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Development of a Method for the Detection and Quantification of 2-Methoxy-3-Isobutylpyrazine in Grape Juice and Wine

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DEVELOPMENT OF A METHOD FOR THE DETECTION AND QUANTIFICATION OF 2-METHOXY-3-ISOBUTYLPYRAZINE IN GRAPE JUICE AND WINE

By

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ABSTRACT

DEVELOPMENT OF A METHOD FOR THE DETECTION AND QUANTIFICATION OF 2-METHOXY-3-ISOBUTYLPYRAZINE IN GRAPE JUICE AND WINE

By

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The expanded utilization of "Bordeaux" red wine cultivars in the cool climate of the Great Lakes Region is limited by the persistence of a group of naturally occurring herbaceous, bell-pepper aroma and flavor compounds in the wine. This group is collectively called methoxypyrazines (MP). The specific compound 2-methoxy-3-isobutylpyrazine (IBMP) is the MP in greatest quantity and human perception threshold is at levels below 20 ng/L (20 parts/trillion). Viticultural and enological efforts to reduce the IBMP to below human perception levels have been hindered by the inadequacies of present methods available to identify and quantify them in complex juice and wine matrices. This thesis concerns a revised assay method employing techniques of head-space solid phase microextration coupled with stable isotope dilution gas-chromatography-mass-spectrometry, following a revised vapor extraction method and purification on a strong acid cation exchange resin column. The method detects IBMP at typical concentrations in both grape juice and wine.

This thesis is dedicated to my mother and father, for whom I have looked to for guidance and wisdom my entire life. I want to thank my loving wife for her unconditional love and support.

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I appreciate the moral support and friendship of Jen Dwyer, Jon Treloar, Marcel Lenz, Kacey Watts, Leah Clearwater, and Bill Nail. These colleagues and friends made life as a graduate student enjoyable and most of all, memorable.

Lastly, I want to acknowledge the long hours of discussion and laboratory time I was accompanied by my father. Because of him, I am a better scientist, and a better person.

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Introduction

Michigan has 13,500 acres of grapes (1,500 of which are devoted to wine grapes), and ranks fourth in the nation for total acres (Michigan Grape and Wine Industry Council, 2002). More than 25 wineries in the state of Michigan produce approximately 200,000 cases annually; the vast majority of these are produced from Michigan grown grapes (Michigan Grape and Wine Industry Council, 2002). For Michigan and other cool climate wine producing regions, competition with the premium viticultural and enological areas of the world such as Australia, California, Chile, France, Germany and Italy, requires that sound wines must be produced that meet the critical standards of the wine consuming public at a competitive price per bottle.

One key element for cool climate areas competitiveness is the production of Bordeaux-style wines from the grape cultivars Cabernet Sauvignon, Cabernet Franc, Merlot, and Sauvignon Blanc. The most significant factor limiting acceptance of the red wine cultivars is the presence of vegetative aromas and flavors in these wines when the grapes are cultured in cool climate regions. A particular group of compounds, 2-methoxy-alkylpyrazines (MP), are responsible for the vegetative and herbaceous aromas (like green bell-peppers) found in some wines produced from the above cultivars in cool climates. 2-Methoxy-3-isobutylpyrazine is the major contributing component of this group of compounds.

The research described in this thesis was designed with the major goal to develop a method of detection and quantification for 2-methoxy-3-isobutylpyrazine that was readily reproducible in a timely fashion and to be applicable to grape juice and wine

samples at any time during the growing season and the fermentation and post-fermentation processing periods for wine.

Literature Review

Buttery, *et al.* (1969) determined that the aromatic, grassy, herbaceous, smelling compound in green bell-peppers (*Capcicum annuum*) was 2-methoxy-3-isobutylpyrazine (IBMP). IBMP is found in a wide variety of other fruits and vegetables including, peas, tobacco, coffee, potatoes, and grapes (Czerny, *et al.*, 2000; Maga, *et al.*, 1973; Murray, *et al.*, 1975). IBMP is a strongly odorous compound with a lower human perception threshold of about 2 parts per trillion (2 ng/L), in water (Buttery, *et al.*, 1969; Seifert, *et al.*, 1970). Bayonove, *et al.* (1975) was the first to detect IBMP in Cabernet Sauvignon (*Vitis vinifera L.)*, suggesting the compound gave the wine its vegetative, green bell-pepper aromas.

IBMP can also be found in other *vinifera* grape cultivars: Cabernet Franc, Merlot, and Sauvignon blanc (Augustyn, *et al.*, 1982; Heymann, *et al.*, 1986; Harris, *et al.*, 1987; Calo, *et al.*, 1991; Allen, *et al.*, 1991, 1993, 1994; Lacey, *et al.*, 1991). Typical herbaceous varietal characters of wines derived from these cultivars have been attributed to the presence of very low levels of IBMP (Harris, *et al.*, 1987). Initial efforts to detect and quantify IBMP in grapes employing the GC-MS proved unsuccessful (Slingsby, *et al.*, 1980) and Allen, *et al.* (1994) suggested that improvement of the selectivity of isolation and analysis was important.

It has been reported that IBMP concentrations in grapes decline during ripening and that this phenomenon is linked to weather, the vine's canopy status and yield, and the vineyard management practices affecting the sun exposure of grape clusters (Lacey, et *al.*, 1991; Allen, *et al.* 1996; Roujou de Boubée, *et al.* 2000). Roujou de Boubée, *et al.*

(2000) reported that, under comparable climactic conditions in Bordeaux vineyards, the methoxypyrazine content of the grapes at veraison and variations during ripening are strongly influenced by environmental and cultural conditions including soil type, pruning and training system, density of plantation and crop load.

The extremely low detection threshold of IBMP requires specialized analytical isolation techniques to enable detection of the compound. Such low levels are below the detection abilities of high performance liquid chromatography (HPLC) (Heymann, *et al.*, 1986) and require the employment of gas chromatography-mass spectrometry (GC-MS) (Allen, *et al.*, 1994).

Detection and Quantification

Several analytical detection and quantification methods have been developed specifically for methoxypyrazines and most have been inadequate to achieve rapid and repeated analysis with great accuracy. The methods developed have mainly been in one of two forms: a) liquid extraction; or b) solid phase microextraction (SPME). Both methods are coupled to GC-MS. Liquid extraction can be a very tedious and time consuming procedure requiring multiple steps employing solvents to extract a target compound from an initial matrix such as wine, into the solvent. SPME (Pawliszyn, 1997; De Fatima Alpendurada, 2000) is a simple and effective adsorption and desorption technique, which eliminates not only the need for solvents and complicated sample preparations, but also the laborious techniques for concentrating and analyzing for volatile or non-volatile compounds in liquid samples or headspace (De Fatima Alpendurada, 2000).

SPME fibers consist of various types of polymer coatings fused to a silica fiber, which can adsorb analytes from a sample by immersion or headspace extraction (Supelco, 2003).

Headspace solid phase microextraction (HS-SPME) analysis, coupled with a GC-MS, lends itself well to analysis of volatile compounds in any number of different types of matrices, including wine. HS-SPME has been employed for extraction and quantification of grape terpenoids (De la Calle Garcia, *et al.* 1998), and the presence of the soil fumigant methyl isocyanate (Gandini, *et al.* 1997), and for the analysis of diacetyl in wine (Hayasaka, *et al.* 1999). The method was also used by American Water Works Association (AWWA) to analyze for methyl-isoborneol (MIB) and geosmin, reported as being responsible for the "musty" odor occurring in drinking water at particular times of the year.

HS-SPME was used with IBMP as an internal standard with the ability to quantify MIB and geosmin down to a concentration of a few parts per trillion (AWWA method 6040). The AWWA method of detection for IBMP and other compounds in the parts-per-trillion range is further reviewed in Volume 19.1 of The Reporter, published by Sigma-Aldrich in conjunction with Supelco. Hartmann, *et al.* (2002) recently conducted a study for the detection and quantification of IBMP in spiked model wines using the techniques of HS-SPME, but reported that the method he developed was sensitive to only the part-per-billion range, lacking the sensitivity to quantify common concentrations found in wine, which are estimated between 10 and 15 ng/L (10-15 ppt) depending on the grape variety (Allen, *et al.*, 1994; Roujou de Boubée, *et al.*, 2000).

Sample Concentration

Several methods of liquid separation with additional concentration of target compound exist for the detection and quantification of IBMP. Harris, *et al.* (1987) developed two assays for the detection of IBMP in Sauvignon blanc grapes and wine that uses a stable isotope (deuterated-isobutylmethoxypyrazine, 2-($^2H_3$)methoxy-3-isobutylpyrazine) as an internal standard. In their process, the analyte itself (IBMP) was isotopically labeled, then added as an internal standard prior to analysis and was carried through all isolation, evaporation, and analytical steps, compensating for any losses throughout isolation and evaporation and for variations in injection volumes and detection efficiency (Allen, *et al.*, 1994).

Allen, *et al.* (1994) developed a similar method that was applicable to red grape juice and red wine as well as white grape juice and white wine. Their assay required a sample of juice or wine (~240 mL) to be distilled at atmospheric pressure. The distillate was then stirred with ion exchange resin, which adsorbed the IBMP. The target compound was then stripped off the resin with a strong base and IBMP was extracted from the aqueous phase with dichloromethane. The dichloromethane containing the IBMP was evaporated down to 20 μL, which concentrated the IBMP, and then a 1 μL injection was made into a GC-MS for analysis.

Roujou de Boubée, *et al.* (2000) modified the above assay further by performing the distillation step in a volatile Cash still, a typical piece of equipment frequently used in an enological laboratory for the determination of volatile acidity and alcoholic strength in wines. Roujou de Boubée, *et al.* (2000) reported that the use of a volatile Cash still extracted between 85 to 100% of IBMP as compared to ~6% by traditional distillation.

Also, rather than stirring the distillate in the presence of ion exchange resin, the distillate was percolated through a micro-column of resin with a peristaltic pump. The ion exchange resin was then placed in a glass micropipette, using a glass wool plug placed in the small end of the pipette to prevent loss of ion exchange resin. The dichloromethane was concentrated to less than 10 μL prior to an injection of sample into the GC-MS for analysis.

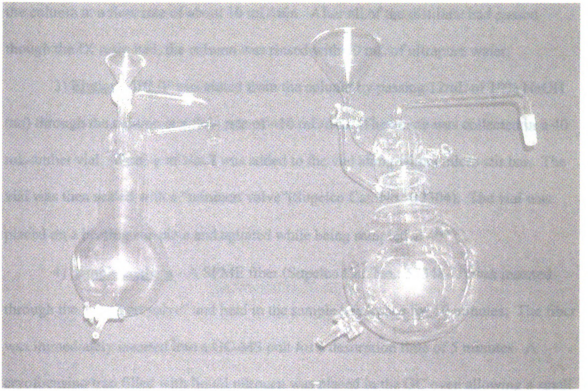
The primary limitations of this approach include: limited sample size, the need for solvents and organic phase extraction, the requirement of a peristaltic pump, and the extended GC-MS run time. From start to finish this method required greater than two hours time. What is needed to achieve the desired result is a method of extraction, concentration, and quantification that is robust and quick. This thesis effort was designed to accomplish that goal.

Materials and Methods

Isolation of 2-methoxy-3-isobutylpyrazine (Final Protocol) Roujou de Boubée, *et al.* (2000) has developed a method for the analysis of the IBMP content in grapes during the ripening period, which was adopted for our analytical procedure as follows:

Ten mL of 10% NaOH plus 20 ng of internal standard [2-($^2H_3$)methoxy-3-isobutylpyrazine] was added to the wine or the centrifuged must sample (300 mL). IBMP was then extracted using the process originally devised by Allen et al. (1994), later modified by Roujou de Boubée, *et al.* (2000), but simplified as follows:

1) Distillation - Distillation of 300 mL of wine was performed by steam extraction using a common enological laboratory apparatus known as a volatile Cash still (Figure 1), typically used to measure the volatile acidity and alcoholic strength of wine. It consists of extracting volatile compounds of wine introduced in the bubble chamber by steam over a period of approximately 15 min. The still used was based on typical designs, but the size was increased to yield a 1 L internal sample chamber and a 3 L external boiling chamber (as compared to 250 mL, and 1 L respectively, in a common still). The added volume eliminated foam-over, which was a problem in the common still's volume. The steam then passed through a glass condensing-column with cold tap water (Temp. ~ 5°C) providing condensation of the distillate. This method was reported to extract between 85 and 100% of the IBMP in the sample compared to about 6% by distillation (Roujou de Boubée, *et al.* 2000). The larger still size is a key improvement, allowing for variable sample sizes; the volume of a low IBMP level sample can simply be increased. Following concentration, IBMP levels in the sample will be within the detection range of the GC-MS.



(Figure 1 – Common and custom made Cash volatile still sizes)

2) Ion Exchange - Instead of either agitating the distillate in the presence of strong acid cation-exchange resin (Allen, *et al.* 1994), or percolating distillate through a small IX resin bed contained in a Pasteur pipet (Roujou de Boubée, *et al.* 2000), the distillate was passed through a column in the following manner. Approximately 1 g of strong acid cation ion exchange (IX) resin (Dowex® 50WX4-200, Aldrich Cat. No.: 42,209-6) was placed in a 50 mL burette with a glass wool plug in the narrow section and a stop cock to control flow rate. Prior to each use the IX resin was cleaned and re-generated (put into the acid form) by passing the following through the column, respectively: 1) 12 mL of 10% NaOH (aq); 2) 25 mL of ~4% HCl (aq); and 3) 100 mL of ultrapure HPLC grade water (Personal Communication, S. Najmy, 2002). After the ion exchange resin was rinsed with 100 mL of ultrapure HPLC grade water, the steam extract was passed through

the column at a flow rate of about 10 mL/min. After all of the distillate had passed though the IX resin bed, the column was rinsed with 10 mL of ultrapure water.

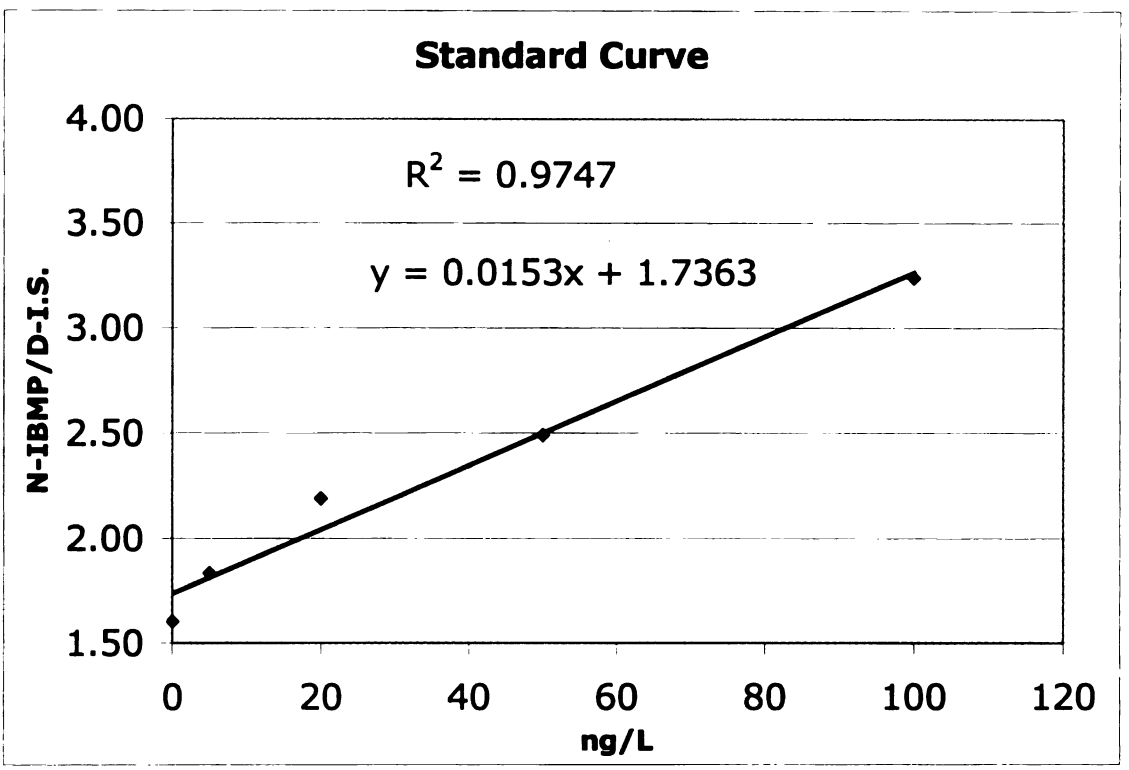
3) Elution - IBMP was eluted from the column by passing 12mL of 10% NaOH (aq) through the column at a flow rate of ~10 mL/min. The eluate was collected in a 40 mL amber vial. Three g of NaCl was added to the vial along with a micro stir bar. The vial was then sealed with a "mininert valve"(Supelco Cat. No.: 33304). The vial was placed on a heating stir-plate and agitated while being sampled at 40° C.

4) Sample analysis - A SPME fiber (Supelco Cat. No.: 57348-U) was inserted through the "mininert valve" and held in the sample headspace for 10 minutes. The fiber was immediately inserted into a GC-MS unit for a desorption time of 5 minutes. A cryofocusing trap filled with liquid nitrogen was placed in the GC oven allowing a small portion of the column to be immersed in the liquid nitrogen during the desorption period. After five minutes desorption time, the cryofocus trap was quickly removed; the GC oven shut, and the GC-MS run started.

5) Gas Chromatography-Mass Spectrometry Method - The system used to analyze for IBMP was a Hewlett Packard 6890 G.C. coupled with a Leco Pegasus II M.S. attached to Dell Dimension computer running Pegasus vs. 1.33 GC-MS analysis software. The capillary column was a Supelcowax 10 (30m x 0.2 mm i.d.; 0.2 μm film thickness; Cat. No.: 24169). The vector gas was helium (Grade 5.0; BOC Gases), with a flow rate of 1.2 mL/min. Injection was made in splitless mode allowing for all of the desorbed compound to migrate through the machine. The temperature gradient from 40 °C to 80°C was 100 °C/min, and then the ramping rate was changed to 50 °C/min until the final temperature of 240 °C was reached, then held for 1 min. Inlet temperature was

set at 220 °C and the transfer line was set at 200 °C. Each injection lasted 4.6 minutes. Ion selection range was from *mass/charge* (*m/z*) 36 and 174.

The system was calibrated by adding increasing concentrations of IBMP (0-100 ng/L for wine) to a Pinot noir wine produced by Michigan State University's research winery following typical production methods. The internal standard concentration was 80 ng/L. Each of the samples prepared in this way were extracted according to the method previously described (Figure 2).



(Figure 2 – Initial standard curve developed in a wine matrix spiked with varying levels of IBMP; x-axis: ng/L IBMP added, y-axis: ratio of IBMP peak area over internal standard peak area)

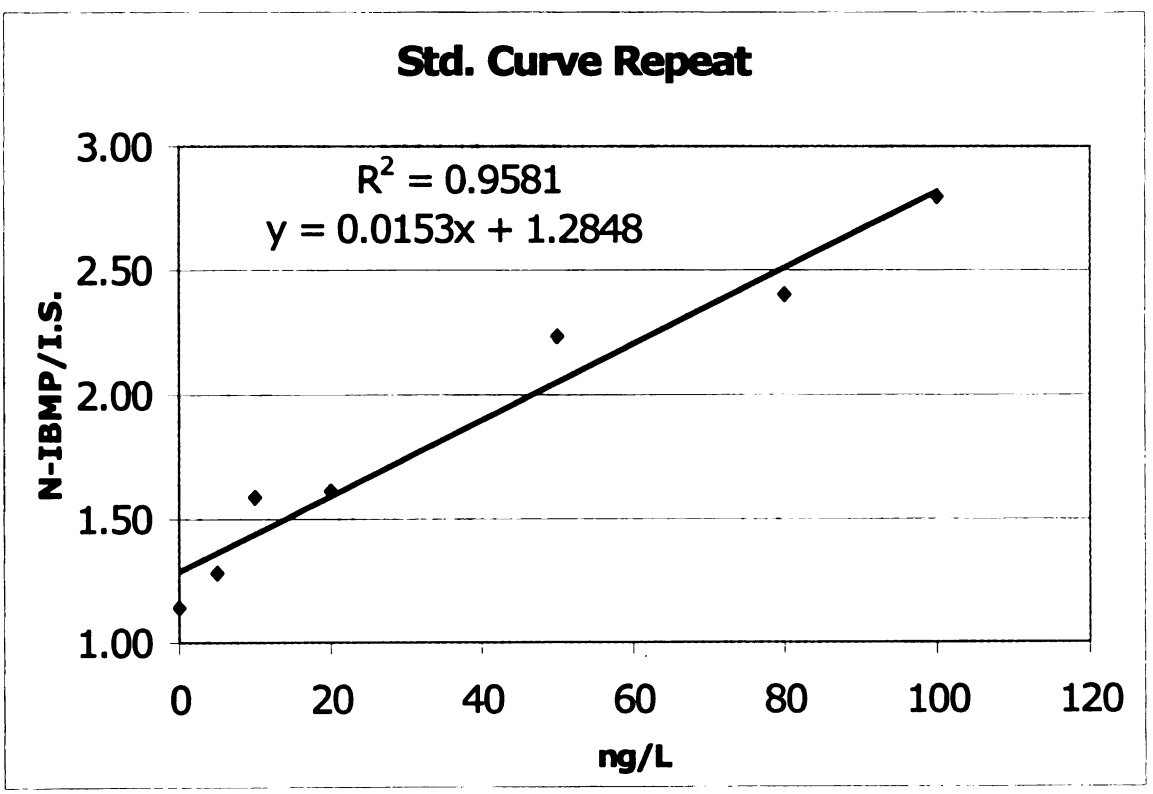
The above standard curve was produced a second time to establish its reproducibility. A 12% ethanol and 88% water matrix was used to produce a curve to establish a difference in standard curves based on matrix type. It was also observed that

after completing a single adsorbtion/desorption and run on the GC-MS, samples were completely taxed. A decay curve was produced by spiking a 12% ethanol/88% water matrix with 1000 ng/L IBMP and repeatedly sampling it to determine the number of sample replications required to tax the sample.

Results and Discussion

Standard Curve #2

The standard curve was reproduced in the same manner as above. The slope of the line was identical to the first curve produced, however the slope intercept was different. The change in slope intercept does not effect the concentration calculation for unknown samples. Because IBMP was added to a wine that contained an initial quantity of IBMP, the y-intercept will be somewhere above zero. When samples with unknown levels of IBMP are run for quantification, it can be assumed that the y-intercept is zero because no addition of IBMP will be made. The regression equation for wine is as follows: IBMP concentration (ng/L) = $(A/A_{is})/0.0153$ (A, area of IBMP peak; A_{is}, area of internal standard peak) (Figure 3).

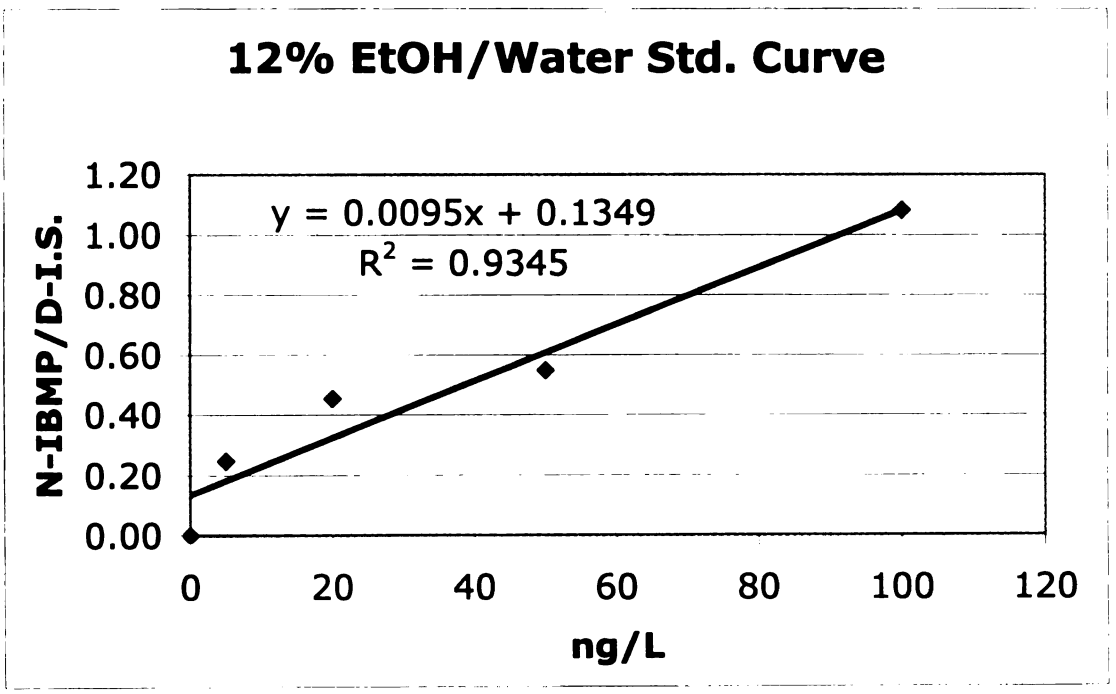


(Figure 3 - Repeated standard curve developed in a wine matrix spiked with varying

levels of IBMP; x-axis: ng/L IBMP added, y-axis: ratio of IBMP peak area over internal standard peak area)

Standard Curve in 12% Ethanol

A standard curve was also developed in a matrix of 12% ethanol and 88% water (Figure 4). Increasing concentrations of IBMP (0-100 ng/L) were added to the ethanol/water solution. The internal standard concentration was 80 ng/L. The slope of the line (y=0.0095x + 0.1349) was different from the previous standard curves developed in a wine matrix. It is important to develop standard curves in a matrix that is similar to the matrix to be analyzed. This is consistent with the work done by Roujou de Boubée, *et al.* (2000) in which a standard curve was developed for both must and wine.



(Figure 4 – A standard curve developed in a 12% ethanol/88% water matrix spiked with varying levels of IBMP; x-axis: ng/L IBMP added, y-axis: ratio of IBMP peak area over internal standard peak area)

Reproducibility (CV)

The reproducibility of the IBMP analysis on a series of eight measurements all spiked with 50 ng/L of IBMP was high (Table 1).

Reproducibility of IBMP Analysis in Wine

Sample #	ng/L
1	56.0
2	52.1
3	56.5
4	46.8
5	57.7
6	56.6
7	51.1
8	45.7
av(*n=8*)	52.8
SD	4.60
CV(%)	8.72

(Table 1)

Double Blind Study

A more rigorous study was conducted in which a neutral party spiked six wine samples with varying levels of IBMP that remained unknown to the analyst until after the quantification data was collected and IBMP concentrations were calculated. The calculated concentrations were compared to the actual levels added to the wine and were found to be in high correlation (Table 2).

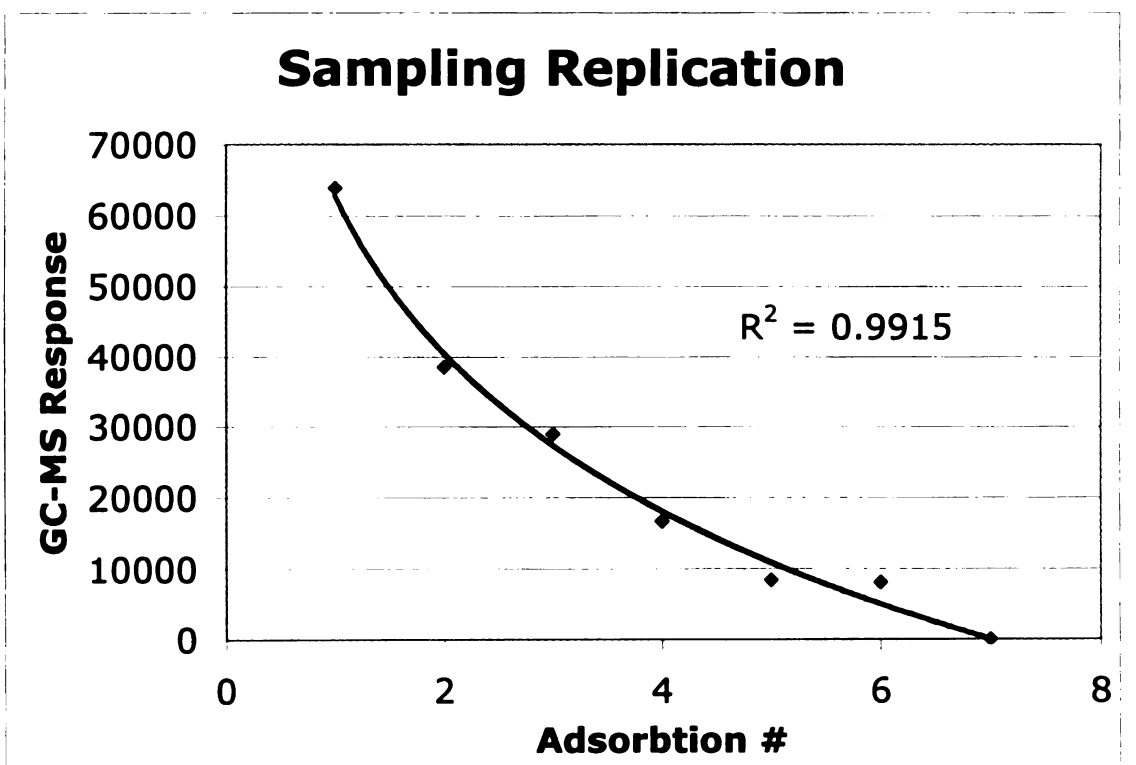
Double Blind Study

Actual IBMP (ng/L)	Calculated IBMP (ng/L)
33	30
67	66
100	101
16	15
33	35

(Table 2 – Double blind study of wine spiked with varying levels of IBMP)

Single Sample Decay Curve

It was observed that after a sample had completed the extraction and concentration preparations, been adsorbed with a SPME fiber, desorbed into the GC-MS and run, that the sample could not be adsorbed for a second run. The quantity of IBMP was taxed from the vial and could not be used for further analysis. An experiment was conducted in which a 12% ethanol/88% water solution was spiked with 1000 ng/L (1000 ppt) of internal standard. This concentration is between 10 and 100 times the concentration range commonly found in wine (10-100 ng/L). The sample was then adsorbed with a SPME fiber and then desorbed and run on the GC-MS multiple times to determine the number of sampling replicates requires to tax a sample of very high concentration, relative to wine samples (Figure 5).



(Figure 5 – Decay curve determining the number of sample replicates required to tax a wine sample spiked with 1000 ng/L IBMP)

In Figure 5, the lowest point on the curve (seventh replicate) is a false data point that has been added to demonstrate a zero response from the GC-MS due to the IBMP levels being lower that the detection limits of the instrument. After the sixth replicated sampling the quantity of IBMP remaining in the vial was below the detection limits of the GC-MS.

Conclusions

This method for detection and quantification of 2-methoxy-3-isobutylpyrazine has good reproducibility and offers the analyst a faster, simpler, less complicated assay than was previously available. The distillation process has been enhanced to accommodate varied sample sizes between zero and 500 mL. The improved method employs solid phase microextraction, eliminating the need for solvents and reducing the labor in concentrating and analyzing volatile compounds in the headspace down to an adsorption/desorption step. The GC-MS run time is estimated to be 40 minutes shorter than previously published methods. The method will work on juice or clarified must samples so that individual wine production steps or vineyard practices may be evaluated for their impact on IBMP concentration in the finished wine.

While this simplified method can be improved, the advantages of the combined analytical techniques in the assay allow for faster sample preparation and reduced analysis time, and should result in expanded research efforts aimed at IBMP reduction through viticultural methods such as clonal selection, crop control, and canopy management and enological approaches such as yeast choice and malolactic strain, fermentation conditions and blending. This is a method of detection and quantification for 2-methoxy-3-isobutylpyrazine that is readily reproducible in a timely fashion and is not only applicable to grape juice and wine at any time during the growing season but also throughout the fermentation and post-fermentation processing of wine. We believe it is a valuable tool for viticulture and enological studies focused on cultivars of red and white Bordeaux wine cultivars in cool climates.

Literature Sited

1. Allen, M.S.; Lacey, M. J. Methoxypyrazine grape flavour: influence of climate, cultivar and viticulture. *Vitic. Enol. Sci.* **1993**, 48, 211-213

2. Allen, M.S.; Lacey, M. J.; Boyd, S. Determination of methoxypyrazines in red wines by stable isotope dilution gas chromatography mass spectrometry. *J. Agric. Food. Chem.* **1994**, 42, 1734-1738.

3. Allen, M.S.; Lacey, M. J.; Boyd, S. Methoxypyrazines in red wines: Occurrence of 2-Methoxy-3-(1-methylethyl)pyrazine. *J. Agric. Food Chem.* **1995**, 43, 769-772.

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. American Water Works Association, Method 6040. Constituent Concentration by Gas Extraction. *Standard Methods for the Examination of Water and Wastewater.*

6. Barker, D. A.; Capone, D. L.; Pollnitz, A. P.; McLean, H. J.; Francis, I. L.; Oakey, H.; Sefton, M. A. Absorption of 2,4,6-trichloroanisole by wine corks via the vapour phase in an enclosed environment. *Australian J. of Grape and Wine Research.* **2001**, 7, 40-46.

7. Bellavia, V.; Natangelo, M.; Fanelli, R.; Rotilio, D. Analysis of benzothiazole in Italian wines using headspace solid-phase microextraction and gas chromatography-mass spectrometry. *J. Agric. Food. Chem.* **2000**, 48, 1239-1242.

8. Boison, J. O. K.; Tomlinson, R. H. New sensitive method for the examination of the volatile flavor fraction of Cabernet sauvignon wines. *J. Chromatography.* **1990**, 522, 315-327.

9. Buttery, R. G.; Seifert, R. M.; Guadagni, D. G.; Ling, L. C. Characterization of some volatile constituents of bell peppers. *J. Agric. Food. Chem.* **1969**, 17, 1322-1327.

10. Csiktusnádi Kiss, G. A.; Forgács, E.; Cserháti, T.; Candeias, M.; Vila-Boas, L.; Bronze, R.; Spranger, I. Solid-phase extraction and high-performance liquid chromatographic separation of pigments of red wines. *J. Chromatography A.* **2000**, 889, 51-57.

11. Czerny, M.; Grosch, W. Potent odorants of raw arabica coffee. Their changes during roasting. *J. Agric. Food Chem.* **2000**, 48, 868-872.

12. De Fátima Alpendurada, M. Solid-phase microextraction: A promising technique for sample preparation in environmental analysis. *J. Chromatography A.* **2000**, 889, 3-14.

13. De la Calle García, D.; Reichenbächer, M.; Hurlbeck, C.; Bartzsch, C.; Feller, K-H. Analysis of wine bouquet components using headspace solid-phase microextraction-capillary gas chromatography. *J. High Resol. Chromatogr.* **1998**, 21, 373-377.

14. Di Tommaso, D.; Calabrese, R.; Rotilio, D. Identification and quantitation of hydroxytyrosol in Italian wines. *J. High Resol, Chromatogr.* **1998**, 21, 549-553.

15. Dubourdieu, D.; Tominaga, T.; Masneuf, I.; Peyrot Des Gachons, C.; Murat, M. The role of yeasts in grape flavor development during fermentation: the example of Sauvignon blanc. *Proc. ASEV 50th Anniversary Annual Meeting, Seattle, Washington.* **June 19-23, 2000.**

16. Edson, C. E.; Howell, G. S.; Flore, J. A. Influence of crop load on photosynthesis of Seyval grapevines I. Single leaf whole vine response pre- and post-harvest. *Am. J. Enol. Vitic.* **1993**, 44, 139-147.

17. Fischer, C.; Fischer, U. Analysis of cork taint in wine and cork material at olfactory subthreshhold levels by solid phase microextraction. *J. Agric. Food. Chem.* **1997**, 45, 1995-1997.

18. Gandini, N.; Riguzzi, R. Headspace solid-phase microextraction analysis of methyl isothiocyanate in wine. *J. Agric. Food. Chem.* **1997**, 45, 3092-3094.

19. Giannakopoulos, P.I.; Markakis, P.; Howell, G.S. The influence of malolactic strain on the fermentation on wine quality of three eastern red wine grape cultivars. *Am. J. Enol. Vitic.* **1984**, 35, 1-4.

20. Harris, R. L. N.; Lacey, M. J.; Brown, W. V.; Allen, M. S. Determination of 2-methoxy-3-alkylpyrazines in wine by gas chromatography/mass spectrometry. *Vitis.* **1987**, 26, 201-207.

21. Hartmann, P.J.; McNair, H.M; Zoecklein, B.W. Measurement of 3-Alkyl-2-Methoxypyrazine by Headspace Solid-Phase Microextraction in Spiked Model Wines. *Am. J. Enol. Vitic.* **2002**, 54, 285-288.

22. Hashizume, K.; Kida, S.; Samuta, T. Effects of steam treatment of grape cluster stems on the methoxypyrazine, phenolic, acid, and mineral content of red wines fermented with stems. *J. Agric. Food. Chem.* **1998**, 46, 4382-4386.

23. Hashizume, K.; Kida, S.; Samuta, T. Green odorants of grape cluster stem and their ability to cause a wine stemmy flavor. *J. Agric. Food Chem.* **1997**, 45, 1333-1337.

24. Hayasaka, Y.; Bartowsky, E. J. Analysis of diacetyl in wines using solid-phase microextraction combined with gas chromatography-mass spectrometry. *J. Agric. Food. Chem.* **1999**, 47, 612-617.

25. 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.

26. Ibañez, E.; Bernhard, A. R. Solid-phase microextraction of pyrazines in model reaction systems. *J. Sci. Food. Agric.* **1996**, 72, 91-96.

27. Jones, R. G.; Pyrazines and related compounds. I. A new synthesis of hydroxypyrazines. *J. Am. Chem. Soc.* **1949**, 71, 78-81.

28. Karmas, G.; Spoerri, P. E. The Preparation of hydroxypyrazines and derived chloropyrazines. *J. Am. Chem. Soc.* **1952**, 74, 1580-1584.

29. Lacey, M. J.; Allen, M. S.; Harris, R. L. N.; Brown, W. V. Methoxypyrazines in Sauvignon blanc grapes and wines. *Am. J. Enol. Vitic.* **1991**, 42, 103-108.

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

31. Matisová, E.; Sedláková, J.; Slezácková, M. Solid-phase microextraction of volatile polar compounds in water. *J. High. Resol. Chromatogr.* **1999**, 22, 109-115.

32. Mestres, M.; Busto, O.; Guasch, J. Headspace solid-phase microextraction analysis of volatile sulphides and disulphides in wine aroma. *Journal of Chromatography A.* **1998**, 808, 211-218.

33. Miller, D. P.; Howell, G. S. The effects of various carbonic maceration treatments on must and wine composition of Marechal foch. *Am. J. Enol. Vitic.* **1989**, 40, 170-174.

34. Miller, D. P.; Howell, G. S.; Michaelis, C. S.; Dickmann, D. I. The content of phenolic acid and aldehyde flavor of white oak as affected by site and species. *Am. J. Enol. Vitic.* **1992**, 43, 333-338.

35. Miller, D. P.; Howell, G. S.; Striegler, R. K. Reproductive and vegetative response of mature grapevines subjected to differential cropping stresses. *Am. J. Enol. Vitic.* **1993**, 44, 435-440.

36. Miller, M. E.; Stuart, J. D. Comparison of gas-sampled ans SPME-sampled static headspace for the determination of volatile flavor components. *Analytical Chemistry*. **1999**, 71, 23-27.

37. Murry, K. E.; Whitfield, F. B. The occurrence of 3-alkyl-2-methoxypyrazines in raw vegetables. *J. Sci. Fd. Aric.* **1975**, 26, 973-986.

38. Noble, A. C.; Arnold, R. A.; Buechsenstein, J.; Leach, E. J.; Schmidt, J. O.; Stern, P. M. Modification of standardized system of wine aroma terminology. *Am. J. Enol. Vitic.* **1987**, 38, 143-146.

39. Pawliszyn, J. Applications of solid phase microextraction (Book), Royal Society of Chemistry, ISBN 0-85404-525-2, **1999**.

40. Roberts, D. D.; Pollien, P.; Milo, C. Solid-phase microextraction method development for headspace analysis of volatile flavor compounds. *J. Agric. Food. Chem.* **2000**, 48, 2430-2437.

41. Rocha, S.; Ramalheira, V.; Barros, A.; Delgadillo, I.; Coimbra, M. A. Headspace solid phase microextraction analysis of flavor compounds in wine. Effects of the matrix volitle composition in the relative response factors in a wine model. *J. Agric. Food. Chem.* **2001**, 49, 5142-5151.

42. Roujou de Boubée, D.; Cumsille, A. M.; Pons, M.; Dubourdieu, D. Location of 2-methoxy-3-isobutylpyrazine in Cabernet sauvignon grape bunches and its extractability during vinification. *Am. J. Enol. Vitic.* **2002**, 53, 1-5.

43. Roujou de Boubée, D.; Van Leeuwen, C.; Dubourdieu, D. Organoleptic impact of 2-methoxy-3-isobutylpyrazine on red Bordeaux and Loire wines. Effect of environmental conditions on concentrations in grapes during ripening. *J. Agric. Food. Chem.* **2000**, 48, 4830-4834.

44. Seifert, R. M.; Buttery, R. G.; Guadagni, D. G.; Black, D. R.; Harris, J. G. Synthesis of some 2-methoxy-3-alkylpyrazines with strong bell pepper-like odors. *J. Agric. Food. Chem.* **1970**, 18, 246-249.

45. Supelco. SPME – A fast and inexpensive approach to trace organic analysis. *The Reporter*. **2001**, Vol. 19.1.

46. Supelco Bulletin 869A – Solid phase microextraction: solventless sample preparation for monitoring flavor compounds capillary gas chromatography. **1998**.

47. Supelco Bulletin 928 – Solid phase microextraction troubleshooting guide. **2001**.

48. Supelco Bulletin 929 - A practical guide to quantitation with solid phase microextraction. **2001**.

49. Urruty, L.; Montury, M.; Braci, M.; Fournier, J.; Dournel J. Comparison of two recent solventless methods for the determination of procymidone residues in wines: SPME/GC/MS and ELISA tests. *J. Argic. Food. Chem.* **1997**, 45, 1519-1522.

50. Wada, K.; Shibamoto, T. Isolation and identification of volatile compounds from wine using solid phase microextraction, gas chromatography, and gas chromatography/mass spectrometry. *J. Agric. Food. Chem.* **1997**, 45, 4362-4366.

51. Weber, J.; Beeg, M.; Bartzsch, C.; Feller, K. Improvement of the chemometric variety characterization of wines by improving the detection limit of aroma compounds. *J. High Resol. Chromatoge.* **1999**, 22, 322-326.

The following papers are written in the format

of the journal in which they are

intended to be submitted for publication.

Technical Brief

A Simplified Method for the Detection and Quantification of 2-Methoxy-3-Isobutylpyrazine in Grape Must and Wine

A simplified method was developed to rapidly and accurately quantify 2-methoxy-3-isobutylpyrazine combining previous techniques of stable isotope dilution gas chromatography-mass spectrometry with headspace solid phase microextraction.

Key words: 2-methoxy-3-isobutylpyrazine, headspace solid phase microextraction, volatile cash still.

A particular group of compounds, 2-methoxy-alkylpyrazines (MP), are responsible for the vegetative and herbaceous green bell-pepper aromas and flavors found in wines produced from cultivars Cabernet Sauvignon, Cabernet Franc, Merlot, and Sauvignon blanc when grown in cool climates. The major contributing component of this group of compounds is 2-methoxy-3-isobutylpyrazine (IBMP) is (Allen et al. 1994). IBMP is a

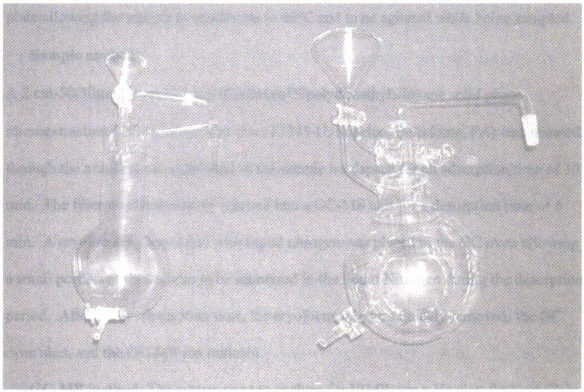
strongly odorous compound with a low human perception threshold of about two parts-per-trillion (ng/L) in water (Allen et al. 1994; Buttery et al. 1969; Seifert et al. 1970). The extremely low detection threshold of IBMP requires specialized analytical isolation techniques in order to be able to detect the compound in wine and berries. In order to implement vineyard management and cellar practices that would ultimately reduce the perception of IBMP in wine, which is commonly organolepticly evaluated as negative, quantification of the target compound is imperative to demonstrate a reduction in concentration. Several analytical detection and quantification methods have been developed specifically for methoxypyrazines and most have been shown to be inadequate to allow for rapid, accurate, or repeated analysis. This report concerns efforts to combine methods of stable isotope dilution gas chromatography-mass spectrometry (GC-MS) (Allen et al. 1994; Roujou de Boubée et al. 2002, 2000) with the simplicity and ease of solid phase microextraction (Pawliszyn, 1997). Using this approach a method has been developed to analyze for IBMP in musts or wines in the cellar and fruit being grown in the vineyard.

Materials and Methods

Distillation. Distillation of 300 mL of wine was performed by steam extraction using a custom-made enological laboratory apparatus known as a 'Cash Volatile Still' which is typically used to measure the volatile acidity and alcoholic strength of wine. The modified still was based on typical designs, but the volume was increased to yield a 1 L internal sample chamber and a 3 L external boiling chamber. The added volume

prevented foam-over in the larger than average sample size and provided additional volume for variable samples sizes (Figure 1).

(Figure 1-Common and custom made Cash volatile still sizes)



Ion Exchange. The distillate was passed through an ion exchange column in the following manner: a) approximately 1 g of strong acid cation ion exchange (IX) resin (Dowex® 50WX4-200, Aldrich, Milwaukee, WI) was placed in a 50 mL burette with a glass wool plug in the narrow section, and a stopcock to control flow rate; b) the ion exchange resin was rinsed with ~100 mL of HPLC grade water, and then the steam extract was passed through the column at a flow rate of about 10 mL/min; c) after all of the distillate had passed though the IX resin bed, the column was rinsed with 10 mL of HPLC water.

Elution. IBMP was eluted from the column by passing 12 mL of 10% NaOH through the column. The eluate was collected in a 40 mL amber vial. Three grams of NaCl were added to the vial along with a micro stir bar. The vial was sealed with a 'mininert valve' (Cat. No.: 33304, Supelco, Bellefonte, PA) and placed on a heat/stir-plate allowing the sample to equilibrate to 40°C and to be agitated while being sampled.

Sample analysis.

A 2 cm-50/30μm divinylbenzene/Carboxen™/polydimethylsiloxane solid phase microextraction (SPME) fiber (Cat. No.: 57348-U, Supelco, Bellefonte, PA) was inserted through the mininert valve and held in the sample headspace for an adsorption time of 10 min. The fiber was immediately inserted into a GC-MS unit for a desorption time of 5 min. A cryofocusing trap filled with liquid nitrogen was placed in the GC oven allowing a small portion of the column to be immersed in the liquid Nitrogen during the desorption period. After 5 min. desorption time, the cryofocus trap was quickly removed, the GC oven shut, and the GC-MS run initiated.

GC-MS method. The system used to analyze for IBMP was a Hewlett Packard (Palo Alto, CA) 6890 GC coupled with a Leco (St. Joseph, MI) Pegasus II MS attached to a Dell (Round Rock, TX) Dimension computer running Pegasus (Leco, St. Joseph, MI) version 1.33 GC-MS analysis software. The capillary column was a Supelcowax 10 (30m x 0.2 mm i.d.; 0.2 μm film thickness; Cat. No.: 24169, Supelco, Bellefonte, PA). The vector gas was Helium, with a flow rate of 1.2 mL/min. Injection was made in splitless mode. The temperature gradient from 40 °C to 80°C was 100 °C/min. The ramping rate was then changed to 50 °C/min until the final temperature of 240 °C was reached, then held for one min. Inlet temperature was set at 220 °C and the transfer line

was set at 200 °C. Each injection lasted 4.6 minutes. Ion selection range was from *mass/charge* (*m/z*) between 36 and 174.

Results and Discussion

Quantitative Assessment. To quantify the naturally occurring concentration of IBMP in a sample, the analyte itself (IBMP) can be isotopically labeled, and added at a known concentration as an internal standard. This labeled internal standard (2-($^2H_3$)methoxy-3-isobutylpyrazine) will be carried through all isolation, distillation, and analytical steps. This compensates for any losses incurred during isolation and evaporation and for variations in SPME fiber adsorption/desorption and detection efficiency as shown effective in preexisting analytical methods (Allen et al. 1994; Harris et al. 1987; Roujou de Boubée et al. 2002, 2000).

Standard Curves and Reproducibility. The standard curves for this assay were developed by the addition of increasing concentrations of IBMP to wine. The area of the IBMP peaks (*m/z* 124) compared to that of the internal standard peaks (*m/z* 127) were in linear correlation with the IBMP concentration added to the wine. The regression equation for wine is as follows: IBMP concentration (ng/L) = $(A/A_{is})/0.0153$ (A, area of IBMP peak; A_{is}, area of internal standard peak) ($r^2 = .975$). The reproducibility of the IBMP analysis on a series of eight measurements all spiked with 50 ng/L of IBMP was high (Table 1).

Reproducibility of IBMP Analysis in Wine	
Sample #	ng/L
1	56.0
2	52.1
3	56.5
4	46.8
5	57.7
6	56.6
7	51.1
8	45.7
av(*n*=8)	52.8
SD	4.60
CV(%)	8.72

(Table 1- Reproducibility of IBMP analysis of 8 samples of wine spiked with 50 ng/L of IBMP)

Double Blind Study. A more rigorous study was conducted in which a neutral party spiked six wine samples with varying levels of IBMP that remained unknown to the analyst until after the quantification data was collected and IBMP concentrations were calculated. The calculated concentrations were compared to the actual levels added to the wine and were found to be in high correlation (Table 2).

Double Blind Study	
Actual IBMP (ng/L)	**Calculated IBMP (ng/L)**
33	30
67	66
100	101
16	15
33	35

(Table 2- Double blind study of wine spiked with varying levels of IBMP)

Conclusions

This simplified method for detection and quantification of 2-methoxy-3-isobutylpyrazine offers the analyst a faster, simpler, less complicated assay with good reproducibility. The distillation process has been enhanced to accommodate varied and larger sample sizes. The improved method employs solid phase microextraction, eliminating the need for solvents and simplifying the labor in concentrating and analyzing volatile compounds in the headspace. The GC-MS run time is also much shorter than previously published methods. The method also will work on juice or clarified must samples so that individual wine production steps or vineyard practices may be evaluated for their impact on IBMP concentration in the finished wine.

Literature Cited

1. Allen, M.S., M. J. Lacey. 1993. Methoxypyrazine grape flavour: influence of climate, cultivar and viticulture. Vitic. Enol. Sci. 48:211-213.

2. Allen, M.S., M. J. Lacey, S. Boyd. 1994. Determination of methoxypyrazines in red wines by stable isotope dilution gas chromatography mass spectrometry. J. Agric. Food. Chem. 42:1734-1738.

3. Buttery, R. G., R. M. Seifert, D. G. Guadagni, L. C. Ling. 1969. Characterization of some volatile constituents of bell peppers. J. Agric. Food. Chem. 17:1322-1327.

4. Harris, R. L. N., M. J. Lacey, W. V. Brown, M. S. Allen. 1987. Determination of 2-methoxy-3-alkylpyrazines in wine by gas chromatography/mass spectrometry. Vitis. 26:201-207.

5. Pawliszyn, J. 1997. Applications of Solid Phase Microextraction: Theory and Practice. John Wiley & Sons, Inc., New York.

6. Roujou de Boubée, D., A. M. Cumsille, M. Pons, D. Dubourdieu. 2002. Location of 2-methoxy-3-isobutylpyrazine in Cabernet sauvignon grape bunches and its extractability during vinification. Am. J. Enol. Vitic. 53:1-5.

7. Roujou de Boubée, D., C. Van Leeuwen, D. Dubourdieu. 2000. Organoleptic impact of 2-methoxy-3-isobutylpyrazine on red Bordeaux and Loire wines. Effect of environmental conditions on concentrations in grapes during ripening. J. Agric. Food. Chem. 48:4830-4834.

8. Seifert, R. M., R. G. Buttery, D. G. Guadagni, D. R. Black, J. G. Harris. 1970. Synthesis of Some 2-Methoxy-3-Alkylpyrazines with Strong Bell Pepper-Like Odors. J. Agric. Food. Chem. 18:246-249.

Analytical Method Development in Wine Chemistry, A Narrative of the Process: Pitfalls and Breakthroughs

A narrative of the process to develop a simplified analytical method for the detection and quantification of 2-methoxy-3-isobutylpyrazine in grape juice and wine.

Key words: Methoxypyrazine, headspace solid phase microextraction, volatile cash still, gas chromatography-mass spectrometry.

The research efforts regarding the impact of methoxypyrazines in wine date back slightly more than a decade. There have been several key improvements in the analytical methods for both detection and quantification. With the advancements in equipment and analytical techniques, scientists are able to apply different and often advantageous practices to their research. This is one such project. The goal of this paper is to give a narrative of the analytical wine chemistry techniques and approaches applied to improving and simplifying the methods of detecting and quantifying 2-methoxy-3-isobutylpyrazine (IBMP) in grape juice and wine.

Previous Research Efforts

The extremely low detection threshold of IBMP requires specialized analytical isolation techniques in order to detect the compound in wine and berries. Such low levels are below the detection abilities of high performance liquid chromatography (HPLC)

(Heymann et al. 1986) and require the employment of gas chromatography-mass spectrometry (GC-MS) (Allen et al. 1994). The abilities of analytical chemistry labs, and especially less equipped enological laboratories, are taxed when striving to detect the lower thresholds of IBMP in wine (10 ng/L). To put it into perspective: ten parts per trillion is analogous to the diameter of a human hair compared to the diameter of the earth, or, stated in terms more closely related to the wine industry, ten drops of vermouth in a pool of gin the size of a football field, 43 feet deep. It is the extremely low naturally occurring level of IBMP that makes detection and quantification difficult.

Detection and Quantification.

Several analytical detection and quantification methods have been developed specifically for methoxypyrazines and most have ultimately been shown to be impractical for rapid and repeated analysis with adequate accuracy. The methods developed have mainly followed one of two approaches: liquid extraction or solid phase microextraction (SPME). Both methods are typically coupled with GC-MS. SPME is a simple and effective adsorption and desorption technique, which eliminates not only the need for solvents and complicated sample preparations, but also the laborious techniques for concentrating and analyzing for volatile or non-volatile compounds in liquid samples or headspace (Pawliszyn 1999). Headspace (HS)-SPME analysis, coupled with GC-MS, lends itself well to analysis of volatile compounds in any number of different types of matrices, including wine. HS-SPME has been employed for a range of extraction efforts such as for terpenoids in wine (De la Calle Garcia et al. 1998), and for the presence of the soil fumigant methyl isocyanate in wine (Gandini et al. 1997). Hayasaka, et al. (1999) used the method coupled with GC-MS for the analysis of diacetyl in wine. The method

was also used by American Water Works Association (AWWA) to analyze for methyl-isoborneol (MIB) and geosmin, reported as being responsible for the "musty" odor occurring in drinking water at particular times of the year. HS-SPME was used with IBMP as an internal standard with the ability to quantify MIB and geosmin down to a concentration of a few parts per trillion (AWWA method 6040). The AWWA method of detection for IBMP and other compounds in the parts-per-trillion range is further reviewed in Volume 19.1 of The Reporter (2001), published by Sigma-Aldrich in conjunction with Supelco. Hartmann et al. (2002) recently conducted a study for the detection and quantification of IBMP in model wines using the techniques of HS-SPME, but reported the method developed was sensitive to only mg/L levels, lacking the sensitivity to quantify common concentrations in wine, which are estimated between 10 and 15 ng/L (10-15 ppt) depending on the grape variety (Allen et al. 1994; Roujou de Boubée et al. 2000).

Concentration.

Several methods of liquid separation with additional concentration of the target compound exist for the detection and quantification of IBMP. Harris et al. (1987) developed two assays for the detection of IBMP in Sauvignon blanc grapes and wine that use a stable isotope (deuterated-isobutylmethoxypyrazine, 2-($^2H_3$)methoxy-3-isobutylpyrazine) as an internal standard. In their process, the analyte itself (IBMP) was isotopically labeled, then added as an internal standard prior to analysis and was carried through all isolation, evaporation, and analytical steps, compensating for any losses throughout isolation and evaporation and for variations in injection volumes and detection efficiency (Allen et al. 1994). Allen et al. (1994) developed a similar method

applicable to red grape juice and red wine as well as white grape juice and white wine. Their assay required a sample of juice or wine (~240 mL) be distilled at atmospheric pressure. The distillate was then stirred with ion exchange (IX) resin, which adsorbed the IBMP. The target compound was then stripped off the resin with a strong base and IBMP was extracted from the aqueous phase with dichloromethane. The dichloromethane containing the IBMP was evaporated down to 20 μL, which concentrated the IBMP, and then 1 μL was injection into a GC-MS for analysis.

Roujou de Boubée et al. (2000) modified the above assay further by performing the distillation step in a Cash volatile still: a typical piece of equipment frequently used in an enological laboratory for the determination of volatile acidity and alcoholic strength in wines. Roujou de Boubée reported that the use of a Cash volatile still extracted between 85 to 100% of IBMP as compared to ~6% by traditional distillation. Also, rather than stirring the distillate in the presence of ion exchange resin, the distillate was percolated through a micro-column of resin with a peristaltic pump. The ion exchange resin was then placed in a glass micropipette, using a glass wool plug placed in the small end of the pipette to prevent loss of ion exchange resin. The dichloromethane was concentrated to less than 10 μL prior to an injection of sample into the GC-MS for analysis.

This was the state of the literature when our efforts were initiated. The goal here was to employ steam distillation, ion exchange resin, solid phase microextraction, and gas chromatography-mass spectroscopy in varying ways to speed-up, simplify and improve accuracy of the analysis for IBMP.

Materials and Methods

Initial Modification Efforts: Improving concentration and extraction.

Initial efforts were focused on direct HS-SPME analysis of wine samples. The naturally occurring levels of IBMP in wine are low in concentration and HS-SPME coupled with GC-MS was found to lack the sensitivity necessary to detect natural levels. Attempts to produce standard curves by spiking wine with IBMP and analyzing by HS-SPME proved unsuccessful due to variation in GC-MS response and the μg/L (part per billion) spiked levels required for detection. For example when the HS of a series of two or more wine samples spiked with the same level of IBMP were analyzed, there was no reproducibility. In some runs the peak area was very small, and very large in others. The simplicity and speed with which a sample can be analyzed with SPME was a logical modification that could eventually be added to an assay to detect and quantify IBMP.

The extraction and concentration procedures originally developed by Allen et al. (1994) have been shown to work but lacked simplicity and ease because their method required liquid phase extraction steps and evaporation techniques, both requiring a relatively high degree of skill. Modifications in their procedures were made, allowing for more rapid and more-easily conducted sample preparation. The first such modifications adopted were the distillation parameters, followed by the ion exchange techniques as follows:

Distillation: Increase the quantity of wine distilled.

Distillation of 300 mL of wine was performed by steam extraction using a common enological laboratory apparatus known as a volatile Cash still, typically used to measure the volatile acidity and alcoholic strength of wine. The still used was based on

typical designs, but the size was increased to yield a 1 L internal sample chamber and a 3 L external boiling chamber (over 3x that of traditional Cash still size). The added volume eliminated foam-over, which was a problem in the common still's volume. Increasing the size of the still also allowed for much larger sample sizes. A wine that contained a low quantity of IBMP, sufficient to tax the detection abilities of the GC-MS could be accommodated by the analyst's flexibility to use more starting material (wine or juice), which contains additional IBMP.

Ion exchange.

Instead of agitating the distillate in the presence of strong acid cation-exchange resin (Allen et al. 1994), or percolating distillate through a small IX resin bed contained in a Pasteur pipet (Roujou de Boubée et al. 2000), the distillate was passed through a column in the following manner: approximately 1 g of strong acid cation IX resin (Dowex® 50WX4-200, Aldrich, Milwaukee, WI) was placed in a 50 mL burette with a glass wool plug in the narrow section and a stop cock to control flow rate. Use of a burette eliminates the need for peristaltic pumps as used by Roujou de Boubée et al. (2000) and Allen et al. (1994). Prior to each use the IX resin was cleaned and regenerated (put into the acid form) by passing the following through the column, respectively: 1) 10 mL of 10% NaOH; 2) 25 mL of ~3.5% HCl; and 3) 100 mL of HPLC grade water (S. Najmy 2002, personal communication). After the IX resin was rinsed with 100 mL of HPLC grade water, the steam extract was passed through the column at a flow rate of approximately 10 mL/min. After all of the distillate had passed though the IX resin bed, the column was rinsed with 10 mL of HPLC water. Regeneration of the

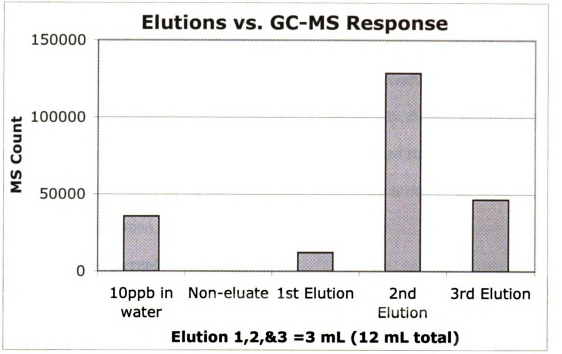
resin allows continual use of the material and eliminates the time needed to load and unload the column between runs.

Second Modification Efforts: Refined methods to elute column.

The distillation and ion exchange modifications made during initial efforts remained in practice throughout the efforts to modify the assay for the detection and quantification of IBMP. The appropriate volume of strong base to elute IBMP from the IX resin was not known. A recovery study was conducted to determine a practical volume and multiple of elutions required to sufficiently elute IBMP at a level within the detection abilities of the GC-MS.

Elution.

Four mL of 10% NaOH was arbitrarily set as a standard elution volume. It was observed that a small percentage of the IBMP elutes from the column with a single elution. To increase the quantity of IBMP eluted, the equivalent of three elutions was deemed practically advantageous (Figure 1).



(Figure 1- Elution volume and replication to efficiently elute IBMP from column)

The following modification was adopted: IBMP was eluted from the column by passing 12mL of 10% NaOH (aq) through the column at a flow rate of ~10 mL/min. The eluate was collected in a 40 mL amber vial. Three grams of NaCl was added to the vial along with a micro stir bar. The vial was then sealed with a "mininert valve"(Cat. No.: 33304,Supelco, Bellefonte, PA). The vial was placed on a heat/stir plate and agitated while being sampled at 40° C.

Sampling.

Attempts were then made to concentrate the eluted IBMP from the aqueous phase with dichloromethane. The process of evaporating the dichloromethane containing the IBMP down to 20 μL, which concentrated the IBMP, and then injecting a 1 μL injection into the GC-MS for analysis was found to be tedious, time consuming and a complex, error producing procedure. At this point HS-SPME analysis seemed a logical alternative

to direct injection, eliminating the need for dichloromethane as well as the slow organic phase extraction and concentration process.

A direct HS-SPME analysis of the eluate proved successful for IBMP detection in spiked ethanol/water solutions, must, and wine. Unfortunately, the problem of variation in GC-MS response from one run to the next also accompanied HS-SPME analysis. At this stage, the assay allowed for the detection of naturally occurring levels of IBMP, but not for its quantification.

Modification – Internal standard.

This hurdle was overcome by the addition of an internal standard (deuterated-isobutylmethoxypyrazine, 2-($^2H_3$)methoxy-3-isobutylpyrazine), as had proved successful for Harris et al. (1987). The internal standard was added prior to analysis and was carried through all isolation, evaporation, and analytical steps, compensating for any losses throughout isolation and evaporation and for variations in injection volumes and detection efficiency (Allen et al. 1994).

The sampling procedure was developed and implemented as follows: A 2 cm-50/30μm divinylbenzene/Carboxen™/polydimethylsiloxane SPME fiber (Cat. No.: 57348-U, Supelco, Bellefonte, PA) was inserted through the "mininert valve" and held in the sample headspace for 10 minutes. The fiber was immediately inserted into a GC-MS unit for a desorption time of 5 minutes. A cryofocusing trap filled with liquid nitrogen was placed in the GC oven allowing a small portion of the column to be immersed in the liquid nitrogen, during the desorption period. After five minutes desorption time, the cryofocus trap was quickly removed; the GC oven was shut, and the GC-MS run started.

GC-MS Reduced Run Time.

Previously developed methods of detection and quantification employed for GC-MS run times were nearly one hour in duration. This seemed unnecessary and inefficient for only two target compounds (IBMP and internal standard). After only a few series of trials, the GC-MS method and run time was successfully modified and reduced to less than five minutes. The protocol is as follows: The system used to analyze for IBMP was a Hewlett Packard (Palo Alto, CA) 6890 GC coupled with a Leco (St. Joseph, MI) Pegasus II MS attached to a Dell (Round Rock, TX) Dimension computer running Pegasus (Leco, St. Joseph, MI) version 1.33 GC-MS analysis software. The capillary column was a Supelcowax 10 (30m x 0.2 mm i.d.; 0.2 μm film thickness; Cat. No.: 24169, Supelco, Bellefonte, PA). The vector gas was Helium, with a flow rate of 1.2 mL/min. Injection was made in splitless mode. The temperature gradient from 40 °C to 80°C was 100 °C/min. The ramping rate was then changed to 50 °C/min until the final temperature of 240 °C was reached, then held for one min. Inlet temperature was set at 220 °C and the transfer line was set at 200 °C. Each injection lasted 4.6 minutes. Ion selection range was from *mass/charge* (*m/z*) between 36 and 174.

Final Protocol.

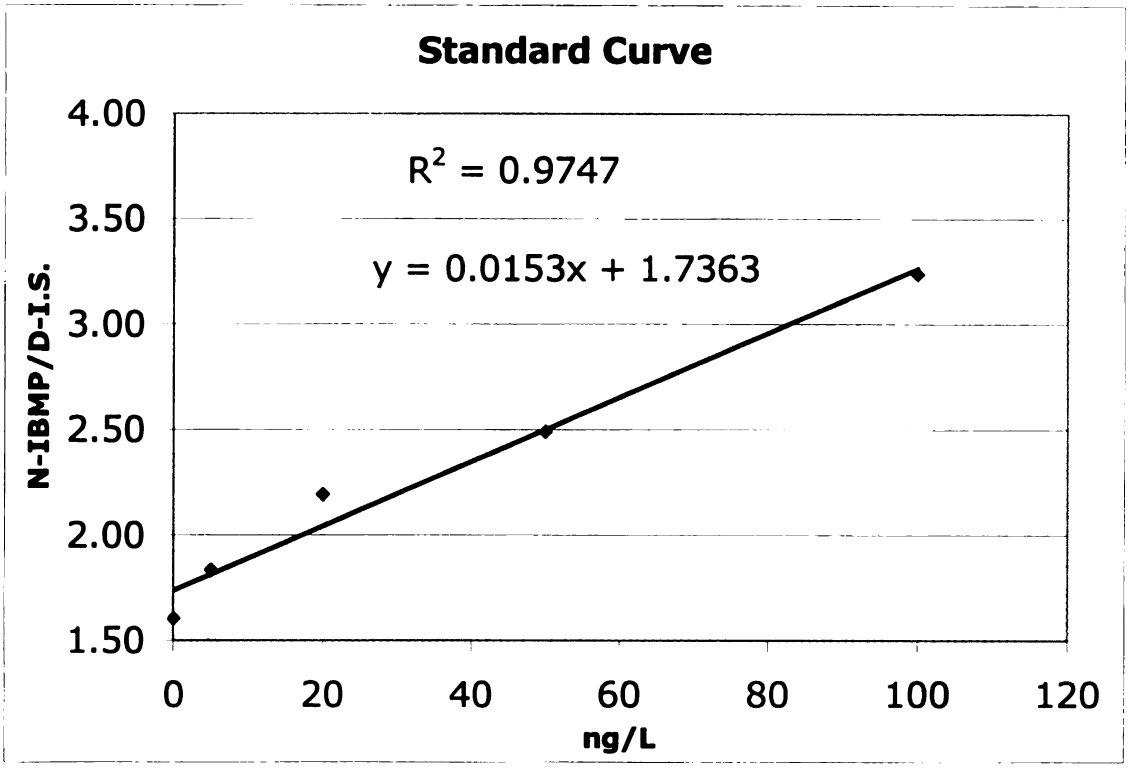
With modifications made to the distillation parameters, the ion exchange techniques, the replacement of liquid injections with HS-SPME, and the shortened GC-MS run time, a method for the detection and quantification of IBMP was in place, taking an average of an hour per sample (Figure 3). Simplifications from previous methods are shown in Table 1.

Standard curve development.

Initial efforts to develop standard curves for the quantification of IBMP were unsuccessful. Originally faux-wines were spiked with increasing amounts of IBMP and run through the sample preparation procedure with the expectation that the curve could be extrapolated to relate directly to wine samples. Several problems caused this approach to fail. Primarily, fluctuations in the GC-MS response caused multiple identical samples to have large variations in peak area, preventing direct comparison from one chromatogram to the next. The addition of an internal standard compensated for response fluctuations, maintaining the deuterated (D)-IBMP in appropriate ratios with the added naturally occurring IBMP. Additionally, wine is a very complex matrix; when wine samples were spiked with D-IBMP as well as the naturally occurring IBMP, the GC-MS responded with consistent peak ratios. It is important to develop standard curves in the matrix to be analyzed.

Standard curves.

The system was calibrated by adding increasing concentrations of IBMP (0-100 ng/L) to wine. The internal standard concentration was 80 ng/L. Each of the samples prepared in this way was extracted according to the method above. The ratio of the area of the naturally occurring IBMP to the D-IBMP was graphed versus the increasing concentration of the naturally occurring IBMP (Figure 2).



(Figure 2 – Initial standard curve developed in a wine matrix spiked with varying levels of IBMP; x-axis: ng/L IBMP added, y-axis: ratio of IBMP peak area over internal standard peak area)

Results and Discussion

The standard curve is the cornerstone of this assay, allowing one to quantify the unknown naturally occurring IBMP in wine. With the added quantity of internal standard known, a ratio of the naturally occurring IBMP over the D-IBMP can be determined and the unknown quantity of IBMP computed based on the equation of the standard curve. Because IBMP was added to a wine that contained an initial quantity of IBMP, the y-intercept will be somewhere above zero. When samples with unknown levels of IBMP are run for quantification, it can be assumed that the y-intercept is zero because no addition of IBMP will be made. The regression equation for wine is as follows: IBMP

concentration (ng/L) = $(A/A_{is})/0.0153$ (A, area of IBMP peak; A_{is}, area of internal standard peak). The curve was reproduced and the slope was identical (results not shown).

Conclusions

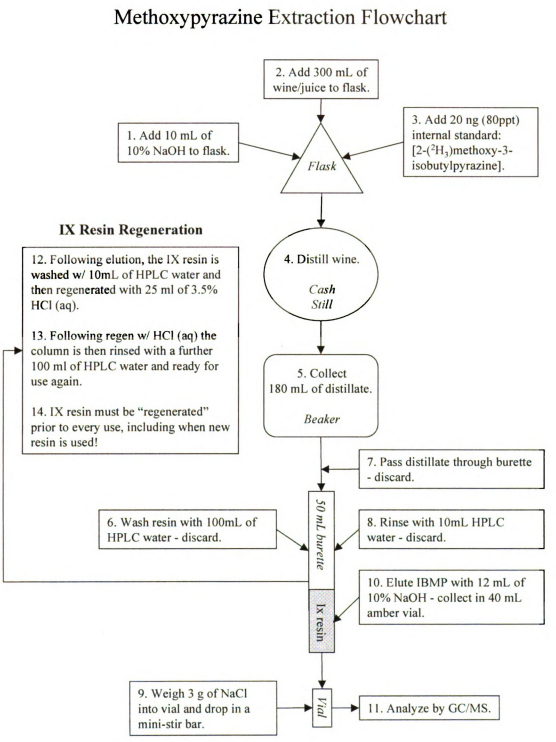
This modified method for detection and quantification of 2-methoxy-3-isobutylpyrazine offers the analyst a faster, simpler, less complicated assay. The distillation process has been enhanced to accommodate varied and larger sample sizes. The improved method employs solid phase microextraction, eliminating the need for solvents and simplifying the labor in concentrating and analyzing volatile compounds in the headspace. The GC-MS run time is much shorter than in previously published methods. This simplified method can be improved. However, the advantages and combined analytical techniques in the above assay allow for faster sample preparation and analysis time, helping to encourage greater efforts related to conditions leading to IBMP reduction into the vineyard and cellar. This is a method of detection and quantification for 2-methoxy-3-isobutylpyrazine that is readily reproducible and can be conducted in a timely fashion.

Summary of Simplifications for the Detection and Quantification of IBMP in Wine

Category	Previous methods' technique	Simplification
Distillation	Distillation/ small Cash still	Custom Cash Still
IX resin	Peristaltic pump, pipette	50 mL burette/ regeneration
Elution	1 mL 10% NaOH	12 mL 10% NaOH
Sample volume	100-240 mL	≥ 300 mL
Sampling	Direct Injection	HS-SPME
GC run time	> 45 min	< 5 min
Total run time	Estimated at > 2 hrs/ sample	≤ 1 hr /sample
Cost / sample	Estimated at $100/ sample	≤ $25/ sample

(Table 1- simplifications made to IBMP detection and quantification)

(Figure 3- IBMP extraction flowchart)



Literature Sited

1. Allen, M.S., M. J. Lacey, S. Boyd. 1994 Determination of methoxypyrazines in red wines by stable isotope dilution gas chromatography mass spectrometry. J. Agric. Food. Chem. 42:1734-1738.

2. American Water Works Association, Method 6040. Constituent Concentration by Gas Extraction. *Standard Methods for the Examination of Water and Wastewater.*

3. De la Calle García, D., M. Reichenbächer, C. Hurlbeck, C. Bartzsch, K-H. Feller. 1998. Analysis of wine bouquet components using headspace solid-phase microextraction-capillary gas chromatography. J. High Resol. Chromatogr. 21, 373-377.

4. Gandini, N., R. Riguzzi. 1997. Headspace solid-phase microextraction analysis of methyl isothiocyanate in wine. J. Agric. Food. Chem. 45:3092-3094.

5. Harris, R. L. N., M.J. Lacey, W.V. Brown, M.S. Allen. 1987. Determination of 2-methoxy-3-alkylpyrazines in wine by gas chromatography/mass spectrometry. Vitis. 26:201-207.

6. Hartmann, P.J., H.M. McNair, B.W. Zoecklein. 2002. Measurement of 3-Alkyl-2-Methoxypyrazine by Headspace Solid-Phase Microextraction in Spiked Model Wines. Am. J. Enol. Vitic. 54:285-288.

7. Hayasaka, Y., E.J. Bartowsky. 1999. Analysis of diacetyl in wines using solid-phase microextraction combined with gas chromatography-mass spectrometry. J. Agric. Food. Chem. 47:612-617.

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

9. Pawliszyn, J. 1999. Applications of Solid Phase Microextraction: Theory and Practice. John Wiley & Sons, Inc., New York.

10. Roujou de Boubée, D., C. Van Leeuwen, D. Dubourdieu. 2000. Organoleptic impact of 2-methoxy-3-isobutylpyrazine on red Bordeaux and Loire wines. Effect of environmental conditions on concentrations in grapes during ripening. J. Agric. Food. Chem. 48:4830-4834.

11. Supelco. 2001. SPME – A fast and inexpensive approach to trace organic analysis. The Reporter. Vol. 19.1.