Quantitation of 3-alkyl-2-methoxypyrazines in Grape Juice and Wine via SPME-GC/MS

by

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Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in the Chemistry Program

YOUNGSTOWN STATE UNIVERSITY

May 2010
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Abstract

This research study involved the development and validation of an analytical method using solid-phase microextraction (SPME) coupled with gas chromatography-mass spectrometry (GC-MS) for the analysis of 3-alkyl-2-methoxypyrazines in grape juice. These methoxypyrazines are naturally found in grapes and may add to the overall aroma of a wine in low concentrations, but may have a negative affect if the concentrations become too large. This research study will develop and validate a method to track the concentration of the methoxypyrazines as the grapes ripen. A method will also be developed and validated to determine the concentration of methoxypyrazines in Cabernet Sauvignon, and Sauvignon Blanc wines.
Acknowledgements

Over the years it has felt like I have had so much work to accomplish. At this time I would like to thank those who played a valuable role in helping me achieve this. I would like to thank my grandparents for always believing in me and pushing me to pursue my goals. My parents for giving me support as I pursued my educational goals. Dr. Brian Leskiw, thank you for all of your help and advice during the past couple years while I was writing this thesis. I would also like to thank Dr. Roland Riesen for sharing his love of enology and for all of his help.

I would also like to thank some of my fellow graduate students who helped me during my time here. To John Milo, thank you for all of your advice and for warning me about ‘obstacles’ that I may come across in my research. For Ashley Wolf and Amanda Kotheimer, thank you for keeping me company as I was writing this. To Chad Miller, Dominic Loiacona, and Tom Rudnicki thanks for providing comic relief in times when I needed to laugh and not take things so seriously. The chemistry box of knowledge shall live on.

I would also like to thank Ray Hoff for helping me every time that something broke and needed to be fixed. I wish to thank Dr. Daryl Mincey for being on my committee and for his comments as I was writing this thesis. I would also like to thank the Youngstown State University Department of Chemistry and the Office of Graduate Studies for support on this research project.
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Chapter I

Literature Review

A. Grapes

a. Growing process

The composition of each variety of grape is dependent on several factors which influence the type of wine that can be produced from the juice. The environment that the grapes are grown in affects the fruit in various ways. Soils are of particular concern as those low in nitrogen do not readily support the fermentation process. However, too much nitrogen has an effect on the wine stability and quality. Soils high in potassium produce grapes that are high in pH and in titratable acid while soils that are unable to absorb precipitation cannot efficiently provide nutrients to the rootstock of the vine. Grapes that are high in potassium will lead to wines with higher pHs. The rootstock on which the vine grows assists the grapes by being resistant to different strains of viruses that may damage crops. However, some viruses are beneficial to the vine.

The leaf canopy also influences grape production and grape maturity by providing energy for vine growth as well as potentially inhibiting the ripening process for those berries hidden from the sunlight. These grapes are particularly troublesome as they do not ripen as quick as the others and thus contain higher concentrations of aroma-altering compounds like, for example, 3-alkyl-2-methoxypyrazines. These hidden berries also have higher concentrations of titratable acids, and are deficient in the sugar content that is necessary for fermentation. To help prevent substantial monetary losses to the wine industry due to the presence of aroma-altering compounds, a rapid solvent-free analysis
would be ideal to identify potential problems. The harvesting process is another important step in the development of a wine.

b. Grape Harvest

In order to collect sufficient juice that can ferment and ultimately produce a desirable product, grapes must be harvested within a particular time frame. The grapes cannot be simply harvested on a particular date each year as the growth rate of each variety of grape depends heavily on the environmental conditions in the region. Sampling of the grapes actually occurs throughout the growth process in order to compile data to monitor changes and to identify when the grapes are to be harvested. Berry sampling was performed at the Ohio Agricultural Research and Development Center (OARDC) vineyard in Kingsville, Ohio located in Ashtabula County on a weekly schedule for this project and will be described further in Chapter 4.

One important variable that can be used to follow the progress of grape development is sugar concentration, or degree Brix, in the grape juice itself. As the grapes ripen, the sugar concentration increases and in California, for example, harvesters regularly see a rise of more than 1.0 Brix each week. In Ohio, however, sugar concentrations increase at a slower rate of less than 1.0 Brix per week, and will be presented in more detail in Chapter 4. Typically a Brix value of 20 to 25 indicates that the grapes are ready to be harvested, depending on the type of wine that the grapes are going to be used to make.

In addition to monitoring the sugar concentration as a measure of harvest date, other analyses are necessary and include, for example, the pH of the juice, the amount of total soluble solids and the amount of titratable acid in the juice of the grapes. Total
titratable acids is not a single acid, but consists of many different acids including tartaric acid, malic acid, amino acids, inorganic acids, lactic and succinic acid along with many others that contribute very little to the titratable acidity of the juice. The pH of the juice is often followed to determine what type of wine may be made. The grape juice for a table wine has a desired pH of 3.0 to 3.3, while lower pHs may be used for sparkling wines.\(^2\) One can also follow berry weight and color as the ripening occurs. For some grape varieties, the berries themselves change color during the ripening process from a green berry to a purple color, giving rise to the possibility of a red wine. This process is often referred to as veraison. Harvesters must also monitor grapes that ripen early, but stay green and remain hard as these are of limited benefit.\(^2\)

Besides following the concentrations of compounds that aid in the quality and development of the grapes, the concentration of compounds, like methoxypyrazines, that alter optimal taste, must also be analyzed at various stages.

c. Fermentation

Fermentation is the process in which the sugars present in grape juice are converted into ethanol. The fermentation process is carried out by yeast, where in most cases, the yeast is either *Saccharomyces cerevisiae* or *Saccharomyces bayanus*. There are other suitable strains of yeast and include *Schizosaccharomyces*, *Zygosaccharomyces*, and *Brettanomyces* depending on the variety of grape and location of the winery. The flora of yeast that grows on the grapes, either wild or those that occur in the vineyard, depends on many different factors and include for example, precipitation, humidity, the spraying regimes of the vineyard, altitude, nitrogen fertilization, and insect vectors. The
fermentation of grape juice may either occur naturally with the yeast strains already on the grapes, or by adding additional yeast to the mixture.

When yeast is added to the grape juice, there are certain criteria that the yeast should meet. For example, the selected yeast should have the ability to conduct a vigorous fermentation and ferment until little or no fermentable sugar is left.\textsuperscript{2} The particular species should behave predictably, tolerate the presence of ethanol and changes in temperature, be tolerant to SO\textsubscript{2}, be easily removable, and produce no off-flavor. The genus \textit{Saccharomyces} however, is fairly limited to the compounds that it may use as an energy source. Monosaccharides such as glucose, fructose, mannose, and galactose are acceptable sources of energy for \textit{Saccharomyces}.\textsuperscript{2}

Yeast takes multiple steps in transforming glucose into ethanol. According to Figure 1.1, one way that yeast may make ethanol is through glycolysis. This process enlists the help of numerous enzymes. The first reaction that takes place is the phosphorylation of glucose to glucose-6-phosphate and is performed by one of three different enzymes: hexokinase PI (A), kexokinase PII (B), or glucokinase. In this reaction, ATP serves as the source of phosphate. Following phosphorylation, glucose-6-phosphate is then converted into fructose-6-phosphate by using the enzyme phosphoglucone isomerase. This compound then becomes phosphorylated again by the enzyme phosphofructokinase to become fructose-1,6-biphosphate. ATP is used again as the source of phosphate for the reaction. Aldolase then catalyzes the hydrolysis of fructose-1,6-biphosphate to dihydroxyacetone phosphate which is then converted into glyceraldehyde 3-phosphate. This last molecule is ultimately converted into pyruvate through multiple enzymatic reactions. Pyruvate is then decarboxylated by pyruvate
decarboxylase to yield acetaldehyde which is then reduced by alcohol dehydrogenase to end with ethanol. This reaction is drawn out in detail in Figure 1.2 including each enzyme that is present in the reactions.

Figure 1.1 Metabolic pathways used by yeast fueled by glucose found in grape juice.
Figure 1.2 Diagram showing the enzymes and the pathways that glucose would take to become ethanol.²

B. Pyrazines

From roasted food to insects and ants, volatile compounds like pyrazines can be found in several unsuspecting places.⁶⁻¹⁷ This class of volatile compounds which is schematically drawn in Figure 1.3, play a role in aroma that in some instances are beneficial, while potentially harmful in others. These compounds have been found in many different species of insects. In one particular species of ants in Madagascar, the *Eutetramorium mocquersyi*, several different pyrazines, are produced in the poison gland located on their bodies. These compounds are then secreted out during foraging and as
an attempt to recruit other ants. At least three different pyrazines, at differing concentrations, have been identified from this particular species and are not the only ants that utilize this class of compounds. Two different species of ant, the *Rhytidoponera metallica* and the *Messor* ants, have also been known to produce these volatile compounds. These ants produce several pyrazines, some that are common to other species as well as a few that are unique.7,8

![Figure 1.3](image)

**Figure 1.3** The general structure for pyrazines where each R group can vary.

There are other insects that produce an appreciable amount of these compounds as well. A group of ladybeetles called *Coccinella septempunctata*, which are known for their seven spots, have been found to produce pyrazines as a means to attract mates. These ladybeetles have also been intentionally distributed in some crops in an attempt to control the aphid population. It has also been shown that these ladybeetles emit 3-isopropyl-2-methoxypyrazine as a defensive mechanism.9 Another beetle, the *Harmonia axyridis*, more commonly known as the multicolored Asian lady beetle (MALB), is not only a regular household pest, but an expensive problem for the wine industry. When these beetles are crushed with grapes, it increases the levels of methoxypyrazines in the must and wine. The beetles can produce at least four different pyrazines: 2,5-dimethyl-3-methoxypyrazine (DMMP), 3-isopropyl-2-methoxypyrazine (IPMP), 3-sec-butyl-2-methoxypyrazine (SBMP), and 3-isobutyl-2-methoxypyrazine (IBMP). The production of pyrazines is approximately picograms per beetle which is equivalent to nanograms per
liter or parts per trillion, ppt.\textsuperscript{10} A liter of wine can be ruined by one single beetle. In Figure 1.4 the structure for the methoxypyrazines is shown along with the structure for the internal standard that was be used for this study. Several species of wasps have also been shown to produce pyrazines in their mandibular gland. It is believed that these compounds are used as a pheromone to attract mates.\textsuperscript{11} A specific bacterium species has also been shown to produce pyrazines. The bacterium species, the \textit{Halomonas venusta} or \textit{Deleya venusta}, is known for having a very strong pea like odor. This odor is the result of production of 2-methoxy-3-(1’-methylpropyl)pyrazine.\textsuperscript{12}

\begin{center}
\begin{tikzpicture}
\node (a) at (0,0) {\includegraphics{structure.png}};
\node (b) at (3,0) {\includegraphics{structure2.png}};
\end{tikzpicture}
\end{center}

\textbf{Figure 1.4} The structure on the left is the generic form for the methoxypyrazines that are investigated in this research. The structure on the right is for an ethoxypyrazine, like the internal standard, where $R$ is the desired functional group.

Pyrazines have also been measured at easily detectable levels in several food items. Interestingly, the processes some food undergo plays a role in the amount of pyrazines found, particularly those that are roasted. One such food item is roasted cocoa beans. While the genetics of the bean itself plays a dominant role in the concentration of the pyrazines, a portion of the total of the concentration of these compounds is due to the roasting process. The storage of the bean, fermentation, and drying also play a role.\textsuperscript{13} Many different processes occur during the roasting process. The pyrolysis of sugars and polysaccharides has been found to give rise to many different volatile compounds.\textsuperscript{14}
Model systems have shown that simple pyrazines are formed from the condensation of sugars and amino acids.\textsuperscript{15} Researchers have also found pyrazines in roasted coconut where overall twenty different pyrazines have been identified.\textsuperscript{16} Interestingly, pyrazines are sometimes added to foods at low concentrations for flavoring.\textsuperscript{17} Pyrazines were initially detected in grape juice in 1975.\textsuperscript{18} To avoid high concentrations of these compounds, many different precautions may be taken.

C. Wine Process

The analysis of wine and its associated aroma can occur at several different stages. As grapes grow and ripen within a vineyard, several processes are occurring. While the young grapes provide the necessary sugars for fermentation, there are many compounds that can become undesirable if their concentration levels become too high. Some of the compounds that are regularly found in higher concentrations in wine include alcohols, organic acids, esters, sugars, and carbohydrates.\textsuperscript{19} Compounds found within wines at low concentrations, yet at very perceivable sensory levels, include methoxypyrazines, which is the subject of the current study. The wine industry loses a substantial amount of product due to methoxypyrazine contamination. In addition to the naturally occurring presence of these compounds in grape juice, the problem is compounded by the fact that ladybeetles desire the environment of a vineyard and are often collected with the harvest of the berries. When these ladybeetles are crushed with the grapes they release methoxypyrazines that they naturally produce. These compounds then end up in the wine, which is often referred to as ladybug taint, and the resulting wine may be undesirable.\textsuperscript{20} With the development of a precise analytical method that can
rapidly assess the quality of the processed wine, steps can be taken to minimize further contamination.

D. Methoxypyrazines

There are many different types of methoxypyrazines. Some of the most common include: 3-sec-butyl-2-methoxypyrazine (SBMP), 3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-2-methoxypyrazine (IPMP), and 3-ethyl-2-methoxypyrazine (EMP). Each one of these compounds has a different odor/taste threshold and a unique odor. For IBMP, SBMP, IPMP, the odor threshold is only 1-2 nanograms per liter (ng/L) in water with a neutral pH. EMP however has an extremely high odor/taste threshold compared to other methoxypyrazines and it has been measured as high as 425 ng/L. Wines with a high concentration level of 3-alkyl-2-methoxypyrazines are described as vegetative, green bell pepper, and herbaceous. The odor that is normally associated with IBMP is more of a bell pepper odor. Even though extreme care can be exercised to remove the Multicolored Asian Ladybeetles during collection, a considerable amount of the detectable methoxypyrazines is produced within the grape themselves.

Methoxypyrazines are found in the skin and the pulp of the grape. When the grapes are crushed, these compounds are collected with the juice that is extracted. The biggest concern is when the grapes are harvested too early as the concentration levels of the methoxypyrazines are often too high, causing the wine to be atypical. For some methoxypyrazines, the concentration might actually be higher in the grapes than in wine depending on when the grapes are harvested. For example, IBMP is found in concentrations of about 10 to 50 ng/L in wine, but may be as high as 80 ng/L in grapes. In wines, these compounds may be playing an important role in the overall taste of the
wine and are often found in the varieties of Cabernet Sauvignon, Merlot, Sauvignon Blanc, and Pinot Noir wines. To determine the concentrations of these compounds in wine, an analysis method must be developed.

E. Analysis Methods in Wine

There are multiple ways to analyze food volatiles using gas chromatographic methods. Since these compounds readily escape the food matrices, one can take advantage of this via headspace sampling. Headspace sampling is simply the analysis of the gas volume immediately above a liquid in a closed container. There are four types of headspace sampling that can be used: static headspace, dynamic headspace, purge and trap, and solid-phase microextraction.

The methods that have been used to analyze methoxypyrazines have evolved over the years as technology improved. Methoxypyrazines were originally analyzed using high performance liquid chromatography (HPLC) in 1986. Using this method, the recovery of the methoxypyrazines was in the range of 43% to 62% with a limit of detection (LOD) at 1.2 µg/liter. Unfortunately, this is higher than the odor and taste threshold for these compounds in wine meaning that the methoxypyrazines can only be detected long after they are tasted. Researchers later used solid phase extraction (SPE) which, when using a longer bed cell, more methoxypyrazines could be extracted. This method has delivered LODs in the range of 2 to 6 ng/L. However, the odor threshold value is still lower.
F. Extraction Methods

More recently, methoxypyrazines have been analyzed using solid-phase microextraction (SPME), which was introduced in 1988 and is a solvent-free method of sample preparation. This technique utilizes a fiber that can sample analytes within the headspace of the vial. There are multiple types of fibers, and based on the polarity of the fiber, different compounds can be targeted. Other parameters also play a role in the concentration of methoxypyrazines detected, and include the vial volume, sample volume to vial volume ratio, pH, adsorption temperature and adsorption time. Using this method, researchers have been able to detect methoxypyrazines in wine at concentrations as low as 1 to 2 ng/L in wine, which is when these compounds can be detected by olfactory methods. As another attempt to minimize the contamination of wine, some researchers have been tracking the concentration of methoxypyrazines during the growing process. The concentration of these compounds is high in the beginning of the process and drops off significantly after the veraison process is completed. This allows for the grapes to be harvested when the concentrations of the methoxypyrazines is low enough to not be detected when the grape juice is converted into wine. Other methods have been developed to increase the limit of detection and limit of quantitation of methoxypyrazines.

Other versions of the original SPME method have been developed and include solid-phase dynamic extraction (SPDE). A recent study comparing SPME, static headspace (S-HS), and SPDE was conducted to determine which method was more efficient at extracting volatiles from various food matrices and showed that SPDE extracted more analytes than SPME. For a majority of the compounds, the SPDE
procedure had a higher concentration factor (CF) given that the coating on the SPDE needle is eight times thicker than on the SPME fibers, thus allowing more analyte to be absorbed.\textsuperscript{34} The SPME method of extraction is often coupled with gas chromatography as a way to separate the different components of the mixture.

G. Chromatography

Chromatography is one of the oldest methods used to separate mixtures. This technique uses a mobile and stationary phase to separate different components of a mixture.\textsuperscript{35} The mobile phase is either an inert gas or a liquid and the stationary phase is either a liquid that has been chemically bonded to inside of a capillary tube or a solid that has been packed inside the column. The mobile phase is moved through the column at a steady flow rate. While the mixture is moving through the column, the components interact with the stationary phase. The amount of time that the components interact with the stationary phase determines when the compound elutes from the column. A compound that spends more time in the stationary phase will emerge at a later time compared with an analyte that does not interact much.

H. Mass Spectrometry

Mass Spectrometry is an extremely powerful tool as an independent instrument and especially when coupled with gas chromatography.\textsuperscript{35} An important part of mass spectrometry is the way that the compounds are ionized. There are several different ionization techniques that can be employed, with some techniques being more harsh than others. The harshest method of ionization is called electron ionization (EI) where a 70 eV beam of electrons ionizes and regularly dissociates the sample of interest. Other
methods for ionization exist, but will not be discussed here. In addition to the method of ionization, mass spectrometers differ by the type of analyzer.

The mass analyzer of every mass spectrometer is responsible for the actual separation of ions based on the $m/z$ ratio. The most common type of analyzer is the quadrupole, which is the analyzer used in this study, and essentially acts as a filter by creating a field where only ions of a specific $m/z$ ratio will pass. Ions traveling through a quadrupole mass analyzer at a different $m/z$ than the correct one will go off axis and not be detected. There are two different types of ion storage that are often used to increase the sensitivity of the method.

Two types of ion storage are select ion storage (SIS) and select ion monitoring (SIM). SIS is different and is not to be confused with select ion monitoring (SIM). Both of the methods have been shown to increase sensitivity when used correctly. SIM is typically used when monitoring a single ion or a small range of ions. The sensitivity of the SIM method is lost however, when the $m/z$ range that is being analyzed is increased. The SIS method was employed for the studies described herein. Mass spectrometry alone is not sufficient to quantitate the methoxypyrazines within a sample.

I. Gas Chromatography/Mass Spectrometry

When gas chromatography (GC) and mass spectrometry (MS) are coupled, the combined instrument becomes an extremely versatile tool. The chromatography section of the instrument induces the separation of a mixture of analytes which allows the mass spectrometer to then analyze each analyte independently. The resulting mass spectrum is very clean and allows for a much easier identification of individual compounds compared to an analysis of all analytes at once. This process turns a complex sample into a much
simpler analysis. The analytical method can also provide the quantitation of analytes in solution.

**J. Solid Phase Microextraction**

Solid phase microextraction (SPME) is a nondestructive, solvent-free sampling technique that has use in a variety of fields. This sampling method, for example, has been used in environmental science, forensic science, botany, toxicology, and entomology. One of the uses for this method is for the detection of volatile components in a solution. Since this sampling technique does not require much work up, very little sample preparation is required to go into making the actual samples. However, in order to perform quantitation, each experimental parameter needs to be carefully controlled and optimized. For example, the optimal pH, extraction temperature, polarity, rate of agitation, the headspace/solution ratio, and extraction time all must be determined. Other factors play a role in recovery of volatiles, including the type of fiber used.

There are several different coatings that may be used on these fibers. Each fiber provides different attributes that may be more efficient during the extraction process for some molecules while quite inefficient for others. A more polar compound is better extracted by a polar fiber. Several variables must be addressed when creating a method of analysis. The method of analysis used in the present work is described further in Chapters 2 and 3.

**K. Need for Present Research**

Numerous studies claim to have accurately determined the concentration of 3-alkyl-2-methoxypyrazines in grape juice and wine. The work presented herein will focus on creating a validated method for analyzing methoxypyrazines by using head
space solid-phase microextraction (HS-SPME) coupled with gas chromatography/mass spectrometry (GC/MS). This work will also measure the concentration of these species as a function of the grape life in the vineyard and establish limits of detection and quantitation.
Chapter II

Experimental

A. Chemicals and Reagents

All standards and reagents were obtained at the highest available purity. Listed below in Table 2.1 and 2.2 are the chemicals with their CAS numbers, purity, and origin.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>CAS number</th>
<th>Percent Purity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol, Absolute</td>
<td>64-17-5</td>
<td>≥99.5</td>
<td>Acros Organics</td>
</tr>
<tr>
<td>Tartaric Acid</td>
<td>87-69-4</td>
<td>99+</td>
<td>Acros Organics</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>7647-14-5</td>
<td>99.9</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Sodium Hydroxide</td>
<td>1310-73-2</td>
<td>95+</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Hydrochloric Acid</td>
<td>7647-01-0</td>
<td>36.5-38</td>
<td>Pharmco-AAPER</td>
</tr>
<tr>
<td>Water, Millipore</td>
<td>-</td>
<td>-</td>
<td>In house</td>
</tr>
<tr>
<td>filtered</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>6381-62-6</td>
<td>≥95</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Dextrose</td>
<td>50-99-7</td>
<td>&gt;99</td>
<td>Fisher Scientific</td>
</tr>
</tbody>
</table>

Table 2.1: Chemical reagents, CAS number, purity, and source
<table>
<thead>
<tr>
<th>Standard</th>
<th>CAS number</th>
<th>Percent Purity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-isobutyl-[^2H_2]-2-methoxypyrazine</td>
<td>-</td>
<td>-</td>
<td>G. Sacks - Cornell</td>
</tr>
<tr>
<td>3-isopropyl-2-methoxypyrazine</td>
<td>25773-40-4</td>
<td>-</td>
<td>Pyrazine Specialties</td>
</tr>
<tr>
<td>3-sec-butyl-2-methoxypyrazine</td>
<td>24168-70-5</td>
<td>-</td>
<td>Pyrazine Specialties</td>
</tr>
<tr>
<td>3-isobutyl-2-methoxypyrazine</td>
<td>24683-00-9</td>
<td>-</td>
<td>Pyrazine Specialties</td>
</tr>
<tr>
<td>3-ethyl-2-methoxypyrazine</td>
<td>25680-58-4</td>
<td>-</td>
<td>Pyrazine Specialties</td>
</tr>
<tr>
<td>3-ethoxy-2-ethylpyrazine</td>
<td>35243-43-7</td>
<td>-</td>
<td>Pyrazine Specialties</td>
</tr>
</tbody>
</table>

**Table 2.2** Chemical standards, CAS number, purity, and source.

**B. Standard Solutions and Samples**

Stock solutions of each pyrazines were made with ethanol at a concentration of 100 mg/L. The stock solutions of 100 mg/L were further diluted with a 9:1 Millipore water/99.5% ethanol solution to a concentration of 1000 µg/L. The 9:1 water/ethanol solutions were used instead of a neat ethanol solution to minimize any altering of the alcohol content during the spiking of the samples. All solutions were stored in darkness at 4°C.

A synthetic grape juice solution, which mimics the behavior of the grape juice, was prepared using 6.0 g/L tartaric acid and adjusting the pH of the solution to 7.0 using sodium hydroxide. A synthetic model wine solution, which mimics the behavior of wine, was also prepared using 6.0 g/L tartaric acid in a hydro-alcoholic solution (10% (v/v)
ethanol). The pH of the solution was then adjusted to 7.0 using sodium hydroxide and reflects the optimization of the recovery of the pyrazines.

C. Preparation of Commercial Wine Samples

To determine the concentration of ethanol in each of the different wines, an Ebuliometer was used. Upon analysis the concentration of ethanol was diluted to an ethanol content of 10.0% (± 0.1%) and the final volume was 40 mL. This wine was then spiked with 24 µL of the internal standard 3-ethyl-2-ethoxypyrazine, EEP, and immediately mixed. From this mixture, 10 mL was taken and placed in a clear 23 x 75 mm vials (MicroLiter Analytical Supplies, Inc.), with 3.0 grams of NaCl and sealed.

D. Equipment

SPME-GC/MS analysis was performed on a Varian 3800 gas chromatograph equipped with a Combi Pal autosampler connected to a Varian Saturn 2000 quadrupole ion trap mass spectrometer. The software the system used was Varian MS workstation version 6.9. The instrument was operated in SIS mode for the selected ions of each analyte.

E. Chromatographic Conditions

The carrier gas for this research was BIP (built in purifier) helium that was supplied by Airgas (Great Lakes) and a gas flow of 1 mL/minute was maintained.

The optimized temperature program was determined and upon injection was: initially held at 40°C for 3 minutes, and then heated to 120°C at a rate of 3°C per minute. The temperature program then ramped the temperature at a rate of 20°C per minute to 250°C. The injector and trap were held at 250°C and 230°C, respectively.
Chapter III

Method Development

In order to obtain a complete and thorough analysis, a systematic method must first be developed. Several different parameters need to be addressed in order to establish an appropriate method. Since GC/MS coupled with SPME was utilized to investigate volatile compounds in this study, several different factors control the efficiency of the extraction. These factors include the length of extraction, extraction temperature, type of fiber, and the matrix of the grape juice or wine. Attempting to account for the wine matrix is extremely difficult given how much the wine matrix actually varies between samples. To account for the variability between the samples, internal standards are regularly used in order to quantify the amount of pyrazines or other analytes in the sample.

In this research, SPME coupled with GC/MS was used to analyze the concentration of 3-alkyl-2-methoxypyrazines near the olfactory threshold. These compounds, as previously stated in Chapter 1, make some wines undesirable if the concentration levels are too high. The level at which the wine becomes undesirable varies for each taster. A method was developed, optimized, and validated to quantitate several species and the conditions of method optimization and validation are described in the following sections.
A. Identification of the Standards

The suspected species leading to tainted wine were all added to a synthetic model wine solution at a concentration of 1 µg/L and included 3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-2-methoxypyrazine (IPMP), 3-ethoxy-2-ethylpyrazine (EEP), 3-ethyl-2-methoxypyrazine (EMP), and 3-sec-butyl-2-methoxypyrazine (SBMP). The retention times of these species were recorded and compared to a model wine blank. The mass range selected was 50 - 200 m/z. The compounds were then identified by comparing the mass spectra of the selected chromatographic peak to the NIST Library. Figure 3.1 is a chromatogram showing the retention times for IBMP, IPMP, SBMP, EEP, and EMP. As illustrated by this chromatogram, EMP was not detected sufficiently and was removed from the list of compounds investigated. Figures 3.2 - 3.5 are the mass spectra of each compound compared to the mass spectra from the NIST library. The results from these experiments also identified the ions that would be collected for select ion storage (SIS) for quantitation purposes.
Figure 3.1 A chromatogram of model wine solution spiked with 1µg/L of each analyte 3-ethyl-2-methoxypyrazine (EMP), 3-isopropyl-2-methoxypyrazine (IPMP), 3-ethyl-2-ethoxypyrazine (EEP), 3-sec-butyl-2-methoxypyrazine (SBMP) and 3-isobutyl-2-methoxypyrazine (IBMP).
Figure 3.2.1 is the chromatographic peak for 3-isopropyl-2-methoxypyrazine (IPMP). Figure 3.2.2 is the mass spectra for the peak shown in Figure 3.2.1 and Figure 3.2.3 is the mass spectra for this compound from the mass spectrometric database.
Figure 3.3 Figure 3.3.1 is the chromatographic peak for 3-ethyl-2-ethoxypyrazine (EEP). Figure 3.3.2 is the mass spectra for the peak shown in Figure 3.3.1 and Figure 3.3.3 is the mass spectra for this compound from the mass spectrometric database.
Figure 3.4 Figure 3.4.1 is the chromatographic peak for 3-sec-butyl-2-methoxypyrazine (SBMP). Figure 3.4.2 is the mass spectra for the peak shown in Figure 3.4.1 and Figure 3.4.3 is the mass spectra for this compound from the mass spectrometric database.
Figure 3.5 Figure 3.5.1 is the chromatographic peak for 3-isobutyl-2-methoxypyrazine (IBMP). Figure 3.5.2 is the mass spectra for the peak shown in Figure 3.4.1 and Figure 3.5.3 is the mass spectra for this compound from the mass spectrometric database.
B. Determination of the Quantitation Ions for SIS mode

As previously discussed in Chapter 1, SIS mode increases the sensitivity of the mass spectrometer by omitting background ions from being stored within the ion trap, which decreases the background noise and allows for the collection of only the ions of interest. In order to maximize the signal-to-noise (S/N) ratios, several ions were investigated to determine which ion has the least amount of interference. Figure 3.6 displays the different ions that were being considered for the quantitation ions for 3-isopropyl-2-methoxypyrazine (IPMP) and the S/N ratio that was calculated for each ion. Since the ion with the $m/z$ ratio of 137 has the highest S/N ratio, this ion was used for the quantitation ion. Figure 3.7 presents the different ions that have the highest response for 3-ethyl-2-ethoxypyrazine (EEP) and the S/N ratio for each of the ions. Based on the calculated S/N ratios, the ion with the $m/z$ ratio of 124 was used. Figure 3.8 displays the chromatographic peak for 3-sec-butyl-2-methoxypyrazine (SBMP) and examining different ions that were under consideration for the quantitation ion. Based on the calculated S/N ratios, the ion with the $m/z$ ratio of 138 was chosen as the quantitation ion. Figure 3.9 displays the chromatographic peak for 3-isobutyl-2-methoxypyrazine (IBMP) and the different ions being considered for quantitation. From the calculated S/N ratios, the ion with the $m/z$ ratio of 124 was chosen as the quantitation ion.
Figure 3.6.1 is a chromatogram of the peak for 3-isopropyl-2-methoxypyrazine (IPMP) with a mass range of 50-200 m/z. Figure 3.6.2 is the ion with an m/z of 137 and Figure 3.6.3 is the ion with an m/z of 138.
Figure 3.7 Figure 3.7.1 is a chromatogram of the peak for 3-ethyl-2-ethoxypyrazine (EEP) with a mass range of 50-200 m/z. Figure 3.7.2 is the ion with an m/z of 123, Figure 3.7.3 is the ion with an m/z of 124, and Figure 3.7.4 is the ion with an m/z of 125.
Figure 3.8.1 is a chromatogram of the peak for 3-sec-butyl-2-ethoxypyrazine (SBMP) with a mass range of 50-200 m/z. Figure 3.8.2 is the ion with an m/z of 137, Figure 3.8.3 is the ion with an m/z of 138, and Figure 3.8.4 is the ion with an m/z of 139.
Figure 3.9 Figure 3.9.1 is a chromatogram of the peak for 3-isobutyl-2-methoxypyrazine (IBMP) with a mass range of 50-200 m/z. Figure 3.9.2 is the ion with an m/z of 123, Figure 3.9.3 is the ion with an m/z of 124, and Figure 3.9.4 is the ion with an m/z of 125.
Table 3.1 contains a summary of the mass spectrum parameters that were used for this research.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Segment Time</th>
<th>Quant. Ion</th>
<th>Mass Range</th>
<th>Mass Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPMP</td>
<td>19.2-19.6 min</td>
<td>137 m/z</td>
<td>50-160</td>
<td>136-138</td>
</tr>
<tr>
<td>EEP</td>
<td>20.5-20.8 min</td>
<td>124 m/z</td>
<td>50-160</td>
<td>123-125</td>
</tr>
<tr>
<td>SBMP</td>
<td>23.1-23.3 min</td>
<td>138 m/z</td>
<td>50-175</td>
<td>137-139</td>
</tr>
<tr>
<td>IBMP</td>
<td>23.5-23.8 min</td>
<td>124 m/z</td>
<td>50-175</td>
<td>123-125</td>
</tr>
</tbody>
</table>

Table 3.1 Mass spectrum parameters.

C. Selection of Internal Standards

Since 3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-2-methoxypyrazine (IPMP), and 3-sec-butyl-2-methoxypyrazine (SBMP) are the suspected methoxypyrazines present in the types of grapes to be investigated, two different internal standards were studied for quantitation. The first internal standard that was used was 3-ethyl-2-ethoxypyrazine (EEP). This was first studied due to the fact that the compound was inexpensive and readily available. The second internal standard that was studied was a deuterated form of IBMP, 3-isobutyl-[^2]H_2]-2-methoxypyrazine. A calibration curve, which ranged from 5 ng/L to 100 ng/L, was completed using both internal standards. The results obtained from using these calibration curves were then compared and since EEP closely mimicked how the other 3-alkyl-2-methoxypyrazines reacted to the changes in the parameters, this molecule was selected as the internal standard for the analysis of both the wine and grape juice.

Another problem with the 3-isobutyl-[^2]H_2]-2-methoxypyrazine is its co-elution with the unlabeled IBMP compound. It was extremely difficult to determine if the compound is actually present in the samples analyzed. Figure 3.10 displays a chromatogram of the model grape juice system that has been spiked with 100 ng/L of
IPMP, SBMP, IBMP, 60 ng/L of EEP (one of the internal standards being investigated) and 30 ng/L of 3-isobutyl-[\textsuperscript{2}H\textsubscript{2}]-2-methoxypyrazine (the second internal standard being investigated). Figure 3.11 displays an enlarged view of the boxed portion of Figure 3.10, showing how the labeled and unlabeled IBMP compounds overlap. Figure 3.12 displays a chromatogram spiked with 10 ng/L of IPMP, SBMP, IBMP, 60 ng/L of EEP, and 30 ng/L of 3-isobutyl-[\textsuperscript{2}H\textsubscript{2}]-2-methoxypyrazine. Figure 3.13 displays an enlarged view of the boxed portion of Figure 3.12, showing how the labeled IBMP and unlabeled IBMP chromatographic peaks overlap.

The calibration curve was generated using 3-isobutyl-[\textsuperscript{2}H\textsubscript{2}]-2-methoxypyrazine as the internal standard in the model wine system and the results are presented in Figure 3.14. A second calibration curve was completed in the model wine system using EEP as the internal standard and the results are presented in Figure 3.15. A calibration curve was also completed using the model grape juice system for each of the internal standards. Figure 3.16 shows the calibration curve generated using the model grape juice system and 3-isobutyl-[\textsuperscript{2}H\textsubscript{2}]-2-methoxypyrazine as the internal standard. Figure 3.17 displays the calibration curve that was generated using EEP as the internal standard in the model grape juice system.
Figure 3.10 A chromatogram showing 3-isopropyl-2-methoxypyrazine (IPMP), 3-sec-butyl-2-methoxypyrazine (SBMP), 3-isobutyl-2-methoxypyrazine (IBMP) at a concentration of 100 ng/L while the internal standards concentration was held at 60 ng/L for 3-ethyl-2-ethoxypyrazine (EEP) and 30 ng/L for 3-isobutyl-[2H2]-2-methoxypyrazine (d-IBMP).
Figure 3.11.1 is a close-up of the separation of Figure 3.10 showing 3-isobutyl-[2H2]-2-methoxypyrazine and IBMP. Figure 3.11.2 is the 124 m/z ion, the quantifying ion for IBMP. Figure 3.11.3 is the 126 m/z ion, the quantifying ion for 3-isobutyl-[2H2]-2-methoxypyrazine (d-IBMP).
Figure 3.12 A chromatogram showing 3-isopropyl-2-methoxypyrazine (IPMP), 3-sec-butyl-2-methoxypyrazine (SBMP), 3-isobutyl-2-methoxypyrazine (IBMP) at a concentration of 10 ng/L while the internal standards concentration was held at 60 ng/L for 3-ethyl-2-ethoxypyrazine (EEP) and 30 ng/L for 3-isobutyl-[²H₂]-2-methoxypyrazine (d-IBMP).
Figure 3.13.1 is a close-up of the separation of Figure 3.10 showing 3-isobutyl-[\(^2\)H\(_2\)]-2-methoxypyrazine and IBMP. Figure 3.13.2 is the 124 \(m/z\) ion, the quantifying ion for IBMP. Figure 3.13.3 is the 126 \(m/z\) ion, the quantifying ion for 3-isobutyl-[\(^2\)H\(_2\)]-2-methoxypyrazine (d-IBMP).
Figure 3.14 Calibration curve generated for the model wine investigating several pyrazines using 3-isobutyl-[2H2]-2-methoxypyrazine as the internal standard.
Figure 3.15 Calibration curve generated for the model wine matrix investigating several pyrazines using EEP as the internal standard.
Figure 3.16 Calibration curve generated for the model grape juice matrix investigating several pyrazines using 3-isobutyl-[\(2^2\)H2]-2-methoxypyrazine as the internal standard.
**Figure 3.17** Calibration curve generated for the model grape juice matrix investigating several pyrazines using EEP as the internal standard.
D. Preliminary Experiments

Preliminary analysis of each analyte was performed before the optimization process began. Each sample was incubated and agitated at a speed of 500 rotations per minute for 5 minutes, followed by a 30 minute extraction at 30°C at the previously mentioned agitation speed. The chromatographic conditions were as described in Chapter 2.

E. Sampling Parameters

a. Model Grape Juice

In order to study the various parameters to optimize the recovery of methoxypyrazines and to minimize effects that could not be accurately ensured and held constant, a model system was necessary. The model grape juice consisted of 6.0 g/L tartaric acid and Millipore water. The pH was then adjusted to 7 using NaOH. To ensure that the data that was collected was reliable, each of the parameters were investigated in triplicate.
i. **Extraction Temperature**

The temperature held during extraction plays a role in the amount of pyrazines that are recovered from the sample. Higher temperatures have been shown to increase the concentration of volatiles in the headspace.\textsuperscript{25} However, too high of a temperature may lead to the desired analytes being unable to find space on the fiber. To identify the optimum temperature that leads to the highest recovery of pyrazines, a series of samples were made at 30, 35, 40, 45, 50, and 55°C and are plotted in Figure 3.18. Each of these samples contained 30 ng/L of SBMP, IPMP, IBMP and 60 ng/L of EEP. A 60 ng/L concentration of EEP was used because a concentration level of 30 ng/L did not provide a sufficient signal. The optimum extraction temperature of 50°C was selected for the analysis of methoxypyrazines in grape juice. This temperature was lower than the literature value of 80°C that was used by Ryona and co-workers to study the concentration of IBMP in grape juice.\textsuperscript{25}

![Figure 3.18](image_url)

**Figure 3.18** The effect of varying extraction temperature on the recovery of selected methoxypyrazines with a 30 minute extraction time with a 0% sugar concentration and a pH of 7.
ii. Extraction Time

By varying the length of time that the fiber is exposed in the headspace, the optimum time that leads to the highest recovery of pyrazines may be determined. The length of extraction time will vary the extent of equilibrium reached on the fiber. To identify the length of time that would lead to the optimum extraction, samples were extracted for 20, 25, 30, 60, 120, and 180 minutes. These samples were spiked with 30 ng/L of SBMP, IBMP, IPMP and 60 ng/L of EEP. As the time length of the extraction increased, so did the recovery of the pyrazines. To conserve experimental time, an extraction time of 30 minutes was selected. Figure 3.19 illustrates the effect of extraction time on pyrazine recovery. This extraction time is consistent with the extraction time that was used in the literature.\textsuperscript{25}

![Figure 3.19 The effect of the length of extraction on the recovery of pyrazines at 30°C with 0% sugar concentration and a pH of 7.](image-url)
iii. pH

Throughout the harvest season the pH of the grape sample varies as the concentration of tartaric acid changes. At a low pH, methoxypyrazines tend to form nonvolatile quaternary ammonium ions. To determine the optimum pH that would lead to the highest recovery of methoxypyrazines, a series of samples were made using 30 ng/L of SBMP, IPMP, IBMP and 60 ng/L of EEP. These samples were adjusted to pH values of 2, 3, 4, 5, 6, and 7 to determine how the pH affects recovery. The role the pH of the sample played in recovery of the methoxypyrazines was identified and is presented in Figure 3.20. A pH of 7 was selected for the analysis of methoxypyrazines in grape juice.

![Figure 3.20](image)

**Figure 3.20** The effect of pH on the recovery of pyrazines at 30°C, an extraction time of 30 minutes and a sugar concentration of 0%.
iv. Sugar Concentration

Over the growing season the sugar concentration of the grape juice has also been observed to change. In order to create a method to determine the concentration of the methoxypyrazines in grape juice, one must also determine if the sugar concentration plays a role in the concentration of the methoxypyrazines recovered. To determine if sugar concentration plays a role in the recovery of methoxypyrazines, a series of sugar solutions were made using 0, 5, 7, 10, 15, and 20% glucose in Millipore water. These samples were then spiked with 30 ng/L of SBMP, IPMP, IBMP, and 60 ng/L of EEP. The concentration of sugar in the grape juice was not found to play a large role in the recovery of pyrazines, as illustrated in Figure 3.21. This is consistent with the literature when no adjustments are needed to be made to analyze the concentration of methoxypyrazines in grapes.25

![Figure 3.21](image)

**Figure 3.21** The effect of sugar concentration on the amount of pyrazines recovered at 30 °C, an extraction time of 30 minutes and a pH of 7.
b. Model Wine

In order to study the various parameters to optimize the recovery of methoxypyrazines and to minimize affects that could not be accurately ensured and held constant, a model system was necessary. The model wine consisted of 6.0 g/L tartaric acid, 10% (v/v) ethanol and Millipore water. The pH was then adjusted to 7 using NaOH and a pH meter.
i. Extraction Temperature

To ensure that the highest amount of analytes has been transferred to the fiber, the temperature of the extraction was investigated. To determine which temperature yields the highest transfer of methoxypyrazines from the headspace of the model wine sample to the fiber, a range of temperatures were investigated. The temperatures range investigated included extraction at 30, 35, 40, 45, 50, and 55°C. Samples were made using 30 ng/L of IPMP, SBMP, IBMP and 60 ng/L of EEP. Based on the results presented in Figure 3.22, an extraction temperature of 45°C was selected. This temperature was determined to be the same as the temperatures used in the literature.40

Figure 3.22 The effect of varying extraction temperatures on the recovery of pyrazines with a 30 minute extraction with a sample pH of 7 and an ethanol concentration of 10%.
ii. Extraction Time

During the development of a method, it is crucial to determine the optimum extraction length. An extraction length that allows for quickest equilibrium with the headspace and the highest recovery of methoxypyrazines is ideal. Six samples were made with 30 ng/L of SBMP, IPMP, and IBMP and 60 ng/L of the internal standard EEP. Six different time lengths were investigated: 20, 25, 30, 60, 120, 180 minutes and are presented in Figure 3.23, where the amount of pyrazines recovered increases with time. To ensure that one methoxypyrazine is not artificially biased, and that the experiments would be completed in a reasonable amount of time, an extraction length of 30 minutes was selected. This extraction time is consistent with the extraction time that is used in the literature.\textsuperscript{33}

![Effect of Extraction Length on Pyrazine Recovery in a Model Wine System](image)

**Figure 3.23** Effect of extraction time on the saturation of the DVB/CAR/PDMS fiber for highest extraction efficiency of analytes at 30°C at pH 7 and 10% ethanol concentration.
iii. pH

The pH of the sample plays an important role in the recovery of the analytes in a sample. In order to determine what the optimum pH for pyrazine recovery, samples were made at a pH of 2, 3, 4, 5, 6, and 7 using the model wine matrix. These samples were made with 30 ng/L of 3-sec-butyl-2-methoxypyrazine (SBMP), 3-isopropyl-2-methoxypyrazine (IPMP), and 3-isobutyl-2-methoxypyrazine (IBMP) and 60 ng/L of the internal standard 3-ethyl-2-ethoxypyrazine (EEP). As displayed in Figure 3.24, the pH of the sample played a role in the recovery of the pyrazines. A pH level of 7 was selected for the model wine and the commercial wines that were analyzed. This pH is consistent with the pH that is used in the literature.40

![Figure 3.24](image)

**Figure 3.24** The effect of pH on sample recovery with 3.0 grams NaCl at an extraction temperature of 30°C with an extraction time of 30 minutes at 10% ethanol concentration.
iv. Ethanol Dilution

The concentration of ethanol in a wine also affects the recovery of the desired analyte. In the case of methoxypyrazines, for example, these compounds are observed to remain in the ethanol as demonstrated by the decrease in the methoxypyrazine extraction as a function of increasing ethanol concentration. In order to determine at what ethanol concentration optimal extraction occurs, a series of model wine solutions were made with 5, 7, 10, 15, 20% ethanol concentrations. Each sample was then spiked with 30 ng/L SBMP, IPMP, IBMP and 60 ng/L of the internal standard of EEP and results are plotted in Figure 3.25. A 10% ethanol model wine was selected over other ethanol concentrations in order to not dilute the sample too much and render the suspected compounds undetectable. Literature reports results from wines with a slightly lower alcoholic content.40

**Figure 3.25** The effect of ethanol on the recovery of the pyrazines at 30°C with an extraction time of 30 minutes and a pH of 7 in the model wine system.
Chapter IV

Results and Discussion

A. Grape Juice

a. Validation of the Method

i. Linearity

A calibration curve was generated using a model grape juice system to determine
the linearity of the method. The pH of each sample was adjusted to 7 to ensure that the
maximum amount of methoxypyrazines were extracted from the grape juice. The grape
juice was then spiked with 60 ng/L of the internal standard, 3-ethyl-2-ethoxypyrazine,
and either 5, 10, 20, 30, 50, 70, or 100 ng/L of 3-sec-butyl-2-methoxypyrazine (SBMP),
3-isopropyl-2-methoxypyrazine (IPMP), and 3-isobutyl-2-methoxypyrazine (IBMP). The
volatiles in each sample were extracted for thirty minutes at 50°C. The results of the
calibration curves are plotted in Figure 4.1 along with the $R^2$ values for each fit.
Figure 4.1 A calibration curve generated for the model grape juice system investigating several pyrazines using EEP as the internal standard.
ii. Precision

Precision is defined as the ability to repeat or reproduce the same result. This is determined by calculating the relative standard deviation, R.S.D. The precision of the method was calculated by spiking eight 10 mL samples of the model grape juice solution with 20 ng/L or 50 ng/L of 3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-2-methoxypyrazine (IPMP), 3-sec-butyl-2-methoxypyrazine (SBMP), and 60 ng/L of 3-ethoxy-2-ethylpyrazine (EEP). These solutions were then extracted at 50°C for thirty minutes. The data for the precision using 20 ng/L is summarized in Table 4.1. The data presented in Table 4.2 summarizes the data for precision measurements using 50 ng/L. The R.S.D. is quite large for IBMP due to the inclusion of two trials that are well outside the other measurements. If these two values were removed, the R.S.D. drops to 11.7%, which is just outside an acceptable range of below 10%.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Model Grape Juice Precision (n=8) R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPMP</td>
<td>20 ng/L</td>
<td>7.8%</td>
</tr>
<tr>
<td>EEP</td>
<td>60 ng/L</td>
<td>4.9%</td>
</tr>
<tr>
<td>SBMP</td>
<td>20 ng/L</td>
<td>4.5%</td>
</tr>
<tr>
<td>IBMP</td>
<td>20 ng/L</td>
<td>20.5%</td>
</tr>
</tbody>
</table>

Table 4.1 The precision of the method using the model grape juice system at a low concentration of methoxypyrazines.
### Table 4.2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Model Grape Juice Precision (n=8) R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPMP</td>
<td>50 ng/L</td>
<td>1.1%</td>
</tr>
<tr>
<td>EEP</td>
<td>60 ng/L</td>
<td>2.5%</td>
</tr>
<tr>
<td>SBMP</td>
<td>50 ng/L</td>
<td>2.4%</td>
</tr>
<tr>
<td>IBMP</td>
<td>50 ng/L</td>
<td>8.8%</td>
</tr>
</tbody>
</table>

*Table 4.2* The precision of the method using the model grape juice system at a high concentration of methoxypyrazines.

### iii. Limit of Detection

The limit of detection (LOD) is defined as the lowest concentration of analyte that can be detected by the method. This concentration needs to generate a signal that is three times the level of the noise. At this concentration, the analyte can be detected in a reproducible manner and not mistaken for noise. Table 4.3 displays the limit of detection for the desired analytes using the described method. While these concentrations are low, a recent study by Kotseridis and co-workers reported the detection of methoxypyrazines at concentrations less than 0.5 ng/L.\(^{33}\)

### Table 4.3

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Optimized Method (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBMP</td>
<td>1.0</td>
</tr>
<tr>
<td>IPMP</td>
<td>1.0</td>
</tr>
<tr>
<td>IBMP</td>
<td>5.0</td>
</tr>
</tbody>
</table>

*Table 4.3* The limit of detection determined by the method.

### iv. Limit of Quantitation

The limit of quantitation (LOQ) is defined as the lowest amount of analyte that can be accurately measured with a signal that is 10 times higher than the noise. This is used to ensure that any error, within an acceptable range, does not have a dramatic effect.
on the quantitative results. Table 4.4 gives a summary of the limit of quantitation for the desired analytes in grape juice. This limit of quantitation is higher than the limit of quantitation by Ryona and co-workers, who was able to quantitate 3-isobutyl-2-methoxypyrazine to a concentration of 1 pg/L.26

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Optimized Method (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBMP</td>
<td>8.0</td>
</tr>
<tr>
<td>IPMP</td>
<td>8.0</td>
</tr>
<tr>
<td>IBMP</td>
<td>10.0</td>
</tr>
</tbody>
</table>

Table 4.4 The limit of quantitation for the desired compounds in grape juice.

b. Analysis of Grapes Harvested

Grapes were harvested once a week from July 7, 2009 to November 4, 2009 from the Ohio Agricultural Research and Development Center (OARDC), which is associated with Ohio State University, located in Kingsville, Ohio. Each weekly harvest consisted of collecting two hundred berries from each of the two types of grapes. One hundred berries were used to determine the sugar, titratable acid concentration, and the pH of the juice. The second group of one hundred berries was used to determine the concentration of methoxypyrazines in the grape juice. For one of the varieties of grape, two different rootstock systems were examined. The grapes that were studied include the Pinot Noir with the 3309 rootstock, Cab Franc with the 3309 rootstock, and Cab Franc with the 101-14 rootstock. To ensure that the entire vineyard is represented in the sample, and not just a select vine, the grapes were randomly selected along several adjacent vines alternating from one side to another. Once the grapes were collected, they were frozen at -80 °C to
ensure that the grapes would not ripen further and that the compounds of interest would not undergo any additional changes.

The amount of precipitation that a vineyard receives influences the growth of the grapes. Water provides essential nutrients to the grapes and for the vine to grow. Near the end of the growing season, when it is almost time to harvest the grapes, mild water stress is not detrimental as this allows for a faster accumulation of sugars.¹ The precipitation activity that occurred at the vineyard during the collection period was recorded and is displayed in Figure 4.2 for each week.

![Precipitation Activity During the Collection Period](image)

**Figure 4.2** Precipitation amounts were recorded at the vineyard where the grapes were harvested during the collection period.
i. Sugar Levels

The sugar level in grapes may serve as an indication of the ripening process. As stated in Chapter 1, a grape that has a sugar concentration of 20 Brix or higher is sufficiently ripe for harvesting. However, the concentration of methoxypyrazines is known to decrease as the grapes ripen.\textsuperscript{39} Grapes were harvested once a week from July 7, 2009 to November 4, 2009. These grapes were then crushed through cheesecloth and the juice was collected in a sealable jar. Verasion, the process in which the grapes change color, is an important time. This process occurred on August 11, 2009 in the Pinot Noir grapes. This same process occurred several weeks later on September 2, 2009 for both rootstocks of the Cab Franc grapes. The grapes were harvested weeks later, when the sugar concentration was high enough to support fermentation and the acid levels were low. The Pinot Noir grapes were harvested on October 14, 2009 and the Cab Franc grapes were harvested on November 4, 2009 to allow for the sugar concentration to increase.

Using a refractometer, the refractive indices for the grape juice from each harvest date was measured. A calibration curve was generated using sugar solutions of varying concentration. These sugar concentrations contained 0, 5, 10, 15, 20, 25, 30, and 35% sugar in Millipore water. One Brix is equivalent to 1% sugar in the solution. These refractive indices were plotted in Figure 4.3 against the percent concentration of sugar in the solution. A line of least squares was fit to the data to allow for the calculation of the sugar content.
Figure 4.3 The calibration curve that was used to determine the sugar content of grapes that were harvested.

Literature describes that as the grapes grow in size and ripen, the concentration of sugar will increase.\textsuperscript{40} A similar result was observed for grapes included in this study. In Figure 4.4, the measured sugar concentration for the Pinot Noir grapes is plotted versus the harvest date. Figure 4.5 displays the sugar concentration versus the harvest date for the Cab Franc grapes with the rootstock 101-14. Figure 4.6 displays the measured sugar concentration for the Cab Franc grapes with rootstock 101-14 during the collection period. As the grapes grow and mature, the weight of the berries also increases. Figure 4.7 displays the weight of 100 Pinot Noir berries that were collected throughout the growing season. Figure 4.8 displays the weight of 100 berries of the Cab Franc with rootstock 3309 with respect to the date that the grapes were harvested. Figure 4.9
displays the weight of 100 Cab Franc berries with rootstock 101-14 plotted versus the date that the berries were harvested. Figure 4.10 displays the weight per berry and the sugar concentration versus the date that the grapes were harvested for the Pinot Noir grapes. Figure 4.11 displays the weight per berry and the sugar concentration that was observed with respect to the harvest date for the Cab Franc grapes with rootstock 3309. Figure 4.12 is the weight in grams along with the observed sugar concentration for the Cab Franc grapes with rootstock 101-14 plotted against the date that the grapes were harvested. Figure 4.13 is the ratio of the Brix level to the weight per berry for the Pinot Noir grapes. The optimum time to harvest these grapes is when the Brix to weight ratio is high. While the ratio is highest earlier on in the season the grapes are too small to produce sufficient juice and contain an insufficient amount of sugar. Figure 4.14 is the ratio of Brix level to the weight per berry observed for the Cab Franc grapes with the rootstock 3309. Figure 4.15 is the ratio of Brix level to the weight per berry for the Cab Franc grapes with the rootstock 101-14. The two different rootstocks that were studied for the Cab Franc grapes did not vary to a large degree. The ratios for the Cab Franc grapes follow the pattern for the Pinot Noir, where the ratio is high initially but decreases as the berries grow in size and the sugar concentration increases.
Figure 4.4 The sugar concentration of Pinot Noir grapes with rootstock 3309 throughout the season.

Figure 4.5 The sugar concentration of Cab Franc grapes with rootstock 3309 throughout the season.
**Figure 4.6** The sugar concentration of Cab Franc grapes with rootstock 101-14 throughout the season.

**Figure 4.7** The weight of 100 berries of Pinot Noir grapes with rootstock 3309 throughout the growing season.
Figure 4.8 The weight of 100 berries of Cab Franc grapes with rootstock 3309 throughout the growing season.

Figure 4.9 The weight of 100 berries of Cab Franc grapes with rootstock 101-14 throughout the growing season.
**Figure 4.10** The weight per berry and sugar concentration of Pinot Noir grapes with rootstock 3309.

**Figure 4.11** The weight per berry and sugar concentration of Cab Franc grapes with rootstock 3309.
Figure 4.12 The weight per berry and sugar concentration of Cab Franc grapes with rootstock 101-14.

Figure 4.13 The Brix concentration to weight per berry ratio for the Pinot Noir grapes.
**Figure 4.14** The Brix concentration to weight per berry ratio for the Cab Franc grapes with rootstock 3309.

**Figure 4.15** The Brix concentration to weight per berry ratio for the Cab Franc grapes with rootstock 101-14.
ii. Total Titratable Acids

The concentration of total titratable acids is another variable that can be readily measured in grapes. The acid that varies in concentration during the ripening process is malic acid and is the primary reason why the titratable acids decrease over time.\textsuperscript{25} Due to the small amount of grape juice that was collected from the grapes harvested on July 7, 2009, the concentration of titratable acid could not be determined from this harvest date. Five milliliters of the grape juice were added to one hundred milliliters of water. The mixture was then titrated using 0.10 M NaOH to a pH of 8.2. A pH of 8.2 is the accepted endpoint by the American Society of Enologists, which is what was used in this study.\textsuperscript{41} In other countries, such as France for example, solutions are titrated to a pH of 7 where the reference acid is sulfuric acid. In the United States, the endpoint of titration occurs at a pH of 8.2 with tartaric acid as the reference acid.\textsuperscript{1} To calculate the amount of total titratable acid in the grape juice from the information gathered, the following equation was used\textsuperscript{41}:

\[
Concentration \ of \ Titratable \ Acid \ \frac{g}{100 \ mL} = \frac{(mL \ of \ NaOH \ used)(0.1)(75)(100)}{(1000)(mL \ of \ grape \ juice)}
\]

In Figure 4.16 the concentration of titratable acids is presented as a function of the harvest date for the Pinot Noir grapes with a rootstock of 3309. Figure 4.17 displays the concentration of titratable acids with respect to the harvest date for the Cab Franc grapes with the rootstock of 3309, while Figure 4.18 represents the concentration of titratable acids as a function of harvest date for the Cab Franc grapes with a rootstock of 101-14. The reason why the titratable acids is closely monitored is because high levels of malic acid may lead to problems in the fermentation process. As malic acid is converted to lactic acid, the acidic taste of the wine decreases. Malic acid plays a role in how some
wine is spoiled after bottling as biogenic amines are produced by spoilage organisms. Figure 4.19 displays the observed titratable acids level and the weight per berry of Pinot Noir grapes with respect to the date that the grapes were collected. Figure 4.20 displays the weight per berry in grams and the observed titratable acids level in the Cab Franc grapes with rootstock 3309. Figure 4.21 displays the concentration of titratable acids that were observed for Cab Franc grapes with rootstock 101-14 and the weight per berry with respect to the harvest date. Figure 4.22 displays the ratio of titratable acid to berry weight for the Pinot Noir grapes. Figure 4.23 displays the ratio of titratable acid to berry weight for the Cab Franc grapes with rootstock 3309. Figure 4.24 displays the ratio of titratable acid to berry weight for the Cab Franc grapes with rootstock 101-14. Figure 4.25 displays the ratio of Brix to titratable acid for the Pinot Noir grapes. The ratio of Brix to titratable acid for the Cab Franc grapes with the rootstock 3309 is displayed in Figure 4.26. Figure 4.27 displays the ratio of Brix to titratable acid for the Cab Franc grapes with rootstock 101-14. Only minor differences between the two different rootstocks that were used in this research study were observed.
**Figure 4.16** The change in titratable acid levels in Pinot Noir 3309 grapes through the growing season.

**Figure 4.17** The change in titratable acid levels in Cab Franc 3309 grapes through the growing season.
**Figure 4.18** The change in titratable acid levels in Cab Franc 101-14 grapes through the growing season.

**Figure 4.19** The observed level of titratable acids and the weight per berry of Pinot Noir grapes with respect to the sampling date.
Figure 4.20 The observed level of titratable acids and the weight per berry of Cab Franc grapes with rootstock 3309 with respect to the sampling date.

Figure 4.21 The observed level of titratable acids and the weight per berry of Cab Franc grapes with rootstock 101-14 with respect to the sampling date.
Figure 4.22 The ratio of titratable acid per weight per berry for the Pinot Noir grapes.

Figure 4.23 The ratio of titratable acid per weight per berry for the Cab Franc grapes with rootstock 3309.
Figure 4.24 The ratio of titratable acid per weight per berry for the Cab Franc grapes with rootstock 101-14.

Figure 4.25 The ratio of Brix to titratable acid for the Pinot Noir grapes.
Figure 4.26 The ratio of Brix to titratable acid for the Cab Franc grapes with rootstock 3309.

Figure 4.27 The ratio of Brix to titratable acid for the Cab Franc grapes with rootstock 101-14.
iii. pH of Sample

The pH of grape juice is known to change over time as the grapes ripen. As the grapes become more mature, the concentration of titratable acid in the grapes decreases leading to an increase in pH. Figure 4.28 shows the pH measured from the grape juice collected from Pinot Noir grapes with the rootstock 3309. The pH of the Cab Franc 3309 grape juice as a function of time is presented in Figure 4.29. The pH measured from grape juice that was collected from Cab Franc 101-14 grapes is presented in Figure 4.30. No pH measurements were available from harvest date July 7, 2009 due to the insufficient volume of juice that was collected. pH measurements were collected from the grape juice over the growing period, and increased, which is consistent with the literature. Titratable acids have different strengths and since the concentration of malic acid decreases over the growing season, the pH of the juice is affected.\(^1\) The pH measurements for both of the rootstocks only differed by slight fluctuations.
**Figure 4.28** The pH of grape juice collected from Pinot Noir grapes with rootstock 3309 over the growing season.

**Figure 4.29** The pH of grape juice collected from Cab Franc grapes with rootstock 3309 over the growing season.
Figure 4.30 The pH of grape juice collected from Cab Franc grapes with rootstock 101-14 over the growing season.

iv. Concentration of Methoxypyrazines

Throughout the growing season, several different measurable variables change for the grape. As was described earlier, as the grapes ripen, the sugar concentration and pH both increase as the amount of tartaric acid decreases. A similar trend for methoxypyrazines is observed. As the grape becomes more mature, the concentration of the methoxypyrazines decreases until the methoxypyrazines are no longer detected. To determine the concentration of the methoxypyrazines in the grape juice, 5 mL of the juice was added to a 20 mL vial with 3 grams of NaCl and 5 mL of 0.1 M EDTA/NaOH with a pH of 7.5. The change in the concentration of methoxypyrazines for the varieties of grapes collected are presented in Figure 4.31 as a function of time. Table 4.5 displays the measured concentrations of Pinot Noir grapes.
The change in the concentration of methoxypyrazines for Cab Franc grapes with a rootstock 3309 is shown in Figure 4.32. Table 4.6 displays the methoxypyrazine concentration for the Cab Franc 3309 grapes for each collection date. Cab Franc grapes with the rootstock 101-14 were also studied and the change in concentration of methoxypyrazines in these grapes is presented in Figure 4.33. Table 4.7 contains the concentration of methoxypyrazines that were detected in the Cab Franc 101-14 grapes. Grapes from harvest date July 7, 2009 were not included in the analysis as insufficient juice was available for collection. The measured concentration of methoxypyrazines were found to vary throughout the season, and decrease overall, as the grapes ripen. Methoxypyrazines break down from exposure to sunlight and throughout the ripening process. Ryona and coworkers also reported long term changes and the overall decrease in 3-isobutyl-2-methoxypyrazine concentrations during the growing season. The most concentrated methoxypyrazine that was observed in this research was 3-isobutyl-2-methoxypyrazine, IBMP. Hashizume also observed the high concentration of IBMP in his studies.

3-isopropyl-2-methoxypyrazine, which was one of the methoxypyrazines investigated in this study, was measured to be almost absent from the sampled grapes. Extreme variations in the concentration of some methoxypyrazines were measured, including IPMP, and could be due to secondary shoots where some grapes only start growing late in the season and do not mature fast enough to go through veraison. These grapes were not removed from the vines to avoid collection until near the end of the growing season.
While the pH, titratable acids, and sugar concentrations observed between the two rootstocks of the Cab Franc grapes were very similar, the measured methoxypyrazine concentration were somewhat different. For the major methoxypyrazine, the IBMP, the rootstock 101-14 grapes started at a low concentration and continued to increase in concentration over several weeks. The rootstock 3309 started at a higher concentration before decreasing slightly and then increasing in concentration for several weeks. The highest concentration of IBMP was observed in the rootstock 3309, and after this peak was reached, the concentration continued to decrease until it could no longer be detected. After the highest concentration of IBMP was observed in rootstock 101-14, the concentration decreased a few weeks before a spike in the concentration of IBMP was observed. The IBMP concentration then decreases almost completely until the end of the growing season when a brief increase is observed before the concentration decreases to the point where it is no longer detected using the developed method.
Figure 4.31 The concentration of methoxypyrazines observed in 5 mL of Pinot Noir grape juice throughout the collection period.
<table>
<thead>
<tr>
<th>Harvest Date</th>
<th>IPMP</th>
<th>SBMP</th>
<th>IBMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>7/7/2009</td>
<td>Not enough juice collected</td>
<td>Not enough juice collected</td>
<td>Not enough juice collected</td>
</tr>
<tr>
<td>7/14/2009</td>
<td>18.1 ± 1.8</td>
<td>Detected</td>
<td>Detected</td>
</tr>
<tr>
<td>7/21/2009</td>
<td>8.37 ± 0.4</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7/28/2009</td>
<td>13.18 ± 1.0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>8/4/2009</td>
<td>14.01 ± 0.5</td>
<td>ND</td>
<td>Detected</td>
</tr>
<tr>
<td>8/11/2009</td>
<td>13.14 ± 4.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>8/18/2009</td>
<td>Detected</td>
<td>Detected</td>
<td>7.08 ± 2.1</td>
</tr>
<tr>
<td>8/26/2009</td>
<td>13.76 ± 2.3</td>
<td>Detected</td>
<td>17.86 ± 2.1</td>
</tr>
<tr>
<td>9/2/2009</td>
<td>Detected</td>
<td>Detected</td>
<td>10.42 ± 3.5</td>
</tr>
<tr>
<td>9/9/2009</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>9/16/2009</td>
<td>ND</td>
<td>ND</td>
<td>24.74 ± 1.3</td>
</tr>
<tr>
<td>9/23/2009</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>9/30/2009</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>10/7/2009</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>10/14/2009</td>
<td>Detected</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>10/21/2009</td>
<td>16.62 ± 2.6</td>
<td>Detected</td>
<td>13.58 ± 2.4</td>
</tr>
<tr>
<td>10/28/2009</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>11/4/2009</td>
<td>Detected</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Table 4.5* The concentration of methoxypyrazines in ng/L for a 5 mL sample from grape juice collected from Pinot Noir 3309 grapes throughout the growing season.
Figure 4.32 The concentration of methoxypyrazines observed in 5 mL of Cab Franc 3309 grape juice throughout the collection period.
<table>
<thead>
<tr>
<th>Harvest Date</th>
<th>IPMP</th>
<th>SBMP</th>
<th>IPMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>7/7/2009</td>
<td>Not enough juice collected</td>
<td>Not enough juice collected</td>
<td>Not enough juice collected</td>
</tr>
<tr>
<td>7/14/2009</td>
<td>83.6 ± 1.4</td>
<td>31.53 ± 0.6</td>
<td>97.3 ± 6.7</td>
</tr>
<tr>
<td>7/21/2009</td>
<td>Detected</td>
<td>ND</td>
<td>53.0 ± 1.9</td>
</tr>
<tr>
<td>7/28/2009</td>
<td>11.06 ± 0.9</td>
<td>ND</td>
<td>56.9 ± 3.6</td>
</tr>
<tr>
<td>8/4/2009</td>
<td>Detected</td>
<td>Detected</td>
<td>91.7 ± 6.1</td>
</tr>
<tr>
<td>8/11/2009</td>
<td>Detected</td>
<td>ND</td>
<td>187.6 ± 9.1</td>
</tr>
<tr>
<td>8/18/2009</td>
<td>Detected</td>
<td>ND</td>
<td>163.1 ± 17.8</td>
</tr>
<tr>
<td>8/26/2009</td>
<td>ND</td>
<td>ND</td>
<td>90.12 ± 7.0</td>
</tr>
<tr>
<td>9/2/2009</td>
<td>Detected</td>
<td>ND</td>
<td>63.04 ± 9.8</td>
</tr>
<tr>
<td>9/9/2009</td>
<td>ND</td>
<td>ND</td>
<td>58.55 ± 2.9</td>
</tr>
<tr>
<td>9/16/2009</td>
<td>ND</td>
<td>ND</td>
<td>46.73 ± 4.6</td>
</tr>
<tr>
<td>9/23/2009</td>
<td>ND</td>
<td>ND</td>
<td>25.71 ± 2.6</td>
</tr>
<tr>
<td>9/30/2009</td>
<td>ND</td>
<td>ND</td>
<td>Detected</td>
</tr>
<tr>
<td>10/7/2009</td>
<td>ND</td>
<td>ND</td>
<td>Detected</td>
</tr>
<tr>
<td>10/14/2009</td>
<td>ND</td>
<td>ND</td>
<td>Detected</td>
</tr>
<tr>
<td>10/21/2009</td>
<td>ND</td>
<td>ND</td>
<td>Detected</td>
</tr>
<tr>
<td>10/28/2009</td>
<td>Detected</td>
<td>ND</td>
<td>Detected</td>
</tr>
<tr>
<td>11/4/2009</td>
<td>ND</td>
<td>ND</td>
<td>Detected</td>
</tr>
</tbody>
</table>

**Table 4.6** The concentration of methoxypyrazines in ng/L for a 5 mL sample from grape juice collected from Cab Franc 3309 grapes throughout the growing season.
Figure 4.33 The concentration of methoxypyrazines observed in 5 mL of Cab Franc 101-14 grape juice throughout the collection period.
<table>
<thead>
<tr>
<th>Harvest Date</th>
<th>IPMP</th>
<th>SBMP</th>
<th>IBMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>7/7/2009</td>
<td>Not enough juice collected</td>
<td>Not enough juice collected</td>
<td>Not enough juice collected</td>
</tr>
<tr>
<td>7/14/2009</td>
<td>Detected</td>
<td>Detected</td>
<td>19.3 ± 0.9</td>
</tr>
<tr>
<td>7/21/2009</td>
<td>Detected</td>
<td>ND</td>
<td>46.7 ± 0.8</td>
</tr>
<tr>
<td>7/28/2009</td>
<td>Detected</td>
<td>ND</td>
<td>42.8 ± 2.9</td>
</tr>
<tr>
<td>8/4/2009</td>
<td>Detected</td>
<td>ND</td>
<td>77.2 ± 4.3</td>
</tr>
<tr>
<td>8/11/2009</td>
<td>Detected</td>
<td>ND</td>
<td>124.5 ± 10.4</td>
</tr>
<tr>
<td>8/18/2009</td>
<td>Detected</td>
<td>ND</td>
<td>96.84 ± 7.3</td>
</tr>
<tr>
<td>8/26/2009</td>
<td>Detected</td>
<td>ND</td>
<td>84.29 ± 7.7</td>
</tr>
<tr>
<td>9/2/2009</td>
<td>35.77 ± 10.4</td>
<td>15.54 ± 2.1</td>
<td>115.46 ± 14.2</td>
</tr>
<tr>
<td>9/9/2009</td>
<td>ND</td>
<td>ND</td>
<td>47.41 ± 2.5</td>
</tr>
<tr>
<td>9/16/2009</td>
<td>ND</td>
<td>ND</td>
<td>33.13 ± 3.1</td>
</tr>
<tr>
<td>9/23/2009</td>
<td>ND</td>
<td>ND</td>
<td>11.88 ± 5.0</td>
</tr>
<tr>
<td>9/30/2009</td>
<td>ND</td>
<td>ND</td>
<td>Detected</td>
</tr>
<tr>
<td>10/7/2009</td>
<td>ND</td>
<td>ND</td>
<td>Detected</td>
</tr>
<tr>
<td>10/14/2009</td>
<td>11.88 ± 2.0</td>
<td>Detected</td>
<td>19.66 ± 1.4</td>
</tr>
<tr>
<td>10/21/2009</td>
<td>ND</td>
<td>ND</td>
<td>Detected</td>
</tr>
<tr>
<td>10/28/2009</td>
<td>ND</td>
<td>ND</td>
<td>Detected</td>
</tr>
<tr>
<td>11/4/2009</td>
<td>Detected</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Table 4.7* The concentration of methoxypyrazines in the grapes in ng/L for a 5 mL sample with respect to their harvest date for Cab Franc 101-14 grapes.
B. Wines

a. Validation of the Method

i. Linearity

A calibration curve was created with a red wine, in this case Jacob’s Creek Shiraz was used, to determine the linearity of the method. Using the Ebuliometer, the ethanol concentration of the wine was determined to be 14.2%. The wine was then diluted to a concentration of 10% ethanol, based on the results from preliminary experiments. The pH was adjusted to 7 to ensure that the maximum amount of methoxypyrazines was extracted from each sample. The wine was then spiked with 60 ng/L of the internal standard, 3-ethyl-2-ethoxypyrazine, and either 5, 10, 20, 30, 50, 70, or 100 ng/L of 3-sec-butyl-2-methoxypyrazine (SBMP), 3-isopropyl-2-methoxypyrazine (IPMP), and 3-isobutyl-2-methoxypyrazine (IBMP). Each sample was extracted for thirty minutes at 45°C. The results of the calibration curves are presented in Figure 4.34 along with the $R^2$ values for the lines. Figure 4.25 displays the calibration curves that were generated for each of the methoxypyrazines to demonstrate the linearity of the method.
Figure 4.34 Calibration curves generated for the methoxypyrazines completed in a model wine sytem.
ii. Precision

Precision is defined as the ability to repeat or reproduce the same result. This is determined by calculating the relative standard deviation, R.S.D.. The precision of the method was calculated by spiking eight 10 mL samples of the model wine solution with 20 ng/L or 50 ng/L of 3-isobutyl-2-methoxypyrazine (IBMP), 3-isopropyl-2-methoxypyrazine (IPMP), 3-sec-butyl-2-methoxypyrazine (SBMP), and 60 ng/L of 3-ethoxy-2-ethylpyrazine (EEP). These solutions were then extracted at 45°C for thirty minutes. The data for the precision using 20 ng/L is summarized in Table 4.8. The data for the precision of the method using 50 ng/L is summarized in Table 4.9. The only compound that is outside the acceptable range is the internal standard EEP in both sets and IPMP in 50 ng/L. For the compound IPMP, there was one value that was well outside the range of the other values and if this value was removed, the R.S.D. would drop to 8.4%. For the internal standard EEP, there were three values for each set that were well outside the range of the other values. If these values were not included in the determination, the R.S.D. would drop to 9.2% and 9.6% for the two trials. The EEP concentration was observed to randomly vary, which then questions whether EEP is an acceptable internal standard.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Model Wine Precision (n=8) R.S.D. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPMP</td>
<td>20 ng/L</td>
<td>9.5</td>
</tr>
<tr>
<td>EEP</td>
<td>60 ng/L</td>
<td>31.6</td>
</tr>
<tr>
<td>SBMP</td>
<td>20 ng/L</td>
<td>6.1</td>
</tr>
<tr>
<td>IBMP</td>
<td>20 ng/L</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Table 4.8 The precision of the method using the model wine system at a low concentration of methoxypyrazines.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Model Wine Precision (n=8) R.S.D. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPMP</td>
<td>50 ng/L</td>
<td>12.4</td>
</tr>
<tr>
<td>EEP</td>
<td>60 ng/L</td>
<td>40.0</td>
</tr>
<tr>
<td>SBMP</td>
<td>50 ng/L</td>
<td>7.6</td>
</tr>
<tr>
<td>IBMP</td>
<td>50 ng/L</td>
<td>8.5</td>
</tr>
</tbody>
</table>

*Table 4.9* The precision of the method using the model wine system at a high concentration of methoxypyrazines.

### iii. Limit of Detection

The limit of detection (LOD) is defined as the lowest concentration of analyte that can be detected by the method, where the measured concentration needs to generate a signal that is three times the level of noise. At this concentration the analyte is reproducible and not mistaken for noise. The limit of detection was completed in a red wine matrix in order to accurately determine the affect of the matrix on the detection of these analytes. *Table 4.10* is the limit of detection for the desired analytes using the described method. These limits of detection are quite high considering that the olfactory threshold is close to 1 ng/L. Other research have been successful in detecting these compounds at 0.3 ng/L in wine samples.\(^{43}\)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBMP</td>
<td>20.0 ng/L</td>
</tr>
<tr>
<td>IPMP</td>
<td>50.0 ng/L</td>
</tr>
<tr>
<td>IBMP</td>
<td>20.0 ng/L</td>
</tr>
</tbody>
</table>

*Table 4.10* The limit of detection of the desired analytes in wine.
iv. Limit of Quantitation

The limit of quantitation (LOQ) is defined as the lowest amount of analyte that can be accurately measured with a signal that is 10 times higher than that of the noise. This is used to ensure that any error, within an acceptable range, does not have a dramatic effect on the quantitative results. The limit of quantitation was completed in the same red wine that was used for the calibration curve to accurately determine at what level the methoxypyrazines may be quantitated. Table 4.11 gives a summary of the limit of quantitation for the investigated analytes in wines. The level of quantitation is extremely high when considering other studies using SPME have quantified methoxypyrazines at a range of 33 to 72 ng/L.23

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBMP</td>
<td>50.0 ng/L</td>
</tr>
<tr>
<td>IPMP</td>
<td>130.0 ng/L</td>
</tr>
<tr>
<td>IBMP</td>
<td>150.0 ng/L</td>
</tr>
</tbody>
</table>

Table 4.11 The limit of quantitation of the desired analytes in wine.

b. Analysis of Commercial Wines

To determine the concentration of methoxypyrazines in real wine, a calibration curve was generated for a red wine. Three different commercial wines, which were believed to be contaminated with at least one of the methoxypyrazines, were tested using the proposed optimized and validated method. The preparation of the wine samples is described in Chapter 2 C where each wine was examined in triplicate to ensure that the results were reproducible.
Presented in Figure 4.35 is the calibration curve used to calculate the concentrations of the methoxypyrazines in wine. A summary of the measured results is displayed in Table 4.12. As the table shows, 3-isopropyl-2-methoxypyrazine (IPMP) was not detected in any of the wines. However, two of the wines tested contained 3-sec-butyl-2-methoxypyrazine (SBMP). One wine, the Markko Riesling, had a measured concentration of SBMP that was outside the range for which the calibration curve was generated. The Barefoot Sauvignon Blanc was determined to have detectable levels SBMP. Two of the tested wines, Barefoot Sauvignon Blanc and Livingston Cabernet Sauvignon, contained 3-isobutyl-2-methoxypyrazine (IBMP) at detectable levels.

<table>
<thead>
<tr>
<th></th>
<th>IPMP</th>
<th>SBMP</th>
<th>IBMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>2009 Markko Riesling</td>
<td>ND</td>
<td>Detected $^a$</td>
<td>ND</td>
</tr>
<tr>
<td>Barefoot Sauvignon Blanc</td>
<td>ND</td>
<td>59.03 ± 6.04</td>
<td>Detected</td>
</tr>
<tr>
<td>Livingston Cabernet Sauvignon</td>
<td>ND</td>
<td>ND</td>
<td>Detected</td>
</tr>
</tbody>
</table>

Table 4.12 Results of the analysis of the commercial wines using the optimized and validated method.

$^a$ This compound was detected at concentrations beyond the calibration curve.
Figure 4.35 A calibration curve completed using a Jacob’s Creek Shiraz red wine solution and EEP as the internal standard to determine the concentration of pyrazines present in commercial wine samples.
Chapter V

Conclusion

A method for the determination of methoxypyrazines in wine and grape juice at ultra trace amounts using solid-phase microextraction (SPME) coupled with gas chromatography-mass spectrometry (GC-MS) was developed and validated. The method underwent an optimization process to increase the limit of detection and limit of quantitation of the method. The two processes that had the greatest influence on the recovery of methoxypyrazines were sample preparation and the extraction procedure. The sample preparation parameters, which gave the highest analyte response, were an ethanol dilution of 10 % v/v with 3.0g of NaCl to 10 mL of wine (final volume) with an adjusted pH of 7. The extraction procedure that yielded the highest sensitivity of analytes was an extraction of 30 minutes at 45°C for wines and 50°C for grape juice.

The applicability of the optimized and validated method was demonstrated by the analysis of grape juice samples and commercial wine samples. The results of the analysis of grape juice show that the method is reliable and efficient for determining the concentrations of methoxypyrazines at extremely low concentrations before the grapes are used for winemaking to avoid the possibility of contamination. The method may also be used for the analysis of random samples of wine to determine if the concentration of methoxypyrazines has reached a level that may adversely affect the wine.

The linearity of the proposed method was tested in synthetic and red wine along with a synthetic grape juice solution and was found to show acceptable values. The chromatographic resolution for 3-isopropyl-2-methoxypyrazine (IPMP), 3-ethyl-2-ethoxypyrazine (EEP), 3-sec-butyl-2-methoxypyrazine (SBMP), and 3-isobutyl-2-
methoxypyrazine (IBMP) was excellent and did not require the mass spectrometer to increase the resolution between peaks. The largest R.S.D. value was 11.7% following the removal of excessively variant measurements. The limit of detection for these compounds in grape juice was near the detection limit of these compounds in wine. However, the limit of detection and quantitation of the methoxypyrazines in wine was above the olfactory thresholds.
Chapter VI

Future Work

Based upon the results of this research using solid-phase microextraction coupled with GC-MS, other avenues of research have been identified and include altering fiber type, varying sample matrices, examining in more detail the dependence on rootstocks as well as identifying other internal standards. This research utilized a DVB/CAR/PDMS fiber for the analysis of methoxypyrazines and while the fiber worked well when detecting these compounds in grape juice, the limit of detection in real wines was higher than olfactory threshold for these compounds. A recent study found that the CAR/PDMS fiber showed better linearity and variability. The CAR/PDMS fiber also had high recoveries of methoxypyrazines which led to the limit of detection of these compounds being lower than the olfactory threshold.44

The methoxypyrazines investigated in this study were found in grapes that are used for both red and white wines. To accurately determine if any of the methoxypyrazines are present, a suitable calibration curve must be generated and should be expanded to also include both wine matrices. A red and white wine calibration could expose any important differences in the detection and quantitation of methoxypyrazines.

This research examined several variables where one was systematically varied to determine the optimum conditions that would lead to the highest recovery of methoxypyrazines. A future project could examine which parameters are linked and to what extent they are dependent on each other. This could then help determine the parameters which would give the highest recovery of methoxypyrazines.
The internal standard that was used in this research, 3-ethyl-2-ethoxypyrazine, was determined to have a large R.S.D. value in a model wine system. Additional research is required to identify more suitable internal standards that are readily detectable, do not co-elute with any of the analytes being studied, or interact with the system.
References:


