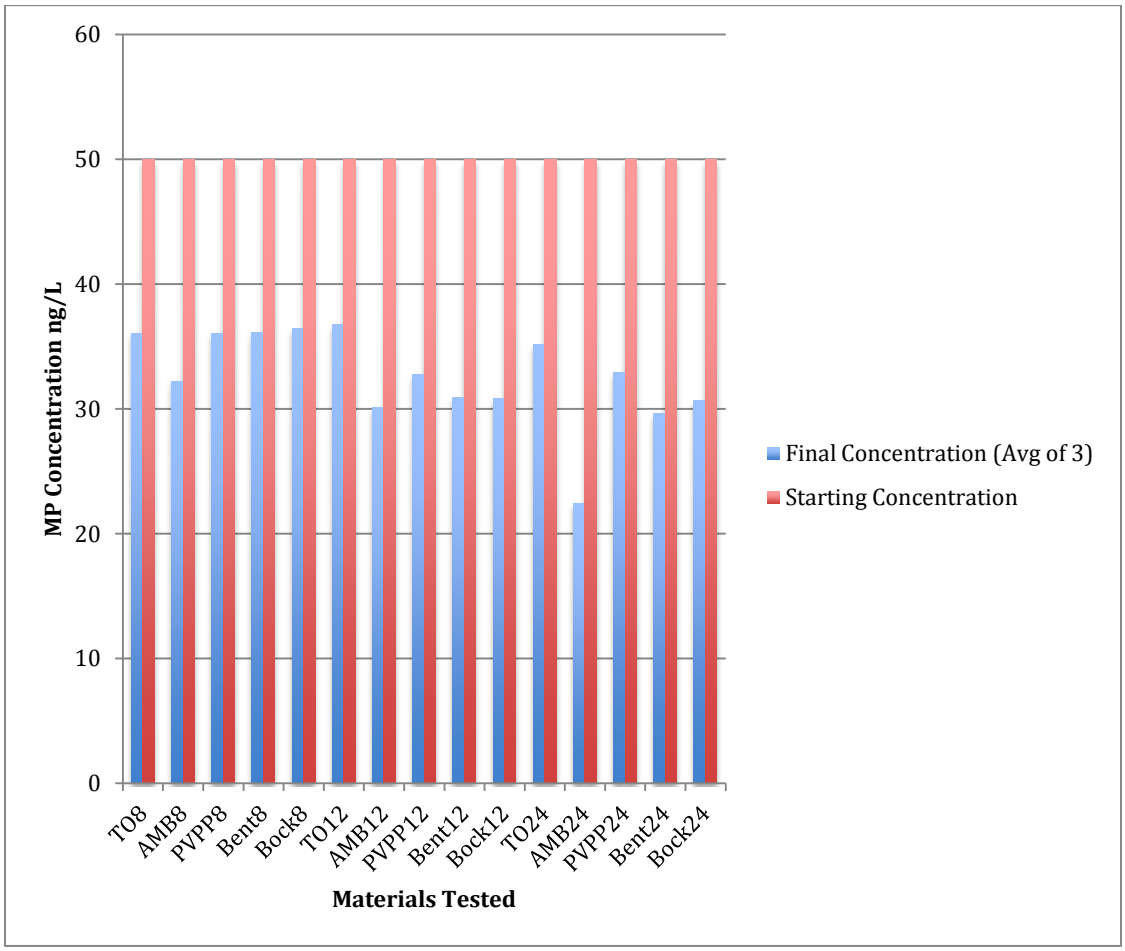

FIGURE 30. Time Trial 186 IBMP.

Time Trial for IBMP binding capacity of PVPP, Bocksin, Bentonite, Ambrelite, and Toasted Oak at 8, 12 & 24 hours. Materials were added at 1g/12mL in MP treated model wine, at 50 ng/L stadards and 40 ng/L isotope.

TABLE 20 Time Trial 186 IBMP

Time Trial 186 IBMP			
Trial	**Final Concentration (Average of Three)**	**Original Concentration**	**Control Concentration**
TO8	36.0734	50	44.34468
AMB8	32.2288	50	44.34468
PVPP8	36.03209	50	44.34468
Bent8	36.1252	50	44.34468
Bock8	36.4295	50	44.34468
TO12	36.72636	50	44.34468
AMB12	30.12797	50	44.34468
PVPP12	32.74569	50	44.34468
Bent12	30.93992	50	44.34468
Bock12	30.8	50	44.34468
TO24	35.13407	50	44.34468
AMB24	22.4385	50	44.34468
PVPP24	32.91538	50	44.34468
Bent24	29.64206	50	44.34468
Bock24	30.69555	50	44.34468

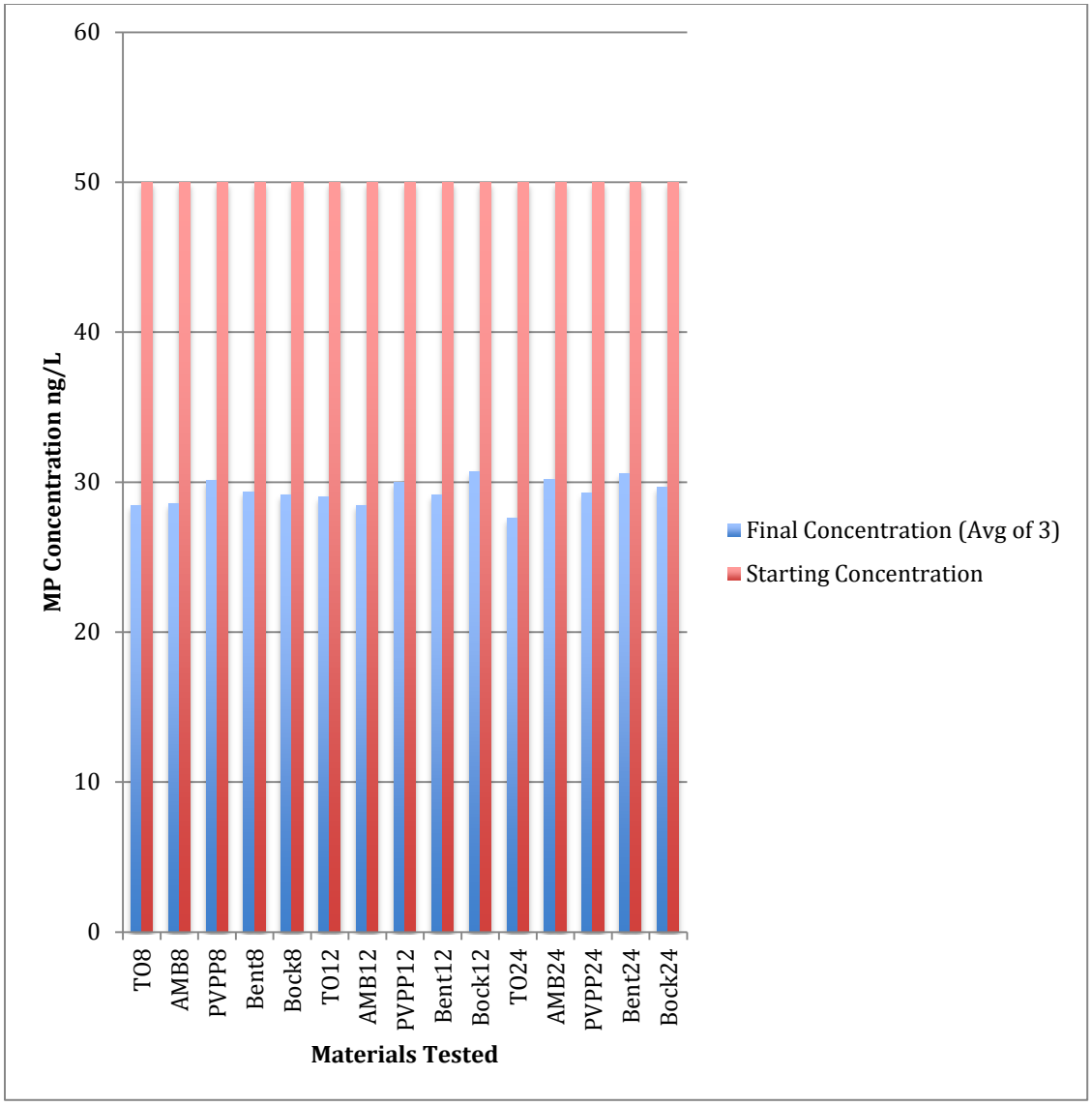

FIGURE 31. Time Trial 186 IPMP.

Time Trial for IPMP binding capacity of PVPP, Bocksin, Bentonite, Ambrelite, and Toasted Oak at 8, 12 and 24 hours. Materials were added at 1g/12mL in MP treated model wine, at 50 ng/L stadards and 40 ng/L isotope.

TABLE 21 Time Trial 186 IPMP

Time Trial 186 IPMP			
Trial	**Final Concentration (Average of Three)**	**Original Concentration**	**Control concentration**
TO8	28.4523	50	46.357
AMB8	28.55654	50	46.357
PVPP8	30.10016	50	46.357
Bent8	29.37167	50	46.357
Bock8	29.17427	50	46.357
TO12	29.03331	50	46.357
AMB12	28.46048	50	46.357
PVPP12	29.96035	50	46.357
Bent12	29.14955	50	46.357
Bock12	30.67251	50	46.357
TO24	27.57058	50	46.357
AMB24	30.20786	50	46.357
PVPP24	29.28095	50	46.357
Bent24	30.53849	50	46.357
Bock24	29.63944	50	46.357

Results from the long term incubation over 8-24h demonstrate that there were no significant changes with the longer time treatments vs. the shorter time treatments. It was also shown that MP behavior in a model wine system remained consistent, not fluctuating significantly, with any specific treatment provided in the time trials (Figure 31, Table 21). A further study to be conducted will be repetition of treatments, with a sequence of treatments using the same or varying materials to further reduce MP in model wine to below sensory threshold.

CHAPTER VII

SUMMARY AND CONCLUSIONS

Elevated concentrations of MP in Texas wine continues to be one of the most problematic issues that arises in the Texas wine industry. Texas winemakers persistently seek out new methods, materials, or techniques to remediate the flaw created by MP concentrations in final wine products. Recently there has been an increase in research specifically pertaining to MP compounds in wines as the problem of high MP concentrations is one that persists in several wine growing regions. If MP concentrations exceed sensory thresholds, the quality of the wine is decreased with causes loss of revenue. Most commonly, wines high in MPs are blended with wines with lower concentrations, diluting MPs to an acceptable threshold. However, blending as a method of remediation compromises the integrity of the grape varietal or vineyard from witch the grapes were harvested. Winemakers either blend with the same varietal from an outside vineyard source or blend with a different varietal containing lower MP concentrations from their own vineyard, having to produce a heritage wine vs. a single varietal.

Currently pre-harvest vineyard practice has proven to be the most effective way to reduce MP concentrations in grapes post verasion. Canopy pruning has shown to be effective in reducing MP concentration in grapes at harvest as modeled by J.J. Scheiner (15). Recent studies have begun to emerge questioning material bind of MP in final

wine products such as the research done by G.S. Howell at the University of Michigan whereby four common cellar practices used to remediate excessive MP concentrations were conducted (30). The trials tested various yeast strains, Malolactic fermentation techniques, soaking wine with various types of oak and finally some enzyme treatments, all commonly believed by winemakers to lower MP concentrations. Later, a study was done where wines with high MP elevations were treated with various enclosures, cork and synthetic materials, to see if MP were being bound by the wine bottle enclosures (31). Another recent study, 2011, was conducted by D. Inglis and G.J. Pickering on removal of MP concentrations due to lady bugs by the addition of binding proteins to wines (32). These studies prompted this investigation of material treatments to finished wines in an effort to reduce MP concentrations.

This research was intended to further investigate a broad scope of materials currently in use and others not in use to garner greater knowledge on the behavior of MP compounds in wine systems when introduced to the material. A model wine was used, free of secondary plant compounds present in wines, in order to focus specifically on MP behavior when treated with various materials.

The findings during the preliminary research showed a reduction of MP concentrations by treating a model wine with AL and PETE. The reduction of MP was 90% using AL and 66% for PETE. These treatments were done for an extended period of time, 16 days, using extremely high volumes of material that would be impartible in winemaking.

These findings prompted further investigation whereby the ratio of material to wine and time of incubation was reduced.

A material screening followed to identify prospective MP binding materials to be used in treating wines with high MP concentrations. Fourteen materials were selected based upon two criteria; a) use in current cellar practices, b) known binding capacity with follow up on preliminary research. The materials were treated for five days and showed a 50 % reduction in MP concentration, respectively. The resulting reduction in MP concentrations seen in the fourteen materials screened led to question how much time was needed for MP binding to occur.

Following the material screening, time-trials were conducted in effort to better understand adsorption rates and methods by which attenuation of MP concentrations may be achieved. Five materials from the previous screening, showing greatest MP binding, were selected, Amberlite, PVPP, Bocksin, Bentonite, and toasted oak. The time trials were divided into two sections the first being shorter time durations of 15 min, 30 min, 1 hour, and 2 hours. Should the shorter times have proven to reduce MP concentrations, longer time trials would not be necessary and repetitive short treatments would be tested. Results from the 15 min to 2 hour time trials showed little reduction in MP concentration with 14% for IBMP and 40% for IPMP. The next time trial was set to include longer treatment durations at eight, twelve, and 24 hours. Results from the longer incubation periods of 8-24h demonstrate that no significant

changes with the longer time treatments vs. the shorter time treatments occurred. It was also shown that MP behavior in a model wine system remained consistent, not fluctuating significantly, with any specific treatment provided in the time trials. A further study to be conducted will be repetition of treatments, will a sequence of treatments using the same or varying materials further reduce MP in model wine to below sensory thresholds.

LITERATURE CITED

1 Pasteur, M.L., Ses maladies, causes qui les provoquent, procedes, novuex pour le consseruer et pour le vieillir. *Etudes sur le vin.* F. Savy: Paris, **1873.**

2 Wine Institute. World statistics, April, 2012. Per capita wine consumption by country (PDF). http://www.wineinstitute.org/resources/statistics

3 Maga, J. A., Sizer, C. E., Pyrazines in foods. *Handbook of flavor ingredients* Vol 1., 2nd edition. CRC Press: Cleveland, **1975**.

4 Allen, M.S.; Lacey, M. J.; Harris R.L.N.; Brown, W. V. Contribution of methoxypyrazines to sauvignon blanc wine aroma. *Am. J. Enol. Vitic.* **1991,** *42,* 109-112.

5 Allen, M.S.; Lacey, M. J.; Methoxypyrazines of grapes and wine. *Chemistry of wine flavor.* American Chemical Society: New York, **1998.**

6 Bailly, S.; Jerkovic, V.; Marchand-Brynaert, J.; Collin, S. Aroma extraction analysis of sauternes wines. Key role of polyfunctional thiols. *J. Agric. Food Chem.*, **2006**, *54*, 7227–7234.

7 Pickering, G. J.; Karthik, A.; Inglis, D.; Sears, M.; Ker, K. Determination of ortho- and retronasal detection thresholds for 2-isopropyl-3-mehtoxypyrazinein wine*. J. Food. Sci.* **2007,** *72*, 468-472.

8 Barlin, G.B., *The pyrazines*. The Chemistry of heterocyclic compounds, John Wiley and Sons, New York, **1982**.

9 Margalit, Y., *Concepts in wine chemistry*. The Wine Appreciation guild. San Francisco, **2004**.

10 Maga, J. A.; Sizer, C. E.. Pyrazines in foods. A review. *J. Agri. Food. Chem.* **1973,** *21*, 22-30.

11 Hartman, P.J. The effect of wine matrix ingredients on 3-Alkyl-2-methoxypyrazines measurements by headspace solid-phase microextraction (HS-SPME). M.S. Thesis, Virginia Polytechnic Institute and State University, 2003**.**

12 Cudjoe, E.; Wiederkehr, T.B.; Brindle, I.D. Headspace gas chromatography-mass spectrometry: a fast approach to the identification and determination of 2-alkyl-

3-methoxypyrazine pheromones in ladybugs. *J. R. Soc. Chem. Perkin. Trans. 1* **2004,** *130*, 152-155.

13 Belancic, A.; Agosin, E., Methoxypyrazines in grapes and wines of *Vitis vinifera* cv. Carmenere. *American Journal of Enology and Viticulture* **2007,** *58*, 462-468.

14 Texas Almanac. County weather records, May 2012. Texas temperature, freeze, growing season and precipitation records by county (PDF). http://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=8&ved=0CE4QFjAH&url=http%3A%2F%2Fwww.texasalmanac.com%2Fsites%2Fdefault%2Ffiles%2Fimages%2Falmanac-feature%2FcountyweatherA.pdf&ei=gGKTUOjbKeH00gHq-oC4BA&usg=AFQjCNFxf3eb4cfLpUAfCVSCSoEdg2Dueg&sig2=vT-vqr7tWQo-S4gJw-aA7g

15 Scheiner, J.J.; Heuvel, J.E.V.; Pan, B.; Sacks, G.L. Modeling impacts of viticultural and environmental factors on 3-isobutyl-2-methoxypyrazine in cabernet franc grapes. *Am. J. Enol. Vitic.* **2012**, *63*, 94-105.

16 Murray, K. E.; Whitfield, F. B., Occurance of 3-alkyl-2-methoxypyrazines in raw vegetables. *Journal of the Science of Food and Agriculture* **1975,** 26, 973-986.

17 Gallois, A.; Kergomard, A.; Adda, J., Study of the biosynthesis of 3-isopropyl-2-methoxypyrazine produced by *Pseudomonas taetrolens*. *Food Chemistry* **1988,** *28*, 299-309.

18 Hashizume, K.; Tozawa, K.; Hiraga, Y.; Aramaki, I., Purification and characterization of a O-methyltransferase capable of methylating 2-hydroxy-3-alkylpyrazine from *Vitis vinifera* L. (cv. Cabernet Sauvignon). *Bioscience, Biotechnology, and Biochemistry* **2001,** *65*, 2213-2219.

19 Hashizume, K.; Tozawa, K.; Endo, M.; Aramaki, I., S-adenosyl-L-methionine-dependent O-methylation of 2-hydroxy-3-alkylpyrazine in wine grapes: a putative final step of methoxypyrazine biosynthesis. *Bioscience, Biotechnology, and Biochemistry* **2001,** *65*, 795-801.

20 Heymann, H.; Noble, A.C.; Boulton, R.B. Analysis of methoxypyrazines in wines. 1. Development of a quantitative procedure. *J. Agric. Food. Chem*. **1986,** *34*, 268-271.

21 Kotseridis, Y.; Baumes, R.L.; Bertrand, A.; Skouroumounis, G.K. Quantitative determination of 2-methoxy-3-isobutylpyrazine in red wines and grapes of

Bordeaux using a stable isotope dilution assay. *J. Chromatogr.* **1999,** *841* 229-237.

22 Pickering, G.; Lin, J.; Reynolds, A.; Soleas, G.; Riesen, R., The evaluation of remedial treatments for wine affected by *Harmonia axyridis*. *International Journal of Food Science & Technology* **2006,** *41*, (1), 77-86.

23 Morales, G., Health Benefits and quality of Texas wine. Ph.D. dissertation, Texas A&M University, 2011.

24 Blake, A.; Kotseridis, Y.; Brindle, I.D. Effect of closure and packaging type on 3-akyl-2-methoxypyrazines and other impact odorants of riesling and cabernet franc wines. *J. Agic. Food. Chem.* **2009**, *57*, 4680-4690.

25 Kotseridis, Y.S.; Spink, M.; Brindle, I.D.; Blake, A.J. Quantitative analysis of 3-alkyl-2-methoxypyrazines in juice and wine using stable isotope labeled internal standard assay. *J.Chroma.* **2008,** *1190*, 294-301.

26 King, E. S.; Kievit, R. L.; Curtin, C.; Swiegers, J. H.; Pretorius, I. S.; Bastian, S. E. P.; Leigh Francis, I., The effect of multiple yeasts co-inoculations on Sauvignon Blanc wine aroma composition, sensory properties and consumer preference. *Food Chemistry* **2010,** *122*, 618-626.

27 Chapman, D.M.; Thorngate, J.H.; Matthews, M.A., Guinard, J.; Ebeler, S.E. Yield effects on 2-Methoxy-3-Isobutylpyrazine concentration in cabernet sauvignon using a solid phase microextraction gas chromatography/mass spectrometry method. *J. Agric. Food Chem.*, **2004**, *52*, 5431–5435.

28 Leater, D.W.; Wilson, B.W. Standardization of alumina and silica adsorbents used for chemical class separations of polycyclic aromatic compounds. *Anal. Chem.* **1985**, *57*, 2979---2984.

29 Tsai, W.T.; Lai, C.W.; Hsien, K.J. Characterization and adsorption properties of diatamacious earth modified by hydrofluoric acid etching. *J. Colloid. Interf. Sci.* **2006**, *297*, 749-754.

30 Howell, G.S., Treloar, J., Beaudry, R., Cellar methods to reduce methoxypyrazines in cabernet franc. Final report, University of Michigan, **2006.** www.michiganwines.com/docs/Research/06howell4.pdf

31 Pickering, G. J.; Blake, A. J.; Soleas, G. A.; Inglis, D.L. Remediation of wine with elevated concentrations of 3-alkyl-2-methoxypyrazines using cork and synthetic closures. *Int. J. Food. Agric. Environ.* **2010**, *8*, 97-101.

32 Inglis, D.; Beh, A., Humes, E.; Pickering, G.J.; Brindle, I. Lady bugs and green bell peppers: methoxpyrazine (MP) removal from grape juice using MP binding proteins. Ph.D. Thesis, Brock University, Ontario, Canada, 2011.