The Effect of Wine Matrix Ingredients on 3-Alkyl-2-methoxypyrazines Measurements by Headspace Solid-Phase Microextraction (HS-SPME)

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(ABSTRACT)

The effect of wine matrix ingredients and conditions on the headspace (HS) sampling of 3-alkyl-2-methoxypyrazines was investigated with solid-phase microextraction (SPME) and capillary gas chromatography, using a nitrogen phosphorus detector. Changes in the recovery of 3-ethyl-, isopropyl-, sec-butyl-, and isobutyl-2-methoxypyrazines from the static headspace of synthetic wine matrices spiked with 5µg/L of each analyte were investigated and reported as a function of SPME fiber type, extraction time, and temperature. The influence of pH, ethanol, phenolics, and ground oak was studied.

DVB/Carboxen™/PDMS SPME fibers at an extraction temperature of 50°C for 30 minutes with 30% (w/v) added sodium chloride resulted in the highest analyte recoveries. Although, PDMS (100 micron) SPME fibers at an extraction temperature of 35°C for 30 minutes with 30% (w/v) added sodium chloride resulted in the lower analyte recoveries, the fiber remained functional after 50 to 75 analyses after other coatings deteriorated. Changing the sample ethanol concentration from 0 to 20% (v/v) resulted in an exponential decrease in the recovered analytes. Below pH 2, there was extensive loss of the analytes in the headspace. No measurable impact on alkylmethoxypyrazine headspace concentrations was observed with exposures to selected phenolics and to ground oak.

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Chapter I: Introduction

Premium wine quality can be achieved only through a favorable balance between fruit, fermentation, and processing-derived aromas and flavors. The 3-alkyl-2-methoxypyrazines produce vegetative odors characteristic of green bell peppers and peas. The olfactory threshold at which these compounds are sensed is extraordinarily low, 1 ng/L in water. Methoxypyrazine concentrations in grapes are influenced by grape variety, fruit maturity, season, climate, and solar exposure of the fruit. These plant metabolites are found in grape varieties including Cabernet Sauvignon and Sauvignon Blanc, and may detract from both quality and varietal character. Measurement of these compounds is, therefore, of significant interest to the winemaker.

The objective of this research was directed at developing an analytical method for the characterization and measurement of alkyl-methoxypyrazines in wine. Research was conducted in the following areas to provide useful information to the winemaker.

The first aim was to develop a gas chromatographic method to separate, identify and characterize the 3-alkyl-2-methoxypyrazines present in wine using both mass spectrometry and a nitrogen selective detector.

The next objective was to optimize headspace solid-phase microextraction (HS-SPME) variables including fiber selection, sample volume, and extraction conditions for the quantitation of selected representatives of this class of compounds.

The influence of wine ingredients including alcohol, phenols, and cations, and conditions such as temperature, ionic strength, and pH on the headspace concentration of nitrogen containing aromas were determined using the most sensitive sample preparation and analytical techniques. Optimizing the analytical method to measure 3-alkyl-2-methoxy-pyrazines with part per trillion sensitivity was an additional objective.

Volatile Compounds Found in Wine

More than 200 volatile active aroma compounds have been identified in wines.[1] Twenty to thirty common volatiles have been determined and studied.[2,3] They represent a broad diversity of organic compounds with the following functionalities:

- alcohols
- organic acids
- esters
- terpenes
- phenols
- lactones
- sulfur compounds
- nitrogen compounds
- heterocyclic compounds

Many of these aroma volatiles are characteristic of specific varietal wines and are desirable at high concentrations. Terpenes are abundant in Rieslings, for example.

Other organic volatiles such as oak lactone are introduced by subsequent processing to further improve the sensory quality of wines.[4]

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1 Vernin, G., C. Boniface, J. Metzger, D. Fraisse, D. Doan, and S. Alamercery. "Aromas of Syrah wines: identification of volatile compounds by GC-MS-spectra data bank and classification by statistical methods". pp 655-685. Frontiers of Flavor, Proceedings of the 5th International Flavor Conference. Porto Karras, Chalkidiki, Greece (1987)

2 Baumes, R., R. Cordonnier, S. Nitz, and F. Drawert. "Identification and determination of Volatile Constituents in wine from different vine cultivars." J. Sci. Food Agric. 37:927-943 (1986).

3 Lopez-Tamammes, E., N. Carro-Marino, Y.Z. Gunata, C. Sapis, R. Baumes, and C. Bayonove. "Potential aroma in several varieties of Spanish grapes." J. Agric. Food Chem. 45:1729-1735 (1997).

Not all organic volatiles in wine contribute to its aesthetic characteristics. Some wine volatiles are detrimental to quality, including the moldy scent of trichloroanisole, associated with cork taint[5]. *Brettanomyces sp* contamination in the fermentation produces 4-ethylphenol and 4-ethylguaiacol[6], which are responsible for off odors in wines.

Other important volatiles, which do not necessarily contribute to the odor of wine, have also been measured. For example, measuring ethyl carbamate, a natural fermentation product which may be carcinogenic, could easily become a priority if it becomes regulated in the future. Methyl isocyanate, a toxic fumigant, has also been successfully detected in wine using solid phase microextraction and gas chromatography.[7]

Table 22, in Appendix A, lists the range of common analytes associated with three varieties of wines, Cabernet Sauvignon, Pinot Noir, and Chardonnay.

The Sample Matrix: Wine

Wine is a complex mixture of ingredients prepared by the fermentation of carbohydrates in grapes by yeasts. Wine composition varies widely, influenced by grape

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4 Pollnitz, A.P., G.P. Jones, and M.A. Sefton. "Determination of oak lactones in barrel aged wines and in oak extracts by stable isotope dilution analysis." J. Chromatogr. A 857:239-246(1999).

5 Dufour, C. and C. Bayonnove. "Influence of wine structurally different polysaccharides on the volatility of aroma substances in model systems." J. Agric Food Chem. 47:671-677(1999).

6 Chatonnet, P., D. Dubourdieu , J.N.Boidron, and M. Pons. "The origin of ethyl phenols in wines." J. Sci. Food Agric. 60:165-178(1992).

7 Gandini, N., and R. Riguzzi. "Headspace solid-phase microextraction of methyl isocyanate in wine." J. Agric. Food Chem. 45:3092-3094 (1997).

source and quality, as well as processing conditions. A representation of typical matrix composition for a red wine[8, 9] is illustrated in Figure 1.

Most wine ingredients are capable of impacting the level of aroma volatiles in the headspace to some degree by a variety of mechanisms that change their solubility or volatility.

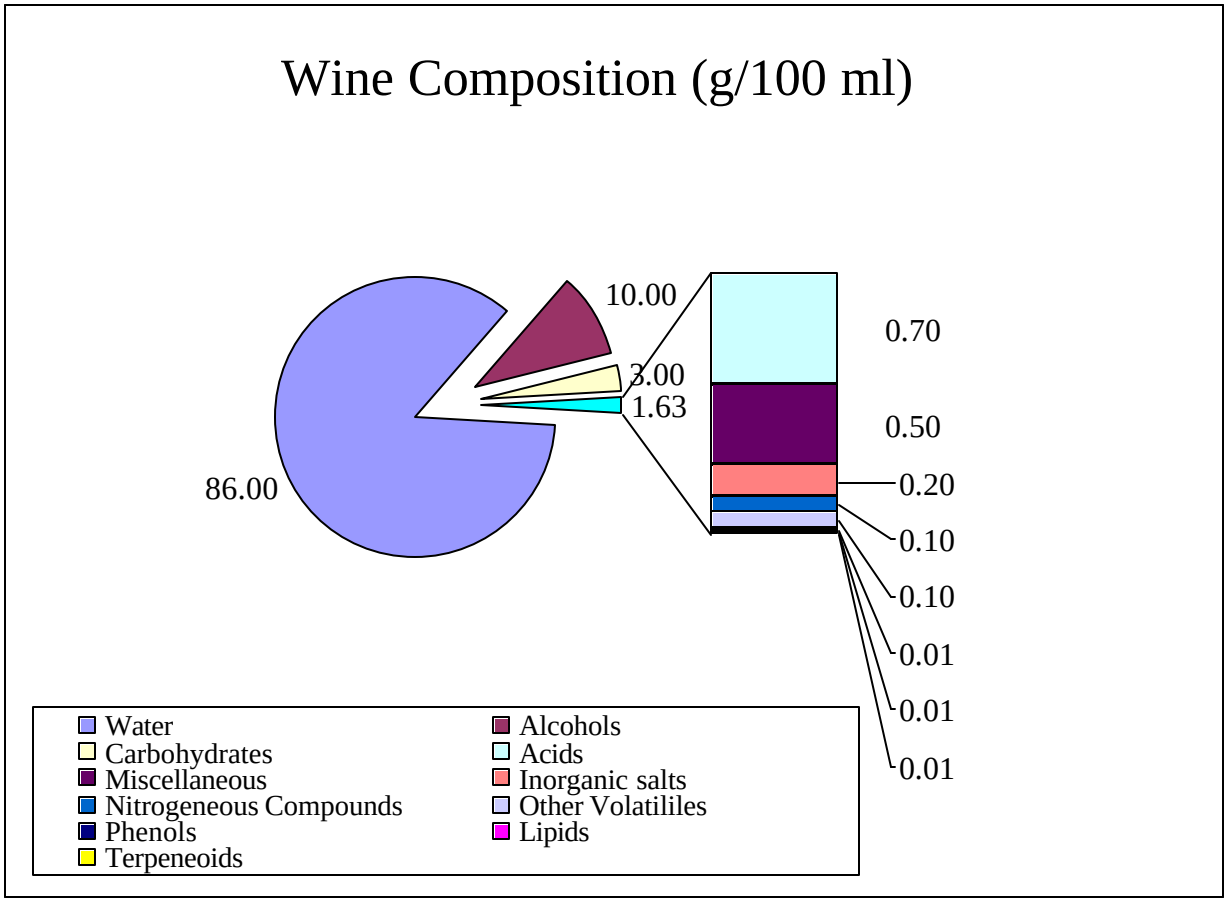

Figure 1 Typical Red Wine Matrix

Water the principle component, solvates odorants to varying degrees related to the polarities and functional groups of these organic molecules.

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8 Amerine, M.A., R.E. Kunkee, C.S. Ough, V.L. Singleton, and A.D. Webb. The Technology of Wine Making. AVI Publishing Company, Inc. Westport, CT, (1979).

9 Zoecklein, B.W., K.C. Fugelsang, B.H. Gump, F.S. Nury. Wine Analysis and Production. Chapman&Hall, New York, NY, 1995.

Altering the ionic strength of the aqueous phase exerts a significant effect on the solubility and volatility of most organics. Sodium chloride is, therefore, added to wine samples to reduce the partition coefficient in the aqueous phase and increase the aroma volatile concentration in the headspace.[10] Without adjustment of ionic strength trace volatiles exhibit low and variable levels in the sample headspace.

The pH of the wine also imparts a significant influence on the concentration of some classes of the aroma compounds in the headspace. In acidic conditions, the ionization of organic acid and phenolics is suppressed. As a result, acidic compounds tend to be more volatile at low pH. Conversely, nitrogen compounds, which are basic, are significantly ionized in acidic matrices, consequently, less volatile at low pH.

In addition to the influence of the aqueous matrix, organic constituents of the wine affect the solubility and volatility of aroma analytes. Ethanol, the second most abundant component of wine, has been reported to affect the recovery of volatiles substantially by reducing the capacity of the SPME fibers and increasing the capacity of the aqueous sample phase.[11,12]

Many aroma ingredients can bind with carbohydrates forming glycosidic bonds. A comprehensive assessment of the quantity of aroma volatiles in wines requires hydrolysis of wine glycosides.[13] Organic acids and lipids, which are odorants

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10 Yang, X., and T.Peppard. "Solid phase microextraction for flavor analysis." J. Agric. Food Chem. 42:1925-1930 (1994).

11 Ferreira, V., M. Ardanuy, R. Lopez, and J. F. Cacho. "Relationship between flavor dilution values and odor unit values in hydroalcoholic solutions: role of volatility and a practical rule for its estimation." J. Agric. Food Chem. 46:4341-4346 (1998).

12 Yang, X., and T.Peppard. "Solid phase microextraction for flavor analysis." J. Agric. Food Chem. 42:1925-1930 (1994).

13 Herraiz, T., g. Reglero, P.J. Martin-Alverez, M. Herriaz, and M. Cabezudo. "Identification of aroma components of Spanish 'Verdejo' wine." J. Sci. Food Agric. 55:103-116 (1991).

themselves, ionize variably depending on pH. They affect the matrix pH and ionization of other organics in the wine.

Phenolics and anthocyanins have been shown to react weakly with some aroma volatiles such as vanillin and syrigealdehyde.[15] Catechin and polyphenols exhibit similar weak affinity to volatiles including esters and aldehydes.[14]

Nitrogen compounds are weak bases capable of reacting with the acidity in wine. When the pH increases above their pKa nitrogenous species are not strongly disassociated into ions. In the organic base form they are more volatile.

The complexity of wine as a matrix and plethora of potential analytical interferences necessitate sample preparation to isolate and concentrate the volatile analytes. Volatiles in the headspace are free from many of the interferences of the liquid matrix and may only require concentration before GC analysis. The headspace of wine is an integral part of the product aesthetics. It is also an alternate sample source containing the aroma analytes. Arguably the headspace of wine may represent the wine aroma quality with better accuracy than the liquid.

The Analytes: Pyrazine Aroma Compounds

Numerous nitrogen compounds are present in wine, as shown in Table 2. They include the range from ammonia and volatile amines, to amides, to nonvolatile amino acids and heterocycles like histamine.[15] One class of heterocyclic compounds, pyrazines are found in many foods and beverages, affecting flavor and odor significantly. The most abundant variety of pyrazine derivatives, more than 60 distinct compounds, occurs in chocolate and coffee.

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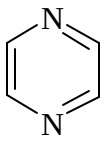
14 Dufour, C., and C.L. Bayonove. "Interactions between wine polyphenols and aroma substances. An insight at the molecular level." J. Agric. Food Chem. 47:678-684(1999).

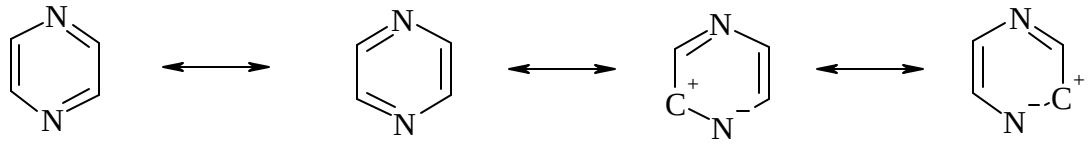
15 Nykanen, l. and H Suomalainen. Aroma of Beer, Wine, and Distilled Alcoholic Beverages. D. Reidel Publishing Company, Boston, MA,1983.

Pyrazine is a planar, six membered, unsaturated ring with nitrogen in the 1 and 4 positions as illustrated in Structure 1. Its chemistry is summarized by Barlin.[16] It has four distinct resonance forms depicted in Structure 2.



80.09
C4H4N2
C 59.99% H 5.03% N 34.98%

Structure 1 Pyrazine



Structure 2 Pyrazine Resonance

The carbons in pyrazine are electron deficient, because the nitrogens exert an inductive effect on their electrons. Consequently it is resistant to electrophilic substitution. The lone electron pairs on the nitrogen in pyrazine are relatively poorly delocalized with a calculated density of 1.88. The electron density of the second nitrogen is 1.37, which is comparable to pyridine. Pyrazine can form relatively stable anions and act as a bidentate ligand with transition metals.

Alkylated pyrazines are solids or semivolatile liquids. Increasing substitution by alkyl groups increases their boiling point and decreases their water solubility. The alkyl groups donate electrons into the pyrazine ring stabilizing some of the resonance

16 Barlin, G.B., The Pyrazines. The Chemistry of Heterocyclic Compounds, John Wiley and Sons, New York, 1982.

structures. A series of alkylkated pyrazines including ethyl, diethyl, 5,6,7,8-tetrahydroquinoxaline, and quinoxaline was selected for chromatographic study.

Alkylpyrazine basic ionization constants (K_b) increase as alkyl substituents are added. The pK_b of pyrazine is 0.65 compared to 5.23 for pyridine, 1.45 for methylpyrazine, 1.90 for 2,3-dimethylpyrazine, and 3.55 for tetramethylpyrazine.

Alkoxypyrazines are potentially more strongly stabilized by the electron donating methoxy function. Several of the compounds were studied, including the principle analytes in wine.

Alkylmethoxypyrazines are found in Cabernet Sauvignon, Sauvignon Blanc and green bell peppers. They are key varietal aroma characteristic of these wines. Excessive levels of these intensely potent compounds easily overwhelm and unbalance the wine. Micro-oxygenation, an accelerated aging process, sometimes appears to increase their perception. An analytical method for determining their level in wines will be useful in determining the nature of the factors governing the release of these potent aromas in such new processes.

Chemical and aroma properties of compounds studied are summarized in Table 1. 3-Isobutyl-2-methoxypyrazine is the predominant pyrazine present in wine representing approximately 80%, of the 5 to 50 ng/L commonly found in wines.[17] Its green bell pepper odor, has a reported threshold of $1x10^{-3}$ ng/L in air.

The remaining pyrazines, 3-isopropyl-2-methoxypyrazine and 3-sec-butyl-2-methoxypyrazine each represent 10% of the pyrazines found in wines. 3-ethyl-2-methoxypyrazine is only present at trace levels.

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17 Allen, M.S. "Stable isotope dilution gas chromatography-mass spectrometry for determination of methoxypyrazines ("green" aromas) in wine." Modern Methods of Plant Analysis. Vol. 19. Springer Verlag, Berlin, (1997).

The 3-isopropyl-2-methoxypyrazine analyte smells like potatoes. 3-sec-butyl-2-methoxypyrazine and 3-ethyl-2-methoxypyrazine both are contributers to the scent of green peas and other vegetables like asparagus.

3-sec-buty-2-methoxypyrazine is chiral at the secondary carbon of the butyl group. This is potentially significant because enantiomers of aroma compounds can vary significantly in olfactory potency. The remaining pyrazines are achiral.

Two compounds, 3-ethyl-2-ethoxypyrazine and 3-isopropyl-2-ethoxypyrazine, were selected as internal standards, because they are chemically similar to the pyrazines found in wine but not present in the samples.

Several functional variations of methoxypyrazines were selected for determination of chromatographic behavior. The acetyl analog, 3-ethyl-2-acetylpyrazine, contains the electron withdrawing carbonyl in contrast to the alkoxypyrazines. Alkoxy groups of the type found in the 3-alkyl-2-alkoxypyrazines are electron donators. 3-ethyl-2-chloropyrazine characterized by the strongly electron withdrawing chlorine atom was .also selected for comparison.

3-Ethyl-2-methylthiopyrazine was selected as the sulfur analog to the 3-alkyl-2-alkoxy pyrazines.

Table 1 Pyrazine Compounds Studied in this Work

Name	CAS	MW	Formula	Bp°C	Mp°C	Odor/flavor MDL (ppb in water)	Odor	Density	RI	Comments
Pyrazine	290-37-9	80.09	$C_4H_4N_2$	115.5	54	0.65				K_{a1}=0.65 K_{a2}=-5.78
2-Ethylpyrazine	13925-00-3	108.1426	$C_6H_8N_2$			6,000-22,000	Nutty, walnut, roasted, buttery, musty, woody, peanut butter			
2-Methoxypyrazine	3149-28-8	110.12	$C_5H_6N_2O$			700				
Quinoxaline	00091-19-0	130.15	$C_8H_6N_2$							
5,6,7,8-Tetrahydroquinoxaline	34413-35-9	134.1804	$C_8H_{10}N_2$	220 – 222, 85.00 C @ 3.00 mm			coffee, cheese, sweet roasted nut	1.079		
2,3-Diethylpyrazine	5910-89-4	136.200	$C_8H_{12}N_2$	180 – 182		5000	Nutty, hazelnut, cereal, earthy, fungi, potato, meaty	0.97	1.496-1.506	
3-Ethyl-2-methoxypyrazine	25680-58-4	138.170	$C_7H_{10}N_2O$			0.4	Roasted nut, hazelnut, earthy			
3-Ethyl-2-chloropyrazine		142.590	$C_6H_7ClN_2$							Chloride potentially hydrolyzes
3-Ethyl-2-acetylpyrazine	32974-92-8	150.1798		55 at 1.10 mm, 220-221			Potato chip, popcorn, nutty, meaty	1.075	1.509 – 1.518	Acetyl is electron withdrawing

Table 1 Pyrazine Compounds Studied in this Work

Name	CAS	MW	Formula	Bp°C	Mp°C	Odor/flavor MDL (ppb in water)	Odor	Density	RI	Comments
3-Isopropyl-2-methoxypyrazine	25773-40-4	152.200	$C_8H_{12}N_2O$	94-100	61.5	0.002	Earthy, bell pepper, potato, vegetable	0.996	1.4940	
3-Ethyl-2-ethoxypyrazine	35243-43-7	152.200	$C_8H_{12}N_2O$				Raw potato			
3-Ethyl-2-methylthiopyrazine	72987-62-3	154.240	$C_7H_{10}N_2S$				Strong roasted or cooked meat			
3-Isopropyl-2-ethoxypyrazine		166.22	$C_9H_{14}N_2O$							
3-Sec-butyl-2-methoxypyrazine	24168-70-5	166.220	$C_9H_{14}N_2O$	218- 219		0.001	green pea, bell pepper	1.005	1.4940	
3-Isobutyl-2-methoxypyrazine	24683-00-9	166.220	$C_9H_{14}N_2O$	120-126		0.002	green bell pepper	0.990	1.4922	

Sample Preparation Alternatives

The high water content of wine as a liquid matrix necessitates a cleanup or concentration step, before volatiles can be subjected to capillary gas chromatographic analysis. Direct injection is impractical because of the adverse effects on column performance and overall system degradation that results from the water and carbohydrates in the sample matrix. The water hydrolyzes carbowax stationary phases at high operating temperatures. Carbohydrates coat the injector and column creating noise and altering the chromatography.

Aroma volatiles have been isolated from wine by solvent extraction with pentane/dichloromethane or Freons. Significant reduction in volume, concentrating the analytes several orders of magnitude, follows, in preparation for GC analysis. This technique both removes the analytes from the aqueous matrix and concentrates them to improve sensitivity. Solvent extraction is often prohibitively expensive and time consuming.

Ion exchange and bonded phase concentration columns have been applied to wine for solid phase extraction of selected aroma volatiles, achieving isolation from the interfering matrix and concentration of the aroma compounds. Nitrogen compounds including alkylmethoxypyrazines have been isolated with this technique.

As an alternative to the analysis of the wine aqueous phase, its headspace has been analyzed to measure its aroma and flavors. Several techniques have been applied including dynamic and vacuum headspace, adsorption-desorption, large volume injection, and SPME.

The composition of the headspace over any fragrant sample, wine in particular, depends on a variety of conditions including temperature, volume of each phase, agitation, equilibration time, purging conditions, partition coefficients, and matrix chemistry.

Accumulated headspace components from flowers have been purged into an adsorbant such as Tenax or carbon, in preparation for GC analysis, using dynamic headspace, or purge and trap. Flowers have also been subjected to vacuum trapping of their aromas off line before chromatographing. It should be noted that the dynamic purging was biased to the more volatile compounds, while the vacuum headspace contains a higher concentration of the high boiling aromas.[18]

Large volumes of the headspace gas have been injected directly on GC columns in lieu of adsorption/desorption or off line collection. The front of these columns are chilled cryogenically, to focus volatile analytes into a narrow band. Temperature programming is then employed to separate the analytes efficiently.

Solid-Phase Microextraction

Silica fibers coated with adsorbing phases such as polydimethylsiloxanes (PDMS), polyacrylates (PA) and carbowax (CW) can be inserted directly into liquid wine samples to isolate aroma components. The fiber is then introduced into the injection port of a gas chromatograph, where the volatile compounds are desorbed and introduced into the analytical column. This technique is called solid-phase microextraction (SPME).[19] It is not particularly robust with the direct insertion mode because the matrix degrades the adsorbing phase rapidly altering the recovery after a small number of samples. SPME fibers are, however, very effective in concentrating the headspace components.[20]

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18 Surburg, H., M. Guentert, and H, Harder. "Investigation of volatiles from flowers analytical and olfactory aspects." Recent Developments in Flavor and Fragrance Chemistry. pp 103-121. VCH, NY (1992).

19 Zhang, Z., M.J. Yang, and, J. Pawliszyn. "Solid-Phase Microextraction." Anal. Chem.66:844A-853A (1994).

20 Butzke, C.E., T.J. Evans, and S.E. Ebeler. "Detection of cork taint in wine using automated solid-phase microextraction in combination with GC/MS-SIM". In: Chemistry of Wine Flavor. A.L. Waterhouse and S.E. Ebeler (Eds.). ACS Symposium Series 714. pp 208-216. Am. Chem. Soc., Washington (1998).

Headspace SPME has received considerable attention because it is very effective in concentrating the analytes and can be used with most injectors without modification. Optimization of conditions is; however, required because there is a bias toward better recoveries of lower boiling, less polar compounds. The alkylmethoxypyrazines are polar semivolatiles, which will benefit from thoughtful selection of analytical conditions. Sala recently reported using HS-SPME in conjunction with GC-NPD to measure part per trillion levels of these compounds in musts.[21] Sala reports that PDMS-Divinylbenzene (DVB) was most effective for the alkylmethoxypyrazines.

Fiber type selection is very important to obtain the maximum recovery from the headspace. CW-PDMS fibers gave higher recoveries than PDMS alone, for nitrogen compounds.[22] For most low molecular weight species in headspace the PDMS-Carboxen fibers, which function by adsorption, offer the best efficiency of extraction.[23]

Other options for improving the recovery of SPME target analytes are to increase the temperature, which frequently improves the recovery. Longer headspace contact times usually help the extraction process. Sodium chloride addition to the aqueous-based liquid is another way of increasing the extraction efficiency. A range of all these conditions needs to be evaluated to obtain the most sensitive reliable analytical method.

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21 Sala, C., M. Mestres, M.P. Marti, O. Busto, and J. Guasch. "Headspace solid-phase microextraction method for 3-alkyl-2-methoxypyrazines in musts by means of polydimethylsiloxane-divinylbenzene fibers." J. Chromatogr. A. 880:93-99(2000).

22 De la Calle Garcia, D., M, Reichenbacher, K. Danzer, C.Hurlbeck, C. Bartzsch, and K.H. Feller. "Investigation of wine bouquet components by solid-phase microextraction capillary gas chromatography using different fibers." J. High Resol. Chromatogr. 20:665-668 (1997).

23 Shirey, R. "Optimizing of extraction conditions for low-molecular-weight analytes using solid-phase microextraction." J. Chromatogr. Sci.. 38:109-116(2000).

Chapter II: Experimental

Instruments

A Hewlett Packard (Palo Alto, CA) Model 6890 gas chromatograph equipped with splitless inlet and 2 mm liner, 30 meter by 0.25 mm ID, 0.25 micron HP-5-MS capillary column, and HP5973 mass spectrometer were used to characterize and confirm the identity of 15 pyrazine compounds studied.

A second HP6890 with splitless inlet, 0.75 mm SPME liner, 30 meter by 0.25 mm ID 0.25 micron HP-5-MS capillary column, and both nitrogen phosphorous (NPD) and flame ionization detector (FID) were used to investigate target analyte SPME recovery.

The other capillary columns and liners used are documented with the discussions of the applicable work.

Standards

Commercially available standard pyrazine compounds and reference materials were purchased from Pyrazine Specialties (Atlanta, GA).

Stock solutions (approximately 250 ppm in concentration) were prepared from the pure compounds diluted in absolute ethanol. These stocks were stored in the dark below 0°C for subsequent dilution to the desired working concentrations.

Model Solutions or Synthetic Matrices

Extraction experiments were performed on a model solution prepared with HPLC grade water containing 12 %v/v absolute ethanol and 2 g/L potassium bitartrate, spiked with 5 ppb target analytes (3-ethyl, 3-isopropyl, 3-sec-butyl, and 3-isobutyl-2-methoxypyrazine) and internal standards (3-ethyl and 3-isopropyl-2-ethoxypyrazine). This model matrix was used in all SPME studies except where

noted. Usually 20 mL of this solution were used, which contained 100 picograms of each analyte.

The water was EM Science (Gibbstown, NJ) OmniSolv HPLC grade. USP grade 200 proof ethanol was supplied by AAPER Alcohol and Chemical Co. (Shelbyville, KY). Potassium bitartrate, catechin, and gallic acid were supplied by the Virginia Tech Food Science and Technology Department (Blacksburg, VA). The sodium chloride and other salts used were analytical reagent grade supplied by Mallincktrodt (Paris, KY).

Solid Phase Microextraction (SPME) Equipment and Methods

Most of the commercially available SPME fibers were evaluated to establish which resulted in the best recovery of nitrogen compounds. Supelco, Inc. (Bellefonte, PA) supplied these fibers, holders, and a heating block.

Unless stated otherwise, the headspace microextractions were conducted in 40 mL headspace vials 25mm in OD and 90mm tall with septum or Teflon™ valved caps.

Sample temperature control was maintained in 50 mm ID by 110mm depth jacketed beakers connected to a circulation water bath filled to the level of sample in the headspace vial. Stirring was provided with a Teflon coated stir bar inside the extraction vial driven with an external magnetic stir plate under the apparatus. The heating block provided by Supelco (Bellefonte, PA) was tried, but provided less precise control of temperature than the water bath.

Sample volumes of 20 mL were used except where noted. All experiments were stirred as rapidly as possible. Normal extraction conditions were 35°C, for 30 minutes, with 30 % added sodium chloride. Where these conditions were not employed the specific differences are documented.

The pH of all samples was measured after each extraction to assure that changes which might affect the analyte concentration had not occurred.

Analyte peak area counts were normalized by dividing with their concentration in the extract, in pats per billion. This relative response provided a basis for comparison independent of analyte concentration.

Chapter III: GC Methods Development

Column Selection

Initially, Carbowax columns were tested for this application, because Sala[21] reported polyethyleneglycols were effective for resolving these analytes. Thirty meter long, 0.25mm ID, 0.25 micron film thickness Perkin Elmer (Norwalk, CT) PE Wax101 and Phenomenex (Torrance, CA) ZB Wax were tested with ten pyrazines. Good separations were achieved. The compounds separated in order of elution, are shown in Figure 2: 2-ethylpyrazine, 3-isopropyl-2-methoxypyrazine, 2,3-diethylpyrazine, 3-sec-butyl-2-methoxypyrazine, 3-ethyl-2-methoxypyrazine, 3-ethyl-2-chloropyrazine, 3-isobutyl-2-methoxypyrazine, 3-ethyl-2-acetylpyrazine, 5,6,7,8-tetrahydroquinoxaline, and 3-ethyl-2-methylthiopyrazine.

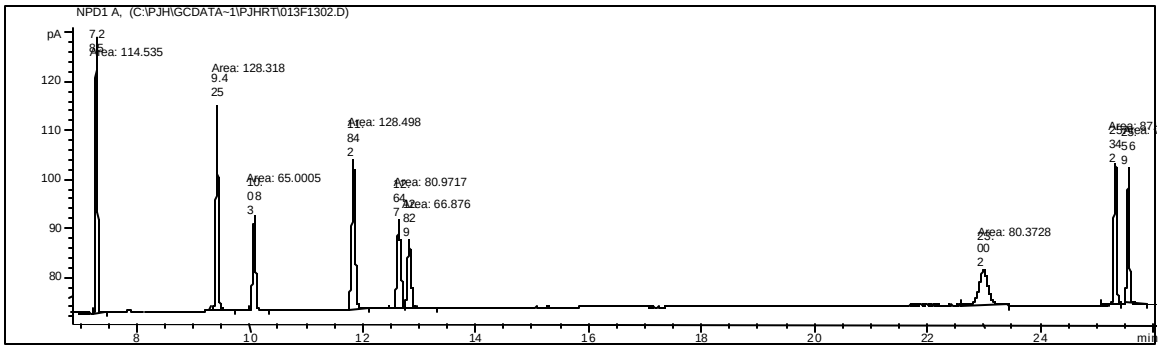


Figure 2 Nine Pyrazines Separated on 30 meter 0.25 mm 0.25 micron PE Wax

Phenomenex (Torrance, CA) ZB-35, 35% phenyl-dimethypolysiloxane, column was then tried as an alternative column or for use in confirmation. These columns were also 30 meters long by 0.28 mm ID with 0.25 micron film thickness. The pyrazine analytes separated in the same order on ZB35 as on ZB Wax.

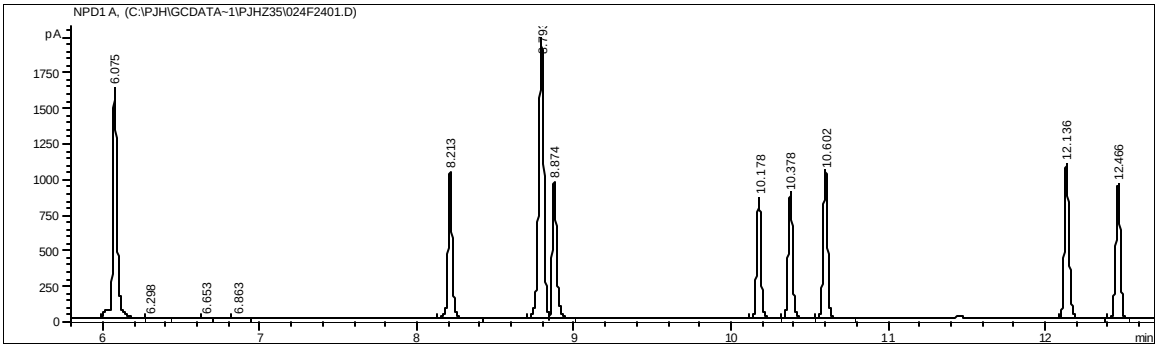


Figure 3 Nine Pyrazines Separated on 30 meter 0.25 mm 0.25 micron Phenomonex ZB35

The best resolution column evaluated was a Hewlett Packard 30 meter, 0.25 mm ID, 0.25 micrometer film thickness, HP-5MS 5% phenyl-dimethylpolysiloxane column which provided excellent results for this analysis. Sample chromatograms are attached Figure 4.

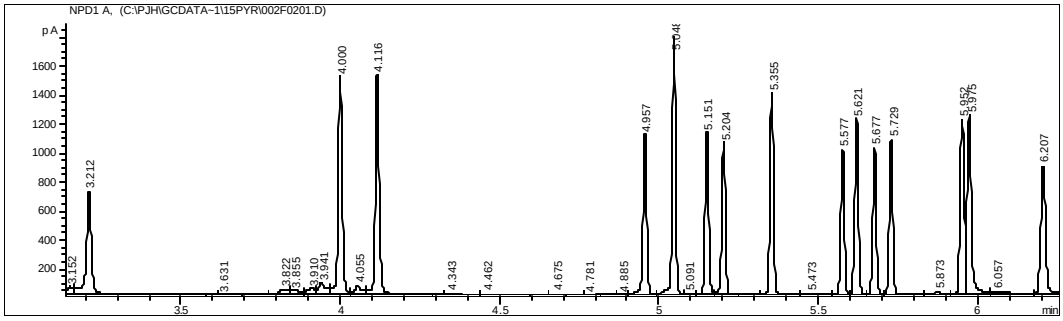


Figure 4 Fifteen Pyrazines Separated with 30 meter 0.25 mm 0.25Micron HP-5MS

Table 2 Pyrazine Elution Order on Selected GC Phases (Minutes)

Analyte	HP 5	ZB 35	PE Wax	ZB Wax
2-Ethylpyrazine	4.12	5.42	6.71	7.28
3-Ethyl-2-methoxypyrazine	4.96	11.65	11.29	11.86
3-Ethyl-2-chloropyrazine	5.05	8.68	11.94	12.66
2,3-Diethylpyrazine	5.15	8.80	9.61	10.10
3-Isopropyl-2-methoxypyrazine	5.2	8.64	8.85	9.43
3-Ethyl-2-acetylpyrazine	5.62	12.86	22.18	23.03
3-Sec-butyl-2-methoxypyrazine	5.68	11.64	11.29	11.85
3-Isobutyl-2-methoxypyrazine	5.73	12.52	12.31	12.85
5,6,7,8-Tetrahydroquinoxaline	5.95	18.64	25.19	25.34
3-Ethyl-2-methylthiopyrazine	6.21	20.47	25.29	25.57

Capacity of the all the pyrazine compounds testes in Table 3 on HP-5 MS exceeded 2. Selectivities between neighboring eluents were usually larger than 1.00 except for 5,6,7,8-tetrahydroquinoxaline and quinoxaline, coeluted.

Table 3 HP-5 MS Chromatographic Parameters

Analyte	Retention time (tR) minutes	Capacity (k)	Selectivity between this and previous peak (α)
Dead time	1.16		
Methoxypyrazine	3.50	2.02	
ethylpyrazine	3.68	2.17	1.08
3-ethyl-2-methoxypyrazine	4.77	3.11	1.43
3-ethyl-2-chloropyrazine	4.86	3.19	1.02
2,3-diethylpyrazine	5.00	3.31	1.04
3-isopropyl-2-methoxypyrazine	5.06	3.36	1.02
ethyl-ethoxypyrazine	5.24	3.52	1.05
isopropyl-ethoxypyrazine	5.49	3.73	1.06
3-ethyl-2-acetylpyrazine	5.53	3.77	1.01
3-sec-butyl-2-methoxypyrazine	5.60	3.83	1.02
3-isobutyl-2-methoxypyrazine	5.65	3.87	1.01
5,6,7,8-tetrahydroquinoxaline	5.89	4.08	1.05
quinoxaline	5.91	4.09	1.00
3-ethyl-2-methylthiopyrazine	6.16	4.31	1.05

Kovats retention indices for TPGC for principal and related analytes were determined for the HP5-MS column. Data for two HP-5 MS columns compared in

Table 4 shows excellent reproducibility with separate columns producing results with 0.1% of each other. Only ethylpyrazine varied more, 1.9%.

Table 4 Kovats Retention Indices of Pyrazine Compounds on Different HP-5 MS Columns

Compound	MS Kovats Index	FID Kovats Index	Difference	% Difference
Methoxypyrazine	904	NA	NA	NA
Ethylpyrazine	928	946	-17	-1.9
3-Ethyl-2-methoxypyrazine	1063	1063	0	0.0
3-Ethyl-2-chloropyrazine	1074	1076	-2	-0.2
2,3-Diethylpyrazine	1092	1090	1	0.1
3-Isopropyl-2-methoxypyrazine	1099	1097	1	0.1
3-Ethyl-2-ethoxypyrazine	1127	NA	NA	NA
2-Isopropyl-3-ethoxypyrazine	1165	NA	NA	NA
3-Ethyl-2-acetylpyrazine	1170	1167	3	0.2
3-Sec-butyl-2-methoxypyrazine	1180	1176	4	0.3
3-Isobutyl-2-methoxypyrazine	1187	1185	3	0.2
5,6,7,8-Tetrahydroquinoxaline	1226	1223	3	0.2
Quinoxaline	1229	NA	NA	NA
3-Ethyl-2-methylthiopyrazine	1270	1267	3	0.2

Calibration and Detection Limits

Internal standard calibrations, using five replicate, 1 µL, direct injections of standards between 6 and 125 ppb (6 and 125 picograms) were performed using the 3-ethyl-2-ethoxypyrazine as the internal standard. Figure 5 illustrates that reasonable linearity with detection limits of 6 picograms or 6 ppb can be achieved.

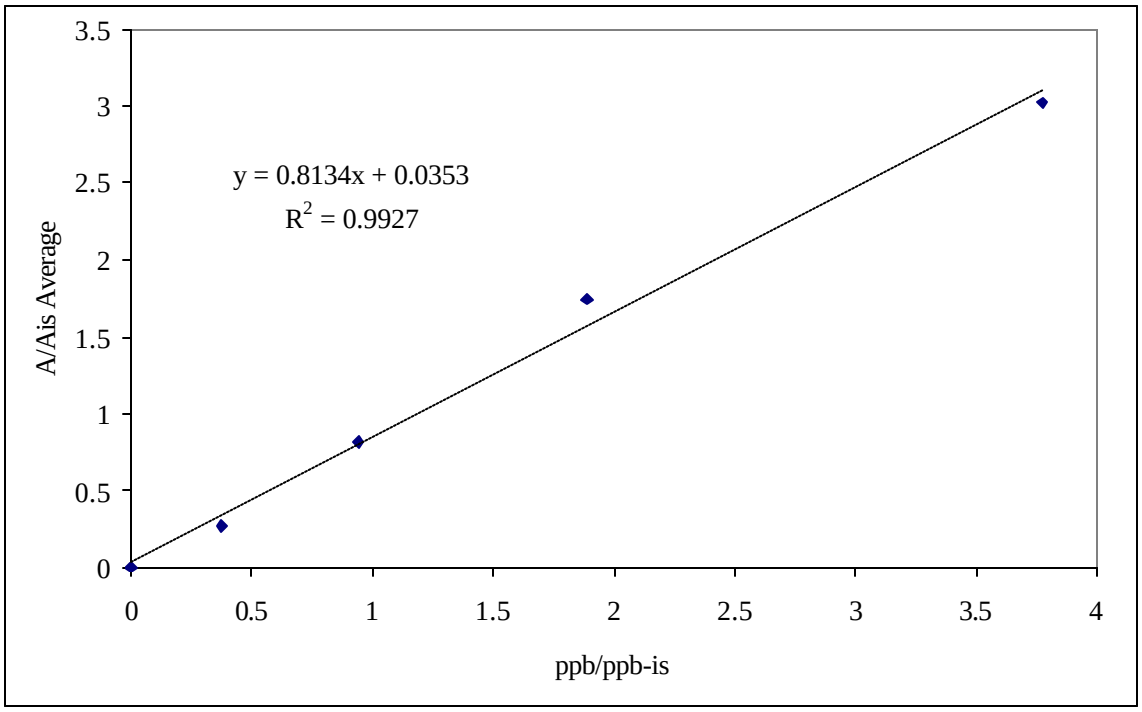


Figure 5 Internal Standard Calibration of 3-Ethyl-2-methoxypyrazine

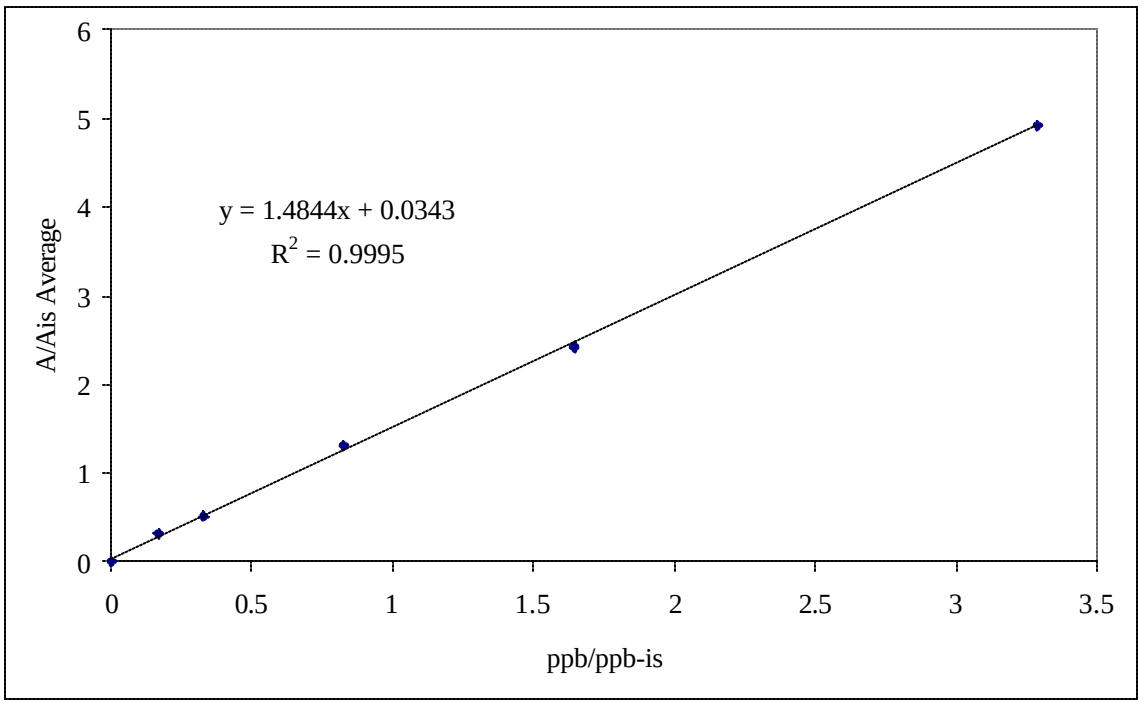


Figure 6 Internal Standard Calibration of 3-Isopropyl-2-methpoxypyrazine

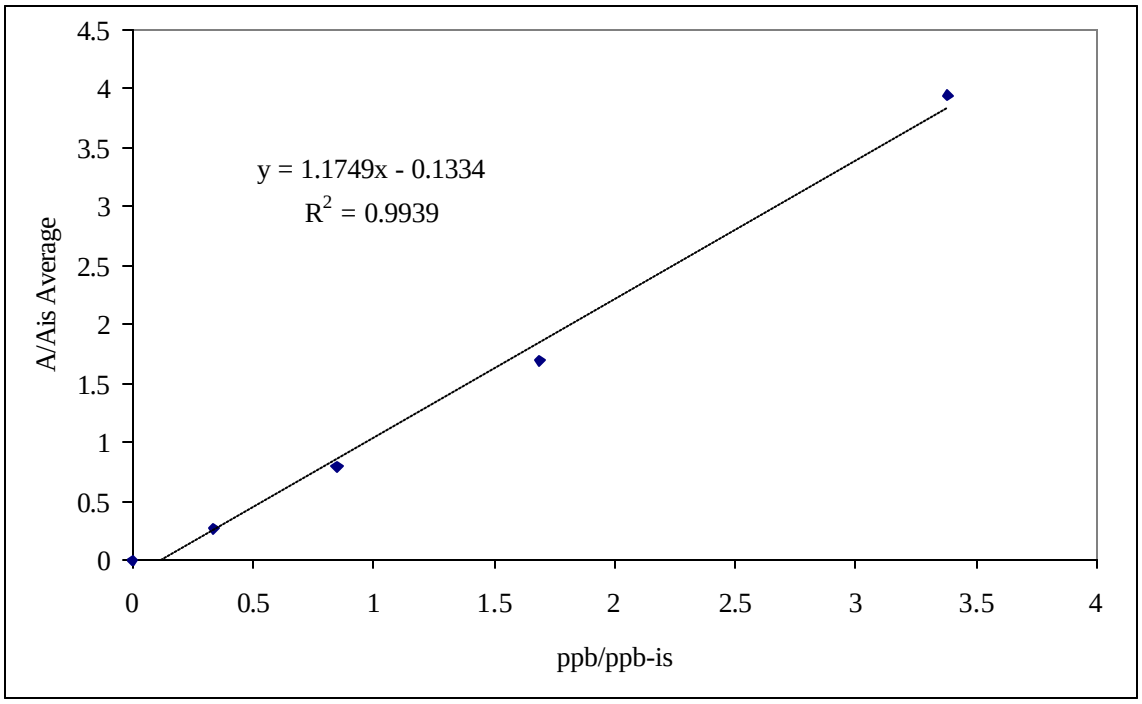


Figure 7 Internal Standard Calibration of 3-Sec-butyl-2-methoxyopyrazine

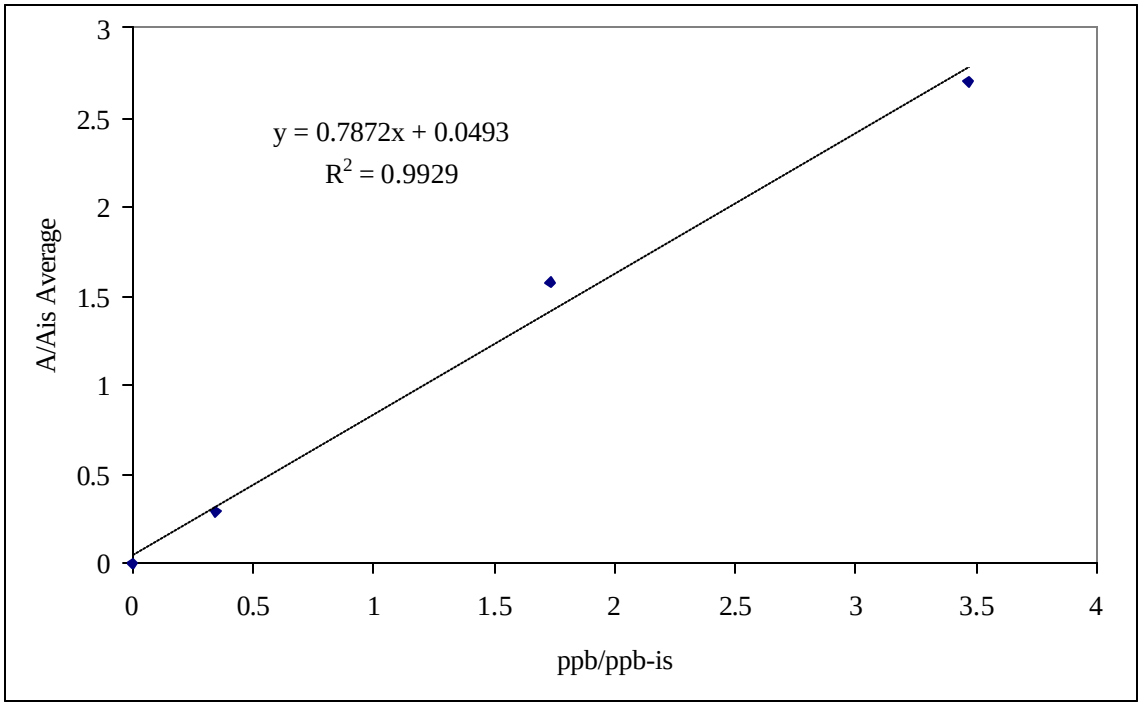


Figure 8 Internal Standard Calibration of 3-Isobutyl-2-methoxypyrazine

A method of standard additions analysis of Silver Ridge (Sonoma, CA) Cabernet Sauvignon as the matrix with analyte additions ranging from 150 to 700 parts per trillion, (2 to 14 picograms) was conducted using PDMS SPME fibers to extract the sample headspace. The extractions were performed for 30 minutes at 35°C in 40 mL headspace vials on 20mL samples with 30% sodium chloride added.

Correlation coefficients for this experiment were between 0.93 and 0.98. The intercepts were all negative suggesting the sample does not contain the analytes or the method does not detect the quantities present in the wine.

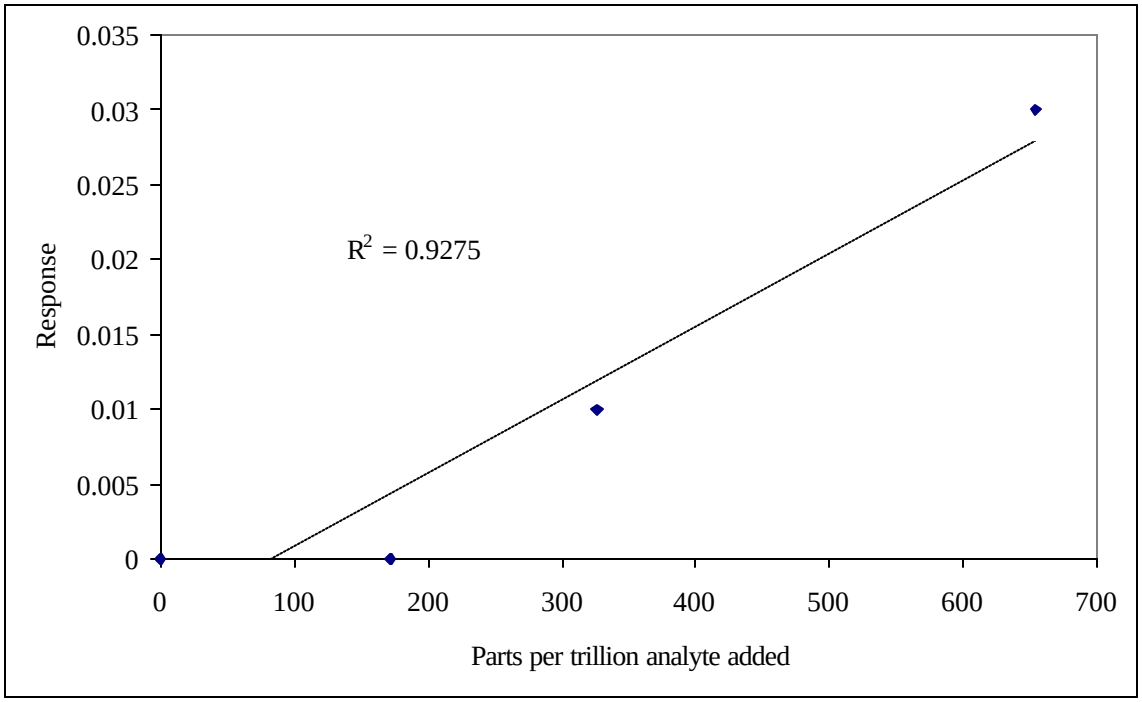


Figure 9 3-Ethyl-2-methoxypyrazine Method of Standard Additions

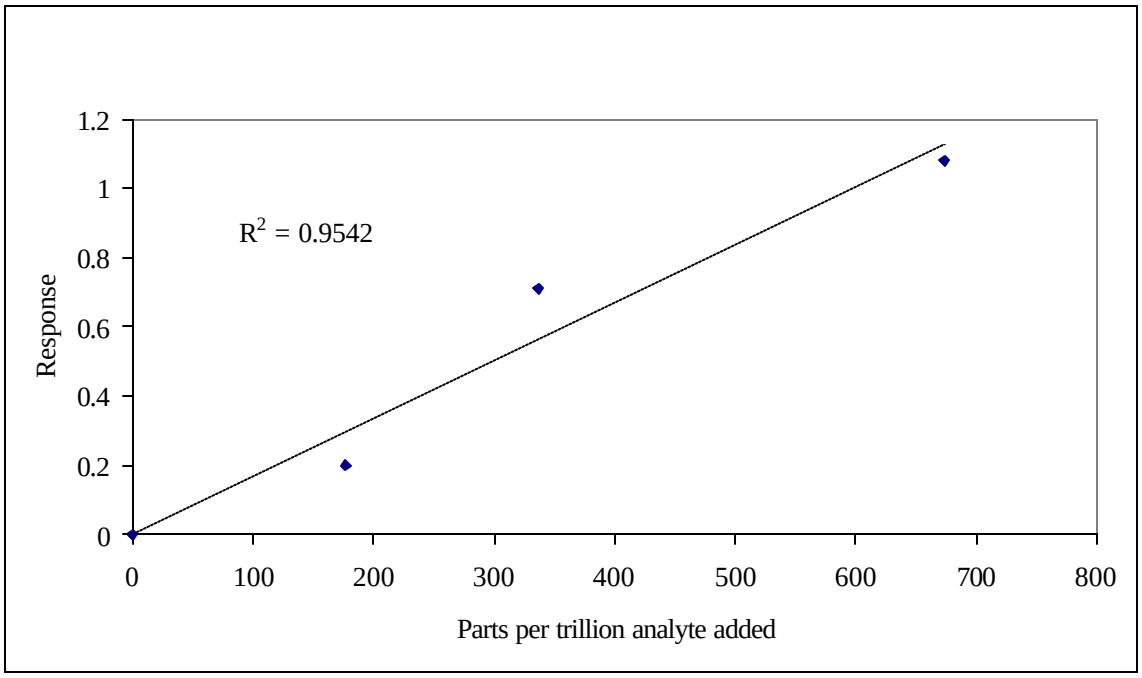


Figure 10 3-Isoprpropyl-2-methoxypyrazine Method of Standard Additions

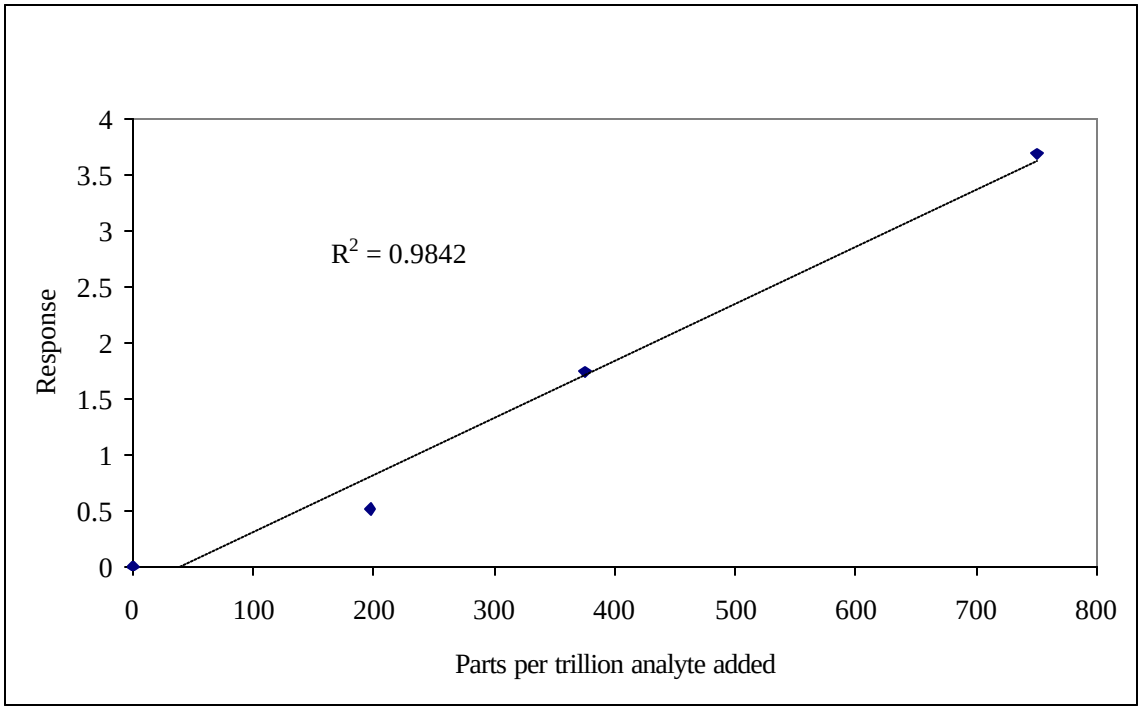


Figure 11 3-Sec-butyl-2-methoxypyrazine Method of Standard Additions

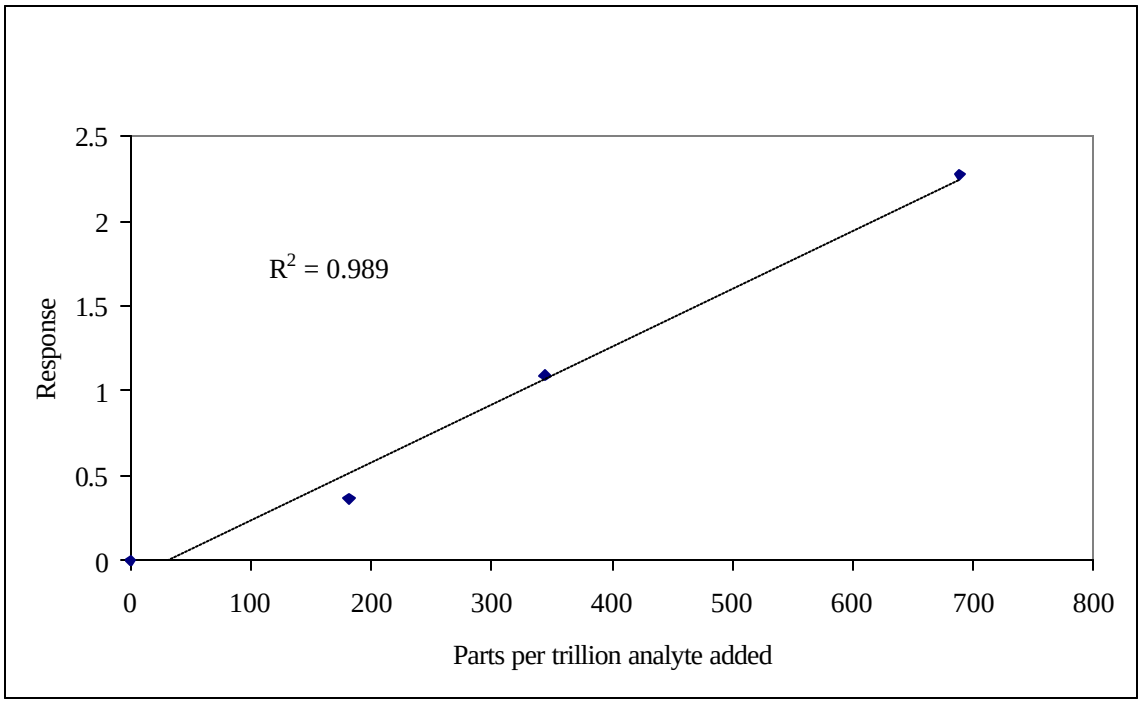


Figure 12 3-Isobutyl-2-methoxypyrazine Method of Standard Additions

Chapter IV: Mass Spectrometry

All samples were analyzed by GC/MS for several reasons. First GC/MS verified the structure and identity of the standards as the desired compounds. It also confirmed the elution order of the analytes for the chromatographic method employed with the NPD/FID system. Additionally the collected mass spectra provide the necessary information about the target compound fragmentation patterns to perform more sensitive selected ion monitoring,

GC/MS Operating Conditions

One microliter of a standard with approximately 1ppm of each of 15 pyrazines in ethanol was injected into an HP6890 gas chromatograph equipped with an HP5973 MSD operating under the following conditions:

- The inlet was maintained at 250°C, operated in split mode 20:1, with 1 mL/minute helium initially at 7.7psi.
- The column was an HP5-MS (5% phenyl-95% dimethlypolysiloxane) 30 meters long and 0.25mm inside diameter with a 0.25 micrometer film thickness.
- Temperature programming started at 50°C; held for 1 minute, followed by a 20°/minute ramp to 80 °, followed by a 25°/minute ramp to 250°, held for 3 minutes.

Analyte Mass Spectra

Figure 13 TIC Chromatogram of 15 Pyrazines Separated on HP-5MS

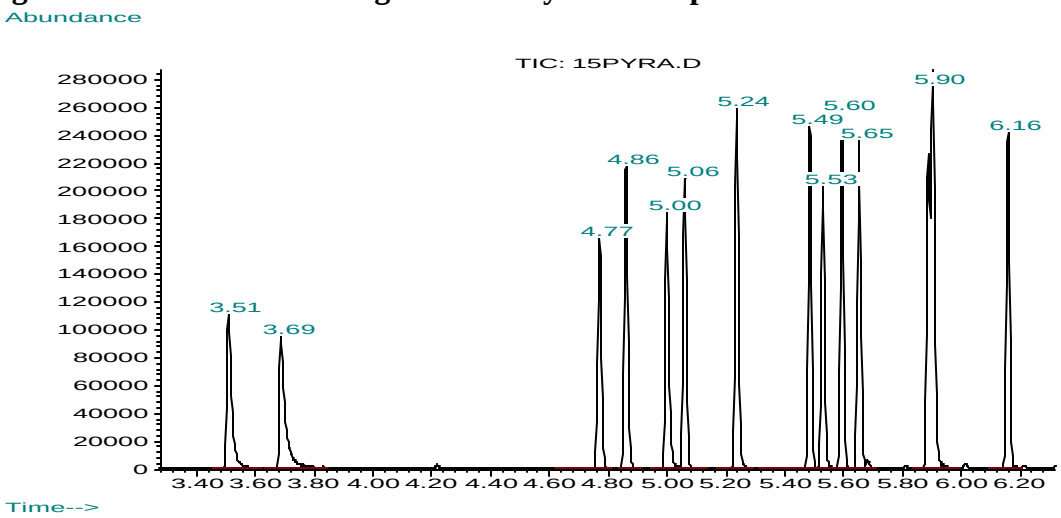


Table 5 Pyrazine Chromatogram Summarized

Peak#	Ret Time	Inte-gration Type	Width (min.)	TIC Area	Start Time (min.)	End Time (min.)	Analyte Identity
Not shown	3.2						Pyrazine
1	3.509	BB	0.018	1301219	3.452	3.618	Methoxypyrazine
2	3.689	BB	0.020	1299584	3.635	3.842	Ethylpyrazine
3	4.770	BB	0.014	1416572	4.617	4.809	3-Ethyl-2-methoxypyrazine
4	4.860	BB	0.014	1885156	4.834	4.908	3-Ethyl-2-chloropyrazine
5	5.000	BV	0.014	1594458	4.942	5.038	2,3-Diethylpyrazine
6	5.061	VB	0.020	1762989	5.038	5.134	3-Isopropyl-2-methoxypyrazine
7	5.237	BB	0.014	2163527	5.168	5.292	3-Ethyl-2-ethoxypyrazine
8	5.486	BV	0.013	1919099	5.311	5.512	3-Isopropyl-2-ethoxypyrazine
9	5.531	VV	0.013	1635149	5.512	5.577	3-Ethyl-2-acetylpyrazine
10	5.596	VV	0.013	2028292	5.577	5.631	3-Sec-butyl-2-methoxypyrazine
11	5.653	VB	0.014	1957491	5.631	5.710	3-Isobuty-2-methoxypyrazine
Coeluted	5.89						5,6,7,8-Tetrahydroquinoxaline
12	5.903	BV	0.022	4384119	5.837	5.995	Quinoxaline
13	6.159	BV	0.014	1979703	6.093	6.206	3-Ethyl-2-methylthiopyrazine

Figure 14 Methoxypyrazine TIC

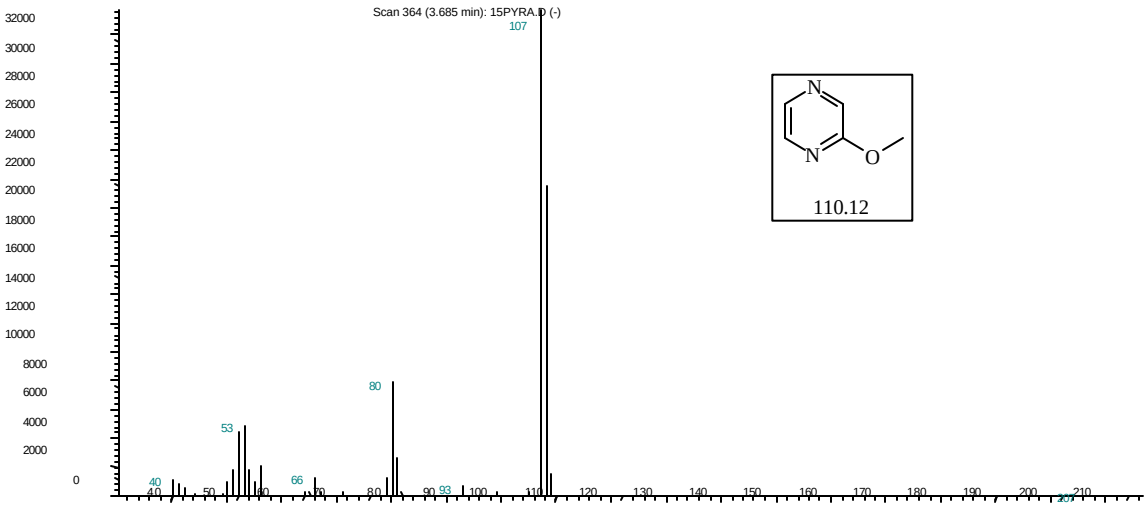


Table 6 Summary Scan 291 (3.518 min) Methoxypyrazine			
003149-28-8 96% Match to WILEY275.L			
m/z	Abundance.	m/z	Abundance.
40.15	5189	67.15	592
41.15	844	68.15	3512
42.15	343	79.15	873
44.05	17	80.15	5680
45.05	84	81.15	4731
51.15	1043	82.05	264
52.15	1688	95.05	744
53.15	2550	107.15	246
54.15	596	109.15	7356
55.15	446	110.15	12455
56.15	1187	111.05	897
66.05	331		

Figure 15 3-Ethylpyrazine TIC

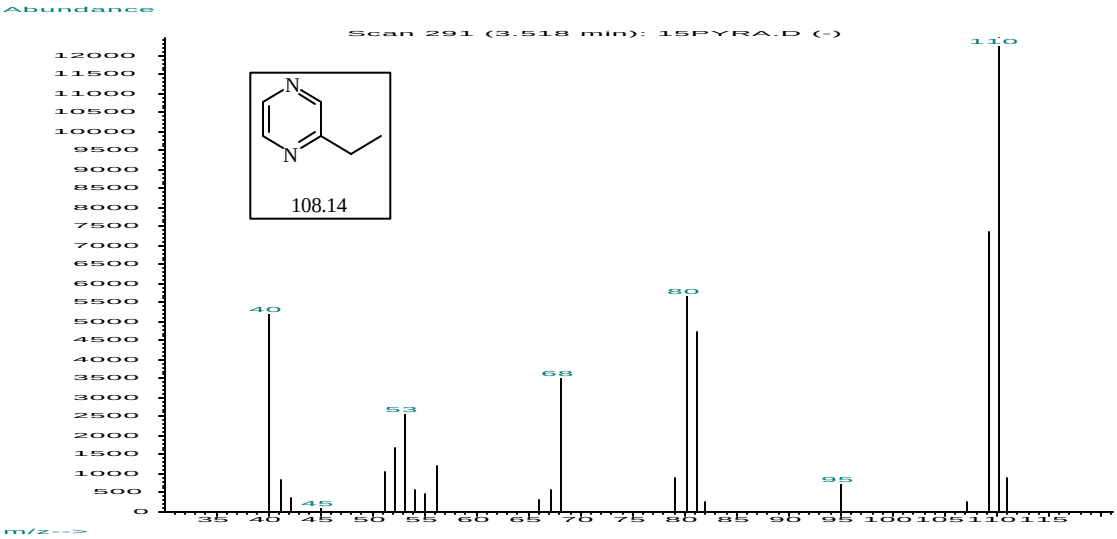


Table 7	**Summary Scan 364 (3.685 min)**		**Ethylpyrazine**
013925-00-3		86% to Match WILEY275.L	
m/z	Abundance.	M/z	Abundance.
40.15	1155	66.15	1247
41.15	869	67.05	242
42.15	533	71c.05	246
44.10	105	79.15	1283
49.15	222	80.15	7828
50.15	945	81.15	2702
51.15	1840	81.95	235
52.15	4407	93.05	609
53.15	4817	99.15	274
54.15	1765	104.95	268
55.15	964	107.15	33696
56.15	2076	108.15	21528
64.05	266	109.15	1590
65.15	266	206.95	6
66.15	1247		

Figure 16 3-Ethyl-2-methoxypyrazine TIC

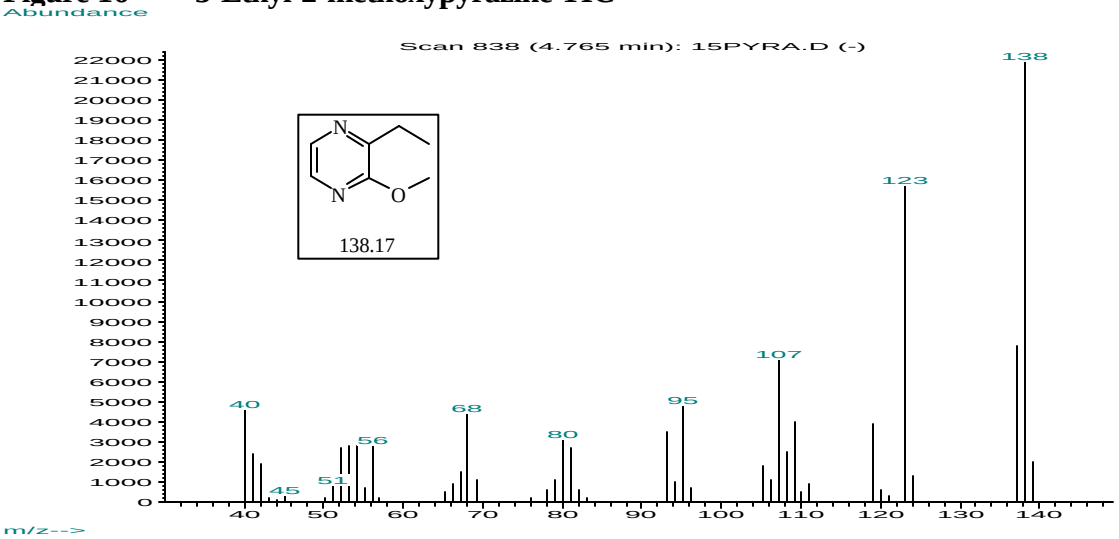


Table 8	**Summary Scan 838 (4.765 min) 3-Ethyl-2-methoxypyrazine**		
025680-58-4		85% Match to WILEY275.L	
m/z	Abundance.	m/z	Abundance.
40.15	4631	81.15	2696
41.15	2369	82.15	572
42.15	1945	83.15	256
43.05	250	93.15	3525
44.05	162	94.15	1052
45.05	322	95.15	4762
50.05	236	96.15	754
51.15	789	105.1	1799
52.15	2653	106.15	1121
53.15	2801	107.15	7034
54.15	2754	108.15	2524
55.15	707	109.15	3953
56.15	2834	110.05	518
57.05	209	111.05	955
65.15	483	119.15	3846
66.15	916	120.05	585
67.15	1456	121.05	338
68.05	4424	123.15	15734
69.15	1149	124.15	1342
76.05	221	137.15	7792
78.05	586	138.15	22424
79.15	1130	139.15	2040
80.15	3144		

Figure 17 3-Ethyl-2-chloropyrazine TIC

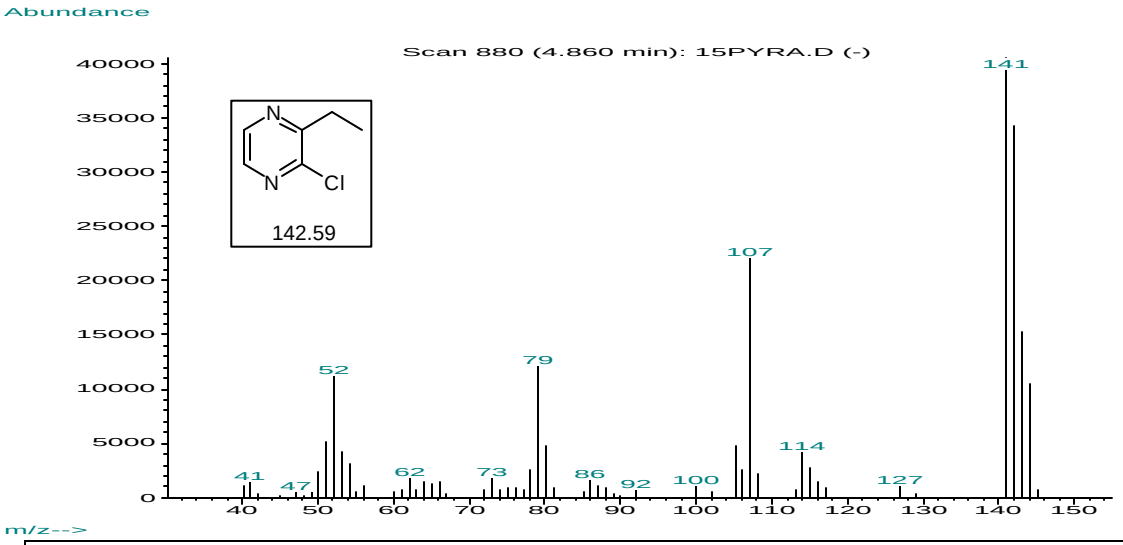


Table 9 Summary Scan 880 (4.860 min) 3-Ethyl-2-chloropyrazine

No satisfactory match to WILEY275.L					
m/z	Abundance	m/z	Abundance	m/z	Abundance
40.15	1094	67.05	409	107.15	22072
41.15	1379	72.05	631	108.15	2144
42.05	390	73.05	1838	113.05	620
44.05	32	74.05	693	114.05	4221
45.05	81	75.05	975	115.05	2803
47.05	556	76.05	856	116.05	1494
48.15	229	77.15	794	117.05	939
49.15	1096	78.15	2522	126.95	1013
50.15	2297	79.15	12217	128.95	312
51.15	5165	80.15	4763	141.05	40576
52.15	11122	81.15	823	142.05	34232
53.15	4215	85.15	454	143.05	15182
54.15	3059	86.05	1663	144.05	10472
55.15	435	87.05	1056	145.05	656
56.15	1064	88.05	852		
60.05	599	89.05	326		
61.15	694	89.95	231		
62.15	1865	92.05	631		
63.05	786	100.05	1132		
64.15	1389	101.95	433		
65.15	1255	105.15	4828		
66.15	1416	106.15	2588		

Figure 18 Diethylpyrazine TIC

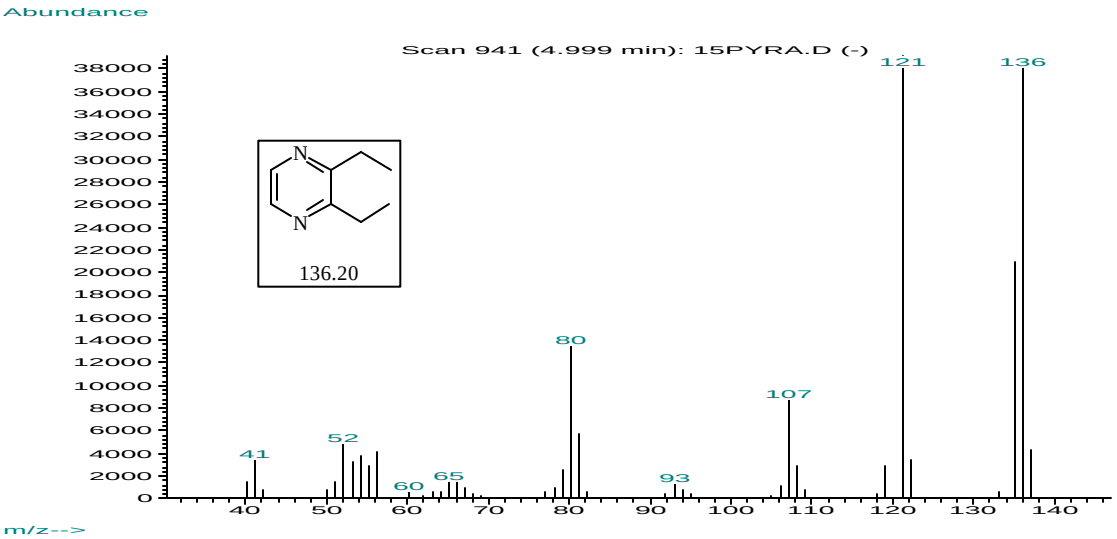


Table 10 Summary Scan 941 (4.999 min) Diethylpyrazine			
015707-24-1		85% Match to WILEY275.L	
m/z	Abundance.	m/z	Abundance.
40.15	1557	80.15	13494
41.15	3452	81.15	5811
42.15	717	82.15	666
50.05	745	91.95	394
51.15	1540	93.15	1290
52.15	4877	94.15	704
53.15	3273	95.15	476
54.15	3730	105.05	240
55.15	2860	106.15	1041
56.15	4175	107.15	8660
60.15	661	108.15	2981
61.95	213	109.05	799
63.05	550	118.15	407
64.05	553	119.15	2918
65.15	1511	121.15	39152
66.15	1493	122.15	3499
67.15	901	133.15	568
68.15	453	135.15	20976
69.05	230	136.15	38984
77.05	650	137.15	4332
78.15	941		
79.15	2541		

Figure 19 3-Isopropyl-2-methoxypyrazine TIC

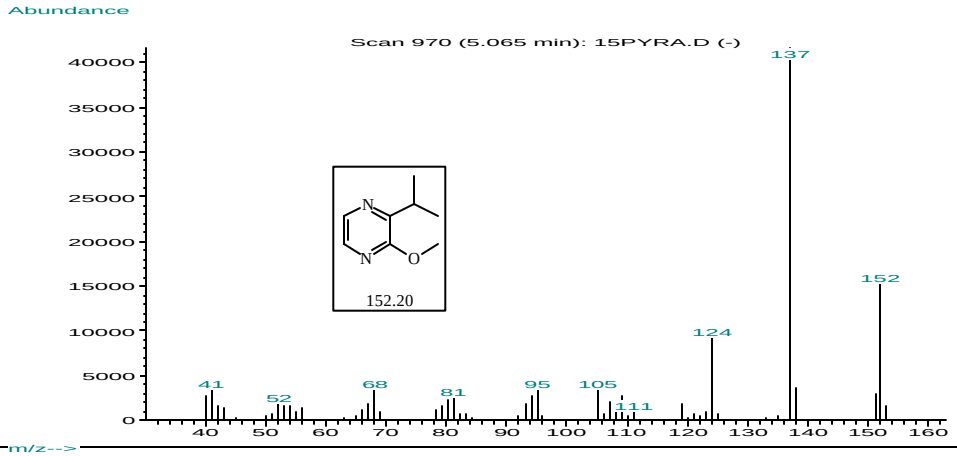


Table 11 Summary Scan 970 (5.065 min) 3-Isopropyl-2-methoxypyrazine

025773-40-4 97% Match to WILEY275.L

m/z	Abundance.	m/z	Abundance.
40.15	2815	93.15	1857
41.15	3394	94.15	2669
42.15	1568	95.15	3501
43.15	1382	95.95	411
45.15	238	105.15	3486
50.05	405	106.15	669
51.15	634	107.15	1987
52.15	1852	108.15	971
53.15	1558	109.15	2719
54.15	1627	110.15	531
55.15	861	111.15	1047
56.15	1511	119.15	1783
63.05	218	120.05	309
65.05	494	121.10	776
66.15	1264	122.15	571
67.15	1765	123.05	950
68.15	3345	124.15	9160
69.15	946	125.05	834
78.15	1165	132.95	203
79.15	1565	135.15	533
80.15	2295	137.15	1480
81.15	2487	138.15	3626
82.15	824	151.25	3032
83.15	748	152.15	15285
84.25	208	153.15	1524
92.05	560		

Figure 20 3-Ethyl-2-ethoxypyrazine TIC

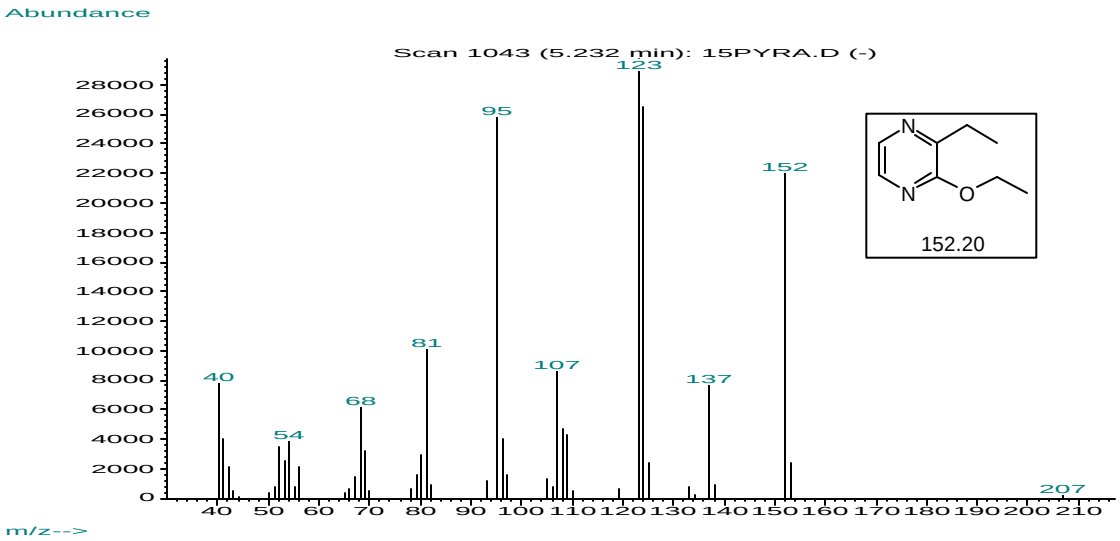


Table 12	**Summary Scan 1043 (5.232 min)**	**3-Ethyl-2-ethoxypyrazine**	
035243-43-7		91% Match to WILEY275.L	
m/z	Abundance.	m/z	Abundance.
41.15	3946	93.15	1191
42.15	2073	95.15	25800
43.05	513	96.15	4048
44.05	82	97.15	1606
50.15	287	105.15	1334
51.15	771	106.15	703
52.15	3422	107.15	8577
53.15	2525	108.15	4723
54.15	3912	109.15	4323
55.15	770	110.05	425
56.15	2164	119.05	624
65.15	414	123.15	29760
66.05	685	124.15	26544
67.15	1401	125.05	2363
68.15	6240	133.15	794
69.15	3233	134.05	273
70.05	486	137.15	7730
78.15	578	138.15	874
79.15	1529	152.15	22040
80.15	2908	153.15	2422
81.15	10160	207.05	210

Figure 21 3-Isopropyl-2-ethoxypyrazine TIC

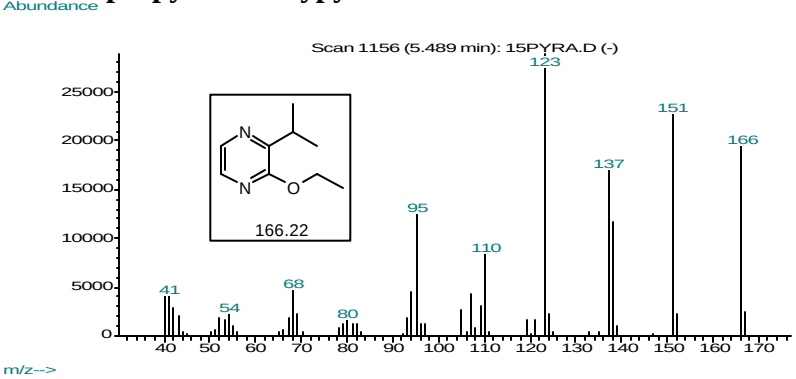


Table 13	**Summary Scan 1156 (5.489 min) 3-Isopropyl-2-ethoxypyrazine**		
No satisfactory match to WILEY275.L			
m/z	Abundance.	m/z	Abundance.
40.15	4004	94.15	4560
41.15	4107	95.15	12595
42.15	2941	96.05	1228
43.15	2092	97.05	1141
44.10	429	105.05	2728
45.15	243	106.15	378
50.05	316	107.15	4290
51.15	507	108.15	820
52.15	1799	109.15	3047
53.15	1541	110.15	8456
54.15	2172	111.05	480
55.15	901	119.15	1650
56.05	437	120.05	270
65.15	390	121.15	1599
66.15	550	123.15	28904
67.15	1764	124.05	2241
68.15	4678	125.05	295
69.15	2132	133.05	382
70.15	468	135.05	346
78.05	748	137.15	16944
79.15	1241	138.15	11661
80.15	1571	139.05	1037
81.15	1140	147.05	267
82.15	1281	151.15	22744
83.05	465	152.15	2204
92.15	234	166.15	19392
93.15	1803	167.15	2526

Figure 22 3-Ethyl-2-acetypyrazine TIC

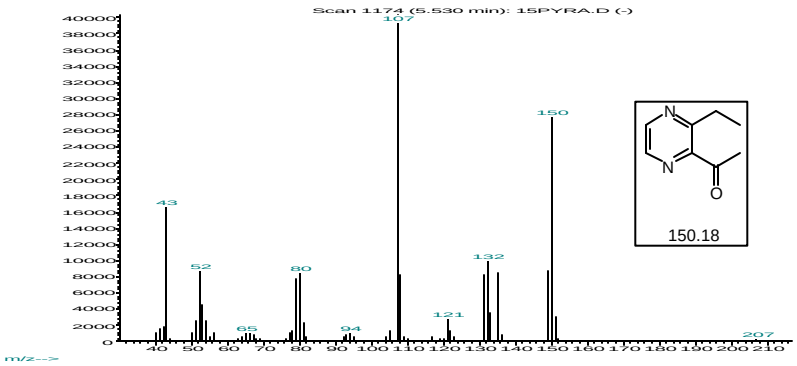


Table 14 Summary Scan 1174 (5.530 min) 3-Ethyl-2-acetylpyrazine			
92% Match to WILEY275.L			
m/z	Abundance.	m/z	Abundance.
40.15	1169	82.05	535
41.15	1584	92.15	498
42.25	1905	93.05	762
43.15	16656	94.15	1199
44.05	264	95.05	710
49.05	212	104.05	650
50.15	1126	105.15	1272
51.25	2590	107.15	40264
52.15	8809	108.15	8208
53.15	4546	109.15	637
54.15	2674	110.05	260
55.05	708	117.05	478
56.15	1106	119.05	267
62.05	220	120.05	394
63.05	375	121.15	2867
64.05	552	122.05	1400
65.05	1131	123.15	705
66.15	1083	131.15	8360
67.15	923	132.15	9951
68.15	381	133.15	3630
69.05	332	135.15	8508
74.95	223	136.15	846
76.15	359	149.15	8635
77.15	1078	150.15	27840
78.15	1366	151.15	3035
79.15	7821	151.95	246
80.15	8576	206.95	314
81.15	2213		

Figure 23 3-Sec-butyl-2-methoxypyrazine TIC

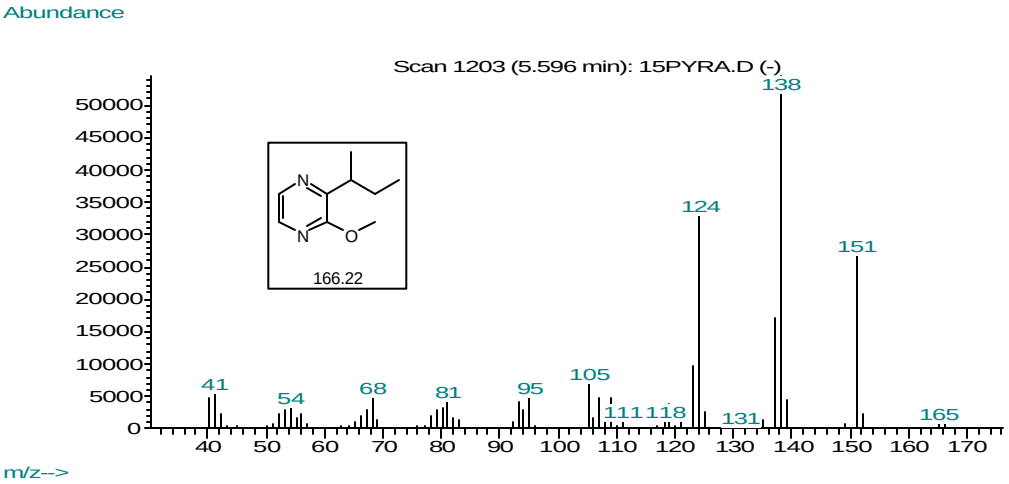


Table 15 Summary Scan 1203 (5.596 min) 3-Sec-butyl-2-methoxypyrazine					
024168-70-5 91% Match to WILEY275.L					
m/z	Abundance.	m/z	Abundance.	m/z	Abundance.
40.15	4615	78.15	2135	120.15	571
41.15	5443	79.15	2844	121.05	2247
42.15	2265	80.15	3230	123.15	9592
43.15	474	81.15	4136	124.15	32976
45.15	426	82.15	1598	125.15	2465
50.05	587	83.15	1236	131.05	264
51.15	838	92.15	1118	133.05	737
52.15	2388	93.15	4098	134.15	449
53.15	2813	94.15	2761	135.05	1506
54.15	3290	95.15	4699	137.15	16984
55.15	1546	96.15	464	138.15	54648
56.15	2239	97.05	278	139.15	4551
57.15	835	105.15	7002	140.05	260
62.95	319	106.15	1797	146.95	206
64.15	325	107.15	4612	149.05	640
65.15	1103	108.15	3081	151.15	26840
66.15	1955	109.15	4645	152.15	2206
67.15	2823	110.15	552	153.15	211
68.15	4610	111.05	1126	165.15	786
69.15	1288	117.05	303	166.15	607
75.95	358	118.15	1089		
77.05	339	119.15	3908		

Figure 24 3-Isobutyl-2-methoxypyrazine TIC

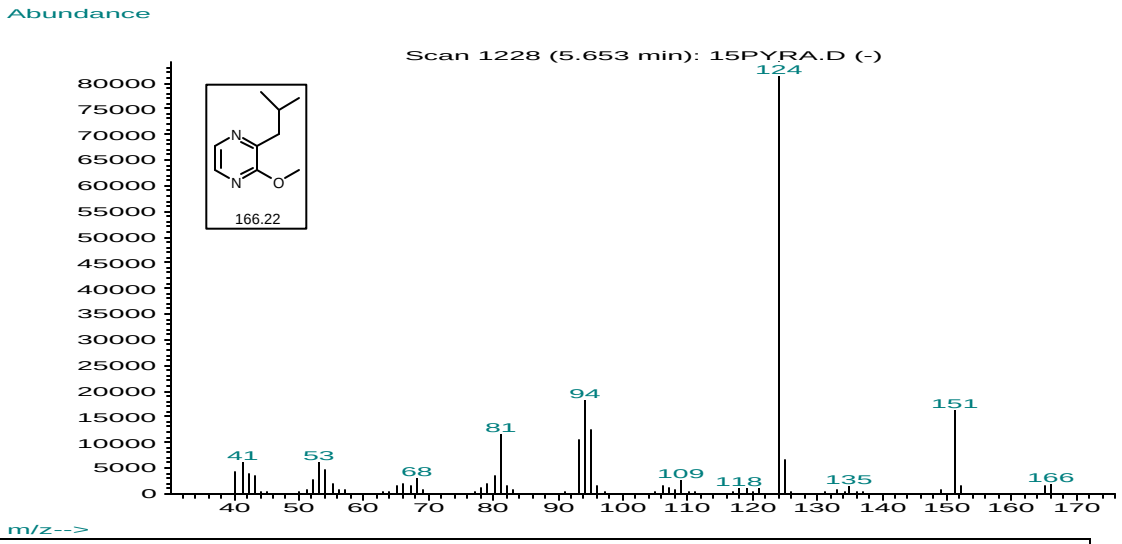


Table 16	**Summary Scan 1228 (5.653 min)**		**3-Isobutyl-2-methoxypyrazine**		
	024683-00-9		93% Match to WILEY275.L		
m/z	Abundance.	m/z	Abundance.	m/z	Abundance.
40.15	4048	69.15	838	111.05	428
41.25	6193	77.15	485	116.95	312
42.15	3953	78.05	922	118.05	946
43.25	3272	79.15	1804	119.15	1321
44.10	265	80.15	3272	120.15	312
45.15	314	81.15	11507	121.05	1040
50.05	430	82.15	1365	124.15	83984
51.15	823	83.15	750	125.15	6384
52.15	2560	90.95	230	126.05	482
53.15	6326	93.15	10412	131.25	248
54.15	4429	94.15	18304	133.05	792
55.15	1711	95.15	12393	134.15	409
56.15	856	96.05	1342	135.05	1684
57.05	665	97.15	249	136.05	328
63.05	242	105.05	338	137.05	268
64.05	405	106.15	1323	149.15	684
65.15	1555	107.15	1194	151.15	16480
66.15	1773	108.05	721	152.15	1571
67.15	1413	109.05	2541	165.15	1695
68.15	2892	110.15	483	166.15	1736

Figure 25 Quinoxaline TIC

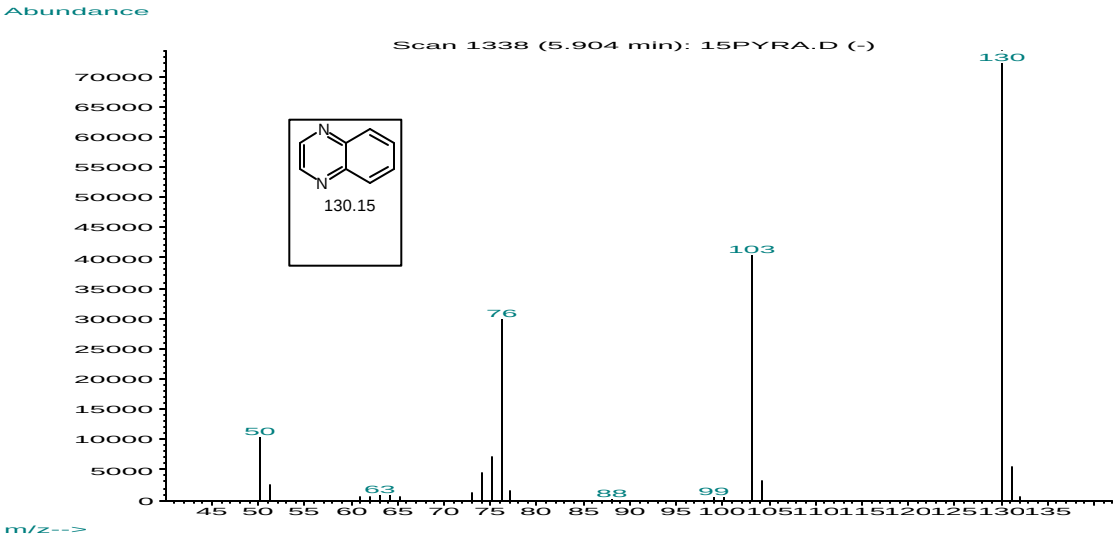


Table 17 Summary Scan 1338 (5.904 min) Quinoxaline	
000091-19-0	91% Match to WILEY275.L
m/z	Abundance.
50.15	10314
51.15	2520
60.95	490
62.05	549
63.10	965
64.15	704
65.15	479
73.10	1076
74.15	4580
75.15	7240
76.15	30000
77.15	1446
88.05	311
99.05	613
100.05	488
103.15	40593
104.15	3079
130.15	74040
131.10	5435
132.05	649

Figure 26 3-Ethyl-2-methylthiopyrazine TIC

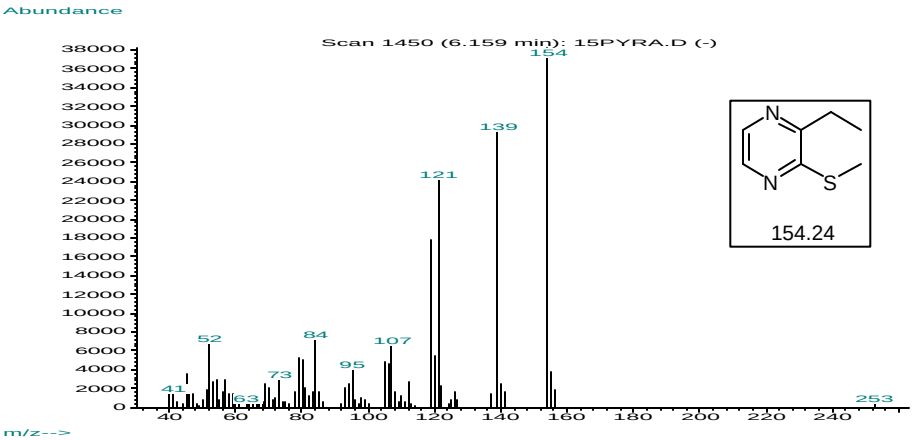


Table 18 3-Ethyl-2-methylthioypyrazine Summary Scan 1450 (6.159 min)

072987-62-3 97% Match to WILEY275.L

m/z	Abundance.	m/z	Abundance.	m/z	Abundance.
40.15	1328	69.05	2497	105.1	4862
41.15	1379	70.05	2021	106.1	4484
42.15	642	71.05	680	107.15	6487
44.15	305	72.05	927	108.15	1591
45.15	3424	73.15	2935	109.15	587
46.15	1339	74.05	627	110.05	1273
47.15	1375	75.05	536	111.05	651
48.05	341	75.95	297	112.05	2754
48.75	205	78.15	1665	113.05	395
50.15	713	79.15	5283	114.05	213
51.15	1838	80.15	5122	119.15	17672
52.15	6797	81.15	2011	120.15	5432
53.15	2576	82.05	1158	121.15	24208
54.15	2791	83.15	1686	122.15	2272
55.15	701	84.05	7078	124.05	295
56.15	1602	85.05	1655	125.05	808
57.05	2878	86.05	570	126.05	1633
58.05	1495	92.05	273	127.05	728
59.15	1312	93.15	1989	137.05	1428
60.05	284	94.15	2378	139.05	29168
61.05	1076	95.15	3913	140.05	2398
63.15	360	96.05	759	141.05	1649
64.05	404	97.05	373	154.15	38248
65.15	648	98.05	1046	155.15	3737
66.15	898	99.05	733	156.05	1733
67.05	568	100.0	340	252.95	266
68.15	583				

Figure 27 5,6,7,8-Tetrahydroquinoxaline TIC

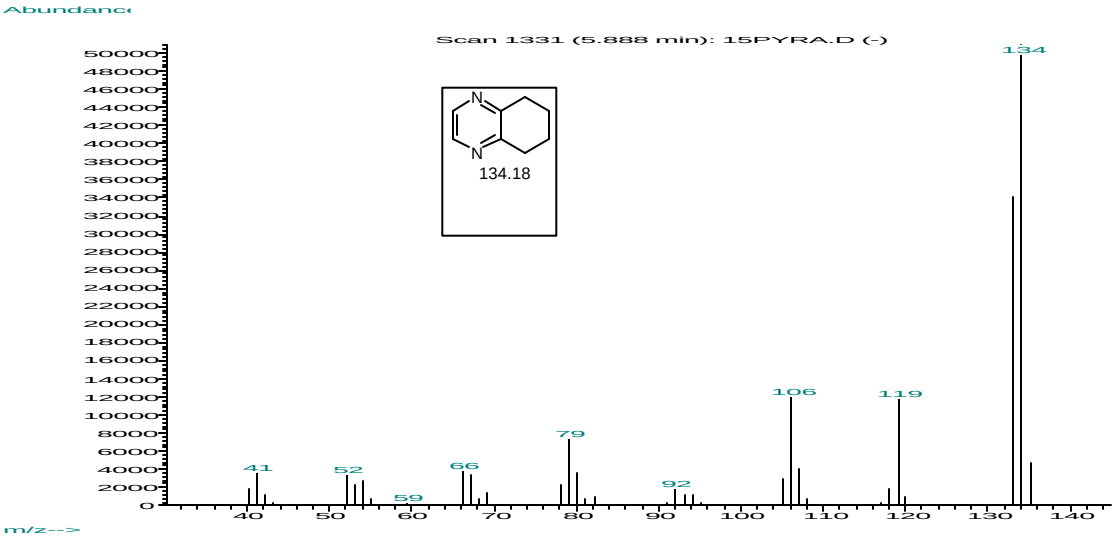


Table 19	**5,6,7,8-Tetrahydroquinoxaline Summary Scan 1331 (5.888 min)**		
34413-35-9		95% Match to WILEY275.L	
M/z	Abundance.	m/z	Abundance.
40.15	1747	82.15	848
41.15	3604	91.05	358
42.15	1120	92.10	1868
43.05	217	93.10	1265
44.05	18	94.15	1104
52.15	3355	95.05	380
53.15	2224	105.15	3035
54.15	2639	106.10	11960
55.15	714	107.15	4130
59.45	262	108.15	838
66.15	3818	116.95	315
67.15	3399	118.05	1951
68.15	703	119.10	11728
69.15	1302	120.05	1000
78.15	2266	133.15	34014
79.15	7352	134.15	50980
80.10	3657	135.15	4618
81.15	763		

Chapter V: SPME Optimization

Fiber Selection

SPME fibers are composed of fused silica, coated with layers of polymeric materials with an affinity for organic compounds. Several types of SPME fibers, with a range of polarities and mechanisms, are commercially available. These fiber types are listed in Table 20Table 20. Six were evaluated to find the best for the trace level analysis of the target compounds. The objective was to establish which fibers reliably achieved the highest recovery of the alkylalkoxypyrazine.

Each fiber was used to extract the model spiked model solution under the normal extraction conditions. The outcomes as area response normalized over actual concentration in ppb are plotted in Figure 28.

Carboxen in PDMS resulted in the highest analyte recoveries. The 2 centimeter dual layer divinylbenzene/carboxen/PDMS fibers produced acceptable yields.

Two types of coating were evaluated. The first type extracts the analytes into the film on the fiber, and is termed absorptive. This includes polydimethylsiloxane (PDMS) and polyacrylate (PA). Of the absorptive fibers, PDMS resulted in the best overall performance in chromatography, desorption characteristics, and analyte recovery. It did not recover low molecular weight 3-ethyl-2-methoxypyrazine as efficiently as the 3-butyl-2methoxypyrazines.

The second type of coating, containing porous particles of divinylbenzene (DVB) or carbon (Carboxen™), adsorb organics on the surface of their pores. The adsorptive coatings evaluated included carbowax/divinylbenzene (CW/DVB), polydimethylsiloxane/ divinylbenzene (PDMS/DVB), and divinylbenzene/Carboxen™/ polydimethylsiloxane (DVB/CAR/PDMS) in standard 1 and 2 cm lengths. The 2 cm DVB/CAR/PDMS resulted in the best recoveries, as well as acceptable chromatography. The larger 3-butyl-2-methoxypyrazine molecules were collected much more effectively than 3-ethyl-2-methoxypyrazine, just as with the PDMS coated fibers.

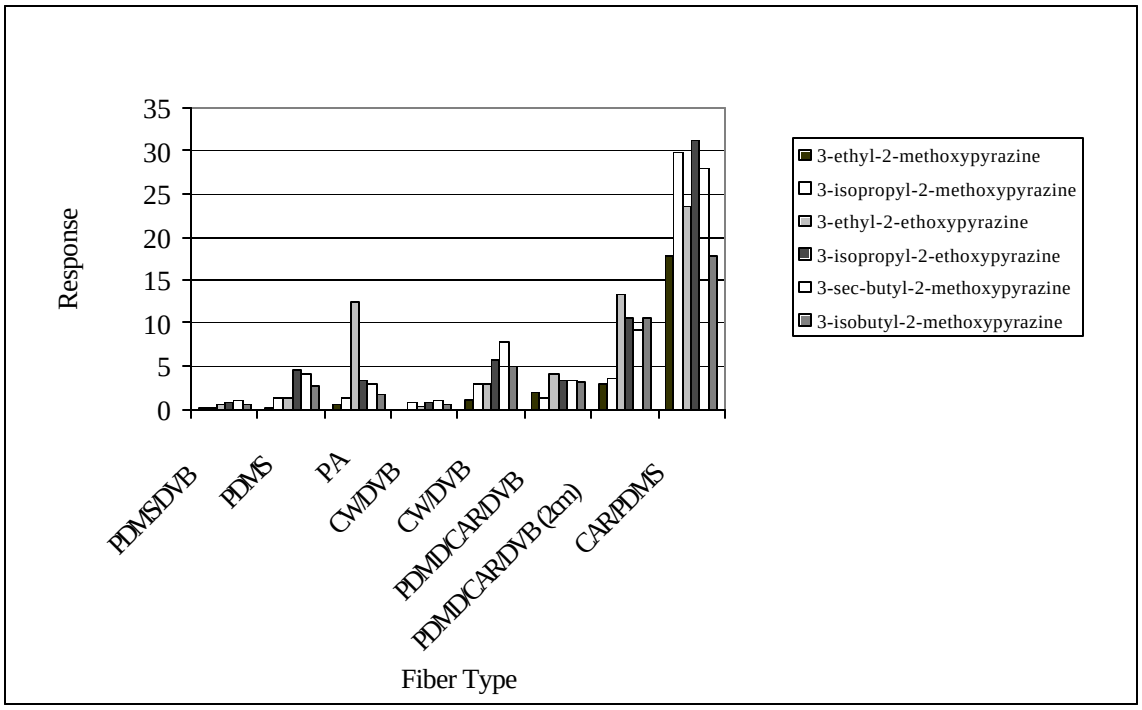


Figure 28 Effect of Fiber Type on HS SPME Alkylalkoxypyrazine Recovery

PDMS fibers were selected because they resulted in best long term stability and overall performance, including chromatography, desoprtion characteristics, and analyte recovery. PDMS coated fibers resulted in ideal peak shapes and excellent selectivities. PDMS was used to determine the influence of extraction conditions and matrix ingredients on HS SPME alkylalkoxyoyrazine recovery.

The symmetrical peaks from a typical HS SPME analysis of the standard spiked matrix, are shown in Figure 29. The PDMS fibers proved very durable retaining their performance after 50 analytical cycles.

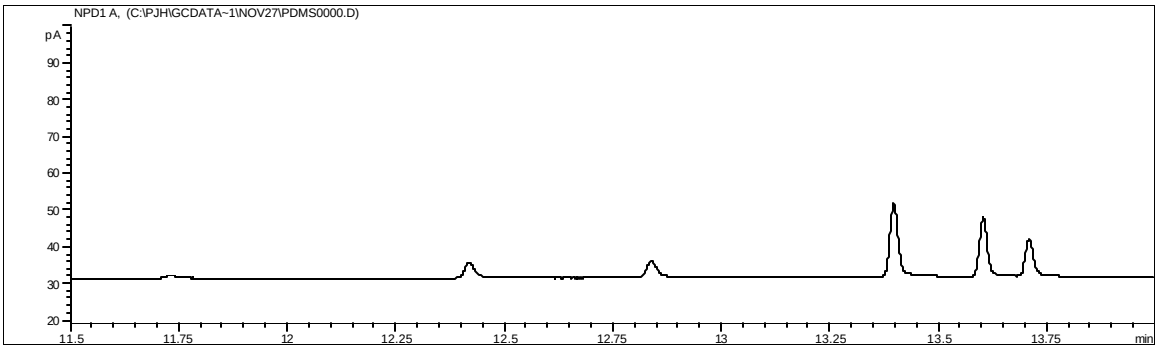


Figure 29 PDMS Fiber HS SPME Analysis of 5ppb Analyte Spiked Model Solution

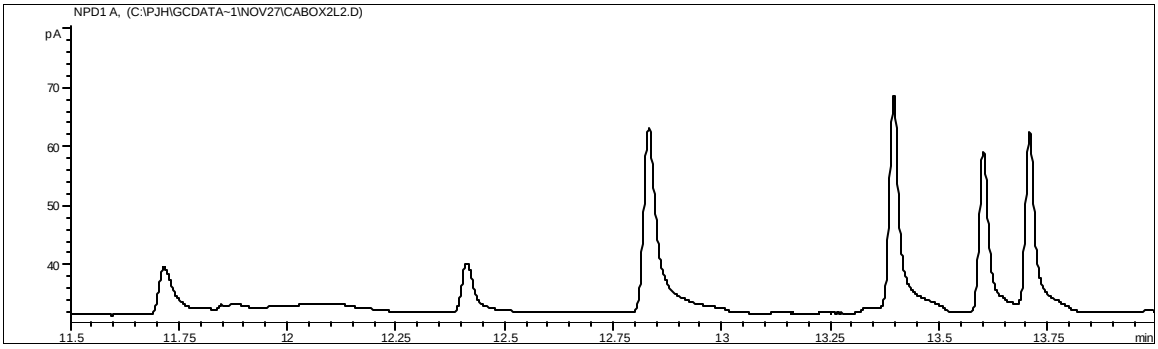


Figure 30 DVB/Carboxen/PDMS Fiber HS SPME Analysis of 5 ppb Analyte Spiked Model Solution

DBV/Carboxen/PDMS was evaluated next because it is manufactured in a longer length (2 cm) for a higher capacity in trace analysis. Temperature and time studies were performed. These fibers were more fragile sometimes swelling and becoming loose from the fused silica core. Their recovery was generally better than PDMS coated fibers.

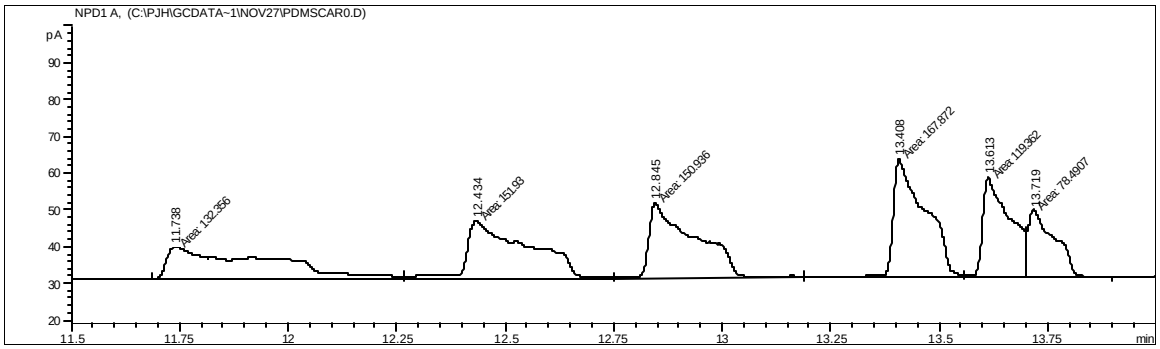


Figure 31 Carboxen/PDMS Fiber HS SPME Analysis of 5 ppb Analyte Spiked Model Solution

Carboxen/PDMS resulted in the highest relative recoveries; unfortunately it yielded unusable distorted and overlapping GC peak shapes, shown in a typical chromatogram in Figure 31.

Analyte concentrations as low as 0.5 ppb were extracted to determine whether sample overloading caused the slow desorption. Even the lowest alkylmethoxypyrazine concentrations under the best conditions were broad and tailed severely.

These asymmetrical tailing peaks result from slow desorption, when the analyte molecules penetrate deep into the carboxen microprores, then desorbs gradually following paths through the carboxen particle instead of directly out. The mixed mechanisms, absorption and adsorption, in effect may also contribute to the slow desorption. Even though the capacities of the analytes was adequate in the range between 3 and 4, excessive tailing reduces the selectivity into the unacceptable range.

Desorption temperatures as high as 310°C were employed to obtain rapid desorptions, but these destroyed the fiber.

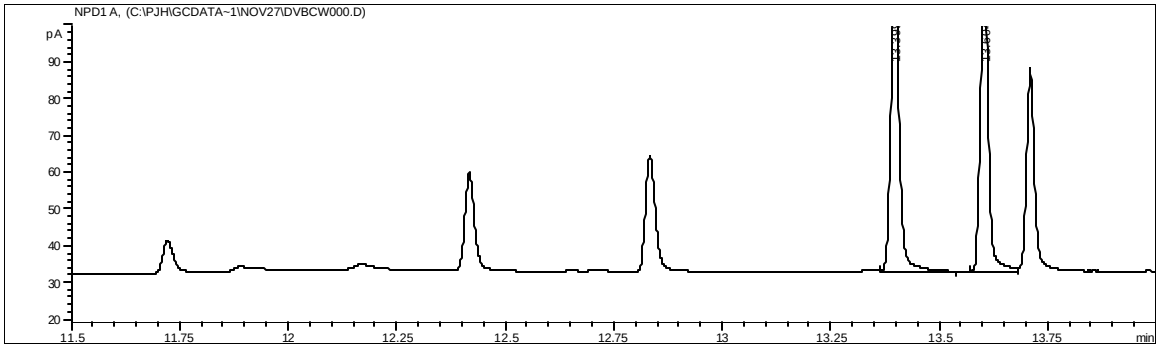


Figure 32 DVB/Carbowax Fiber HS SPME Analysis of 5 ppb Analyte Spiked Model Solution

High recoveries with symmetrical peaks were initially obtained from the spiked standard model solutions. The recoveries deteriorated very quickly with use. The coatings remained bonded to the fibers for only a few cycles with model wine solutions, because the ethanol ultimately condensed on the fibers, even in the headspace and swelled the coatings.

Table 20 Summary of Fiber Types Evaluated for Trace Level Analysis of Alkylalkoxypyrazines

Fiber	Mechanism	Coating identity	Coating thickness um	Length cm	Crosslinkling	Polarity	Operating Temperature °C	Performance with Alkylalkoxypyrazines
PDMS/ DVB	absorption	Polydimethylsiloxane/ divinylbenzene	65	1	partial	bipolar	200-270	Low recovery
PDMS	absorption	Polydimethylsiloxane	100	1	non-bonded	nonpolar	200-270	Consistent recovery
PA	absorption	Polyacrylate	85	1	partial	polar	220-310	High background
CW/DVB	adsorption	Carbowax/divinylbenzene	65	1	partial	polar	200-260	Variable recovery
CW/DVB	adsorption	Carbowax/divinylbenzene	70	1	highly	bipolar	200-260	
DVB/ CAR/ PDMDS	adsorption	Divinylbenzene/Carboxen/ Polydimethylsiloxane	50/30	1	highly	bipolar	230-270	High recovery
DVB/ CAR/ PDMDS	adsorption	Divinylbenzene/Carboxen/ Polydimethylsiloxane	50/30	2	highly	bipolar	230-270	Consitent recovery
CAR/ PDMS	adsorption	Carboxen/Polydimethylsiloxane	75	1	partial	bipolar	240-300	Very high recovery, very distorted peak shape

Salting Out Studies

Selected salts added at 30% were compared to sodium chloride using PDMS fibers to extract the headspace of spiked model solutions at the normal experimental conditions. HS SPME extractions of solutions with potassium and sodium chloride resulted in similar peak areas. Magnesium sulfate, sodium sulfate, sodium iodide, and calcium chloride resulted in lower analyte level recoveries than sodium chloride.

Sodium chloride yielded the highest recoveries probably because of its high solubility and low formula weight relative to the other salts. If all salts were 30%, sodium chloride would have the highest molar concentration. The "salting out" effect is related directly to ionic strength.

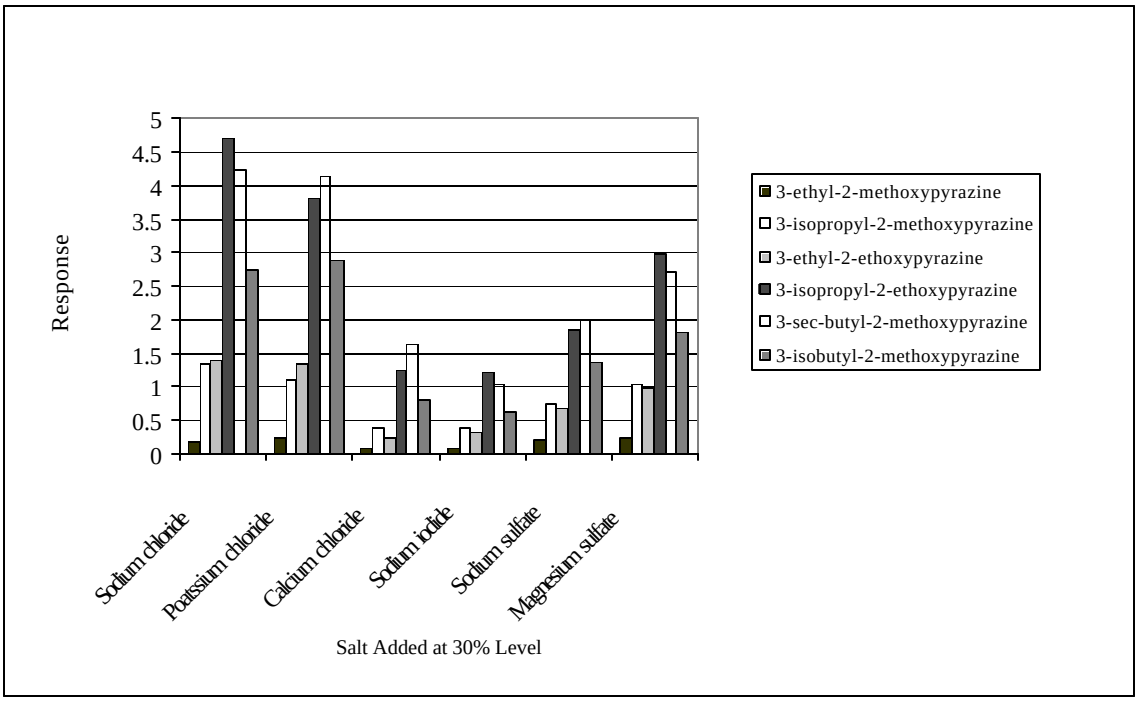


Figure 33 Effect of Salt Type on HS SPME Alkylalkoxypyrazine Recovery on PDMS

Added Sodium Chloride Concentration:

Sodium chloride concentrations were incrementally raised from 0 to 30% to determine the optimum concentration of added salt. These studies were conducted at standard conditions.

Extracted alkylmethoxypyrazine concentrations from the spiked model matrix increased up to threefold. This illustrates the "salting out" effect's direct relation to the solution's ionic strength.

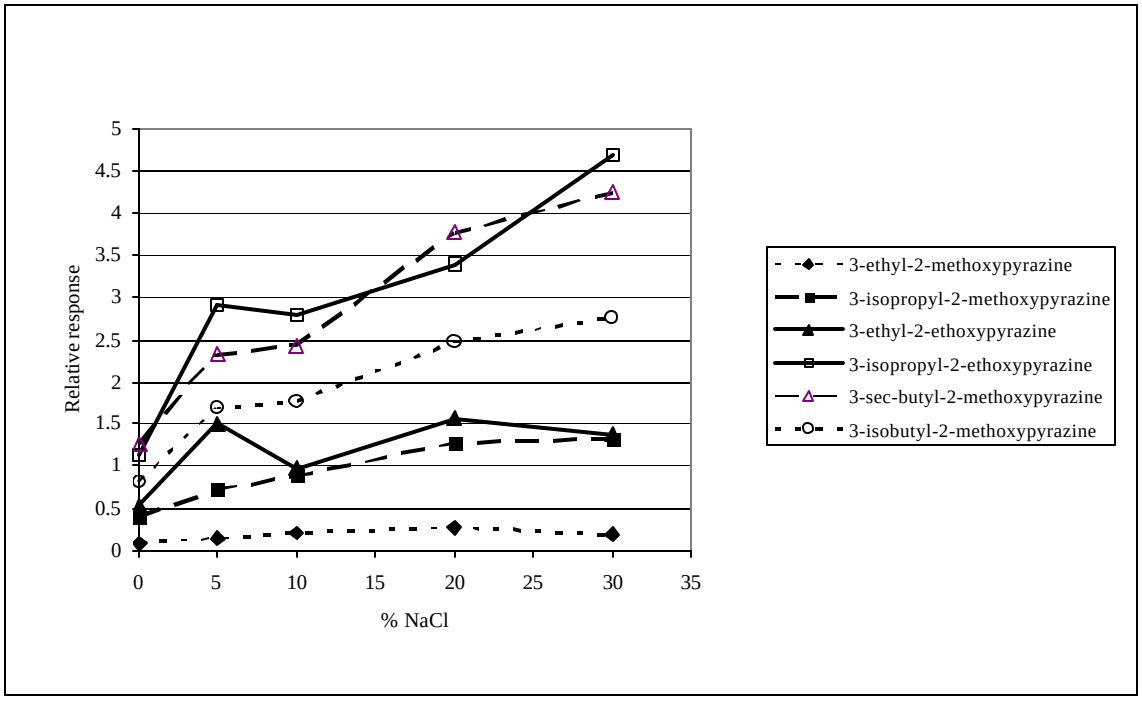


Figure 34 Effect of NaCl Concentration of HS SPME Recovery of Alkylalkoxypyrazines on PDMS

Extraction Time:

Periods of time varying from 0.25 to 4.5 hours were evaluated to establish the optimum extraction time for PDMS coated fibers. Sample temperature was held at 35° with PDMS.

As illustrated in Figure 35, PDMS fibers saturate fairly quickly with alkylmethoxyoyrazines from the headspace at these analytical conditions.

Significant improvement in analyte peak areas were not observed after 30 minutes.

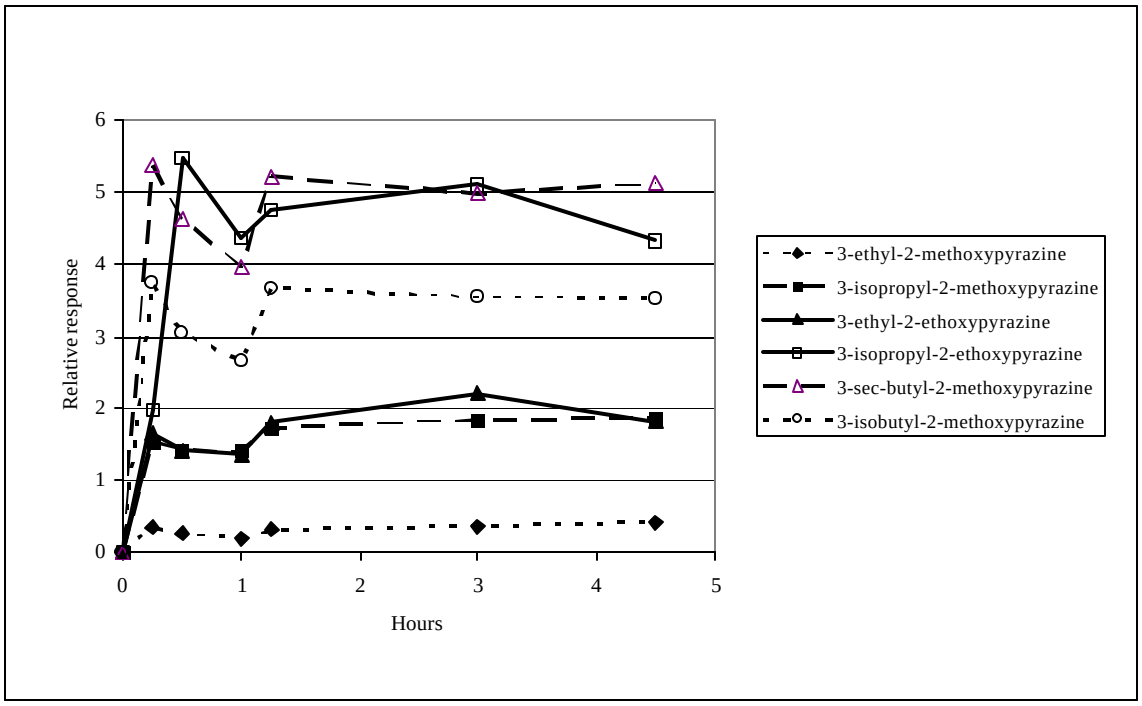


Figure 35 Effect of ExtractionTime on HS SPME on Alkylalkoxypyrazine Recovery on PDMS

Similar optimum extraction times were observed using a different fiber type, divinylbenzene/caboxen/polydimethylsiloxane. The DVB/CAR/PDMS fiber was evaluated using conditions identical to PDMS with the exception of the sample temperature, which was maintained at 50°C. This fiber also became fully saturated with analyte in 30 minutes.

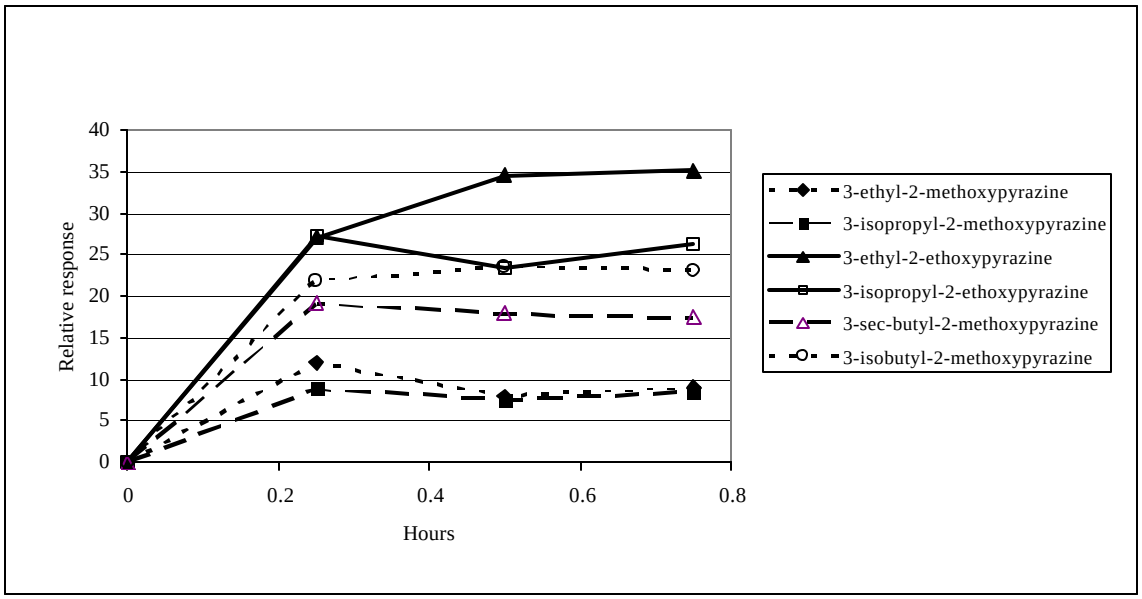


Figure 36 Effect of Extraction Time on Alkylalkoxypyrazine Recovery on DVB/CAR/PDMS

Extraction Temperature:

Sample temperature, a key method parameter, needed to be optimized for each fiber. PDMS fibers were used to extract the headspace of model solutions at a range of temperatures. Sample temperature controls the kinetics and equilibrium between the aqueous sample and the headspace as well as the headspace and PDMS fiber. As temperature increases, the liquid and gas phases equilibrate faster. Increasing the temperature drives more of the high-boiling compounds, such as the pyrazine analytes, into the vapor phase. Increasing fiber temperature causes a competitive effect, the evaporation of volatile analytes from the SPME fiber, ultimately limiting its analytical sensitivity.

Recoveries using PDMS fibers appear to increase to a maximum at 35°C, then drop rapidly as the temperature exceeds 35°C, as illustrated in Figure 37. Elevating the temperature initially increases the alkylmethoxypyrazine volatility. Beyond this temperature, maximum analyte recovery becomes increasingly subject to the reduced solubility in the PDMS fiber.

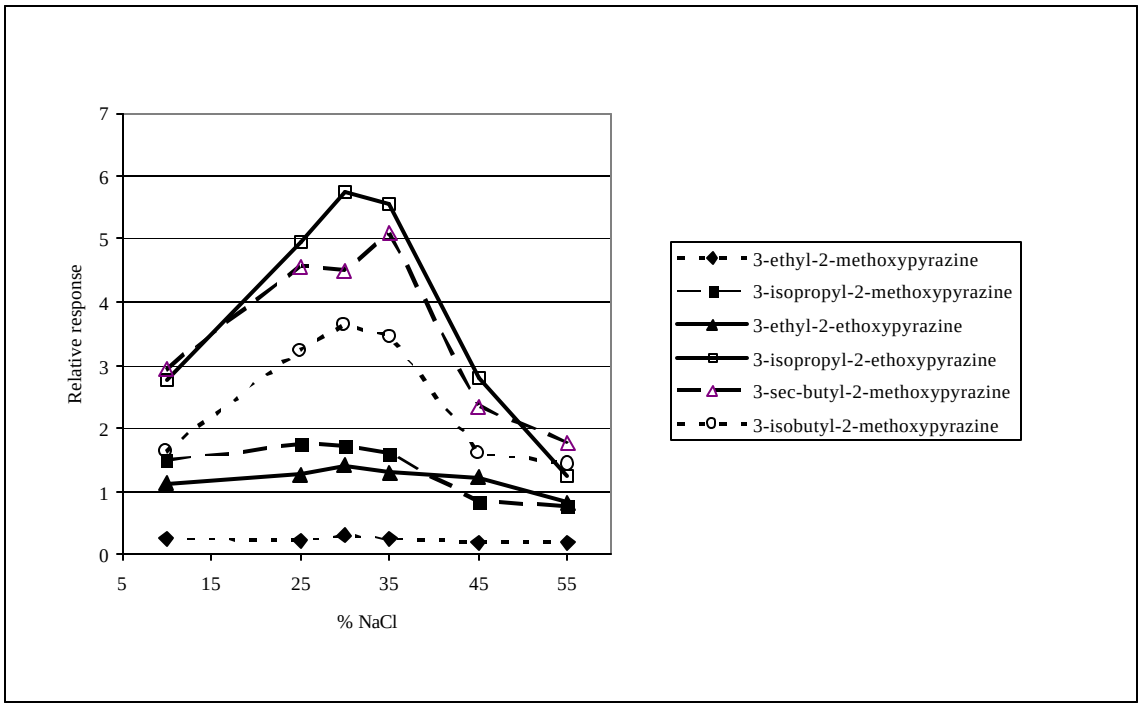


Figure 37 Effect of Extraction Temperature on HS SPME Alkylalkoxypyrazine Recovery on PDMS

DVB/Carboxen/PDMD fibers, which achieve slightly higher recoveries than PDMS at the same conditions, exhibit a higher temperature maximum, 55°C, before analyte solubility in the fiber declines.

Model solutions were extracted at temperatures ranging from 10°C to 65°C with both PDMS and DVB/CAR/PDMS fibers for 30 minutes. The optimum temperatures for most analytes, shown in Figures 4 and 5, was 30°C using PDMS, and 40°C to 50°C with DVB/CAR/PDMS.

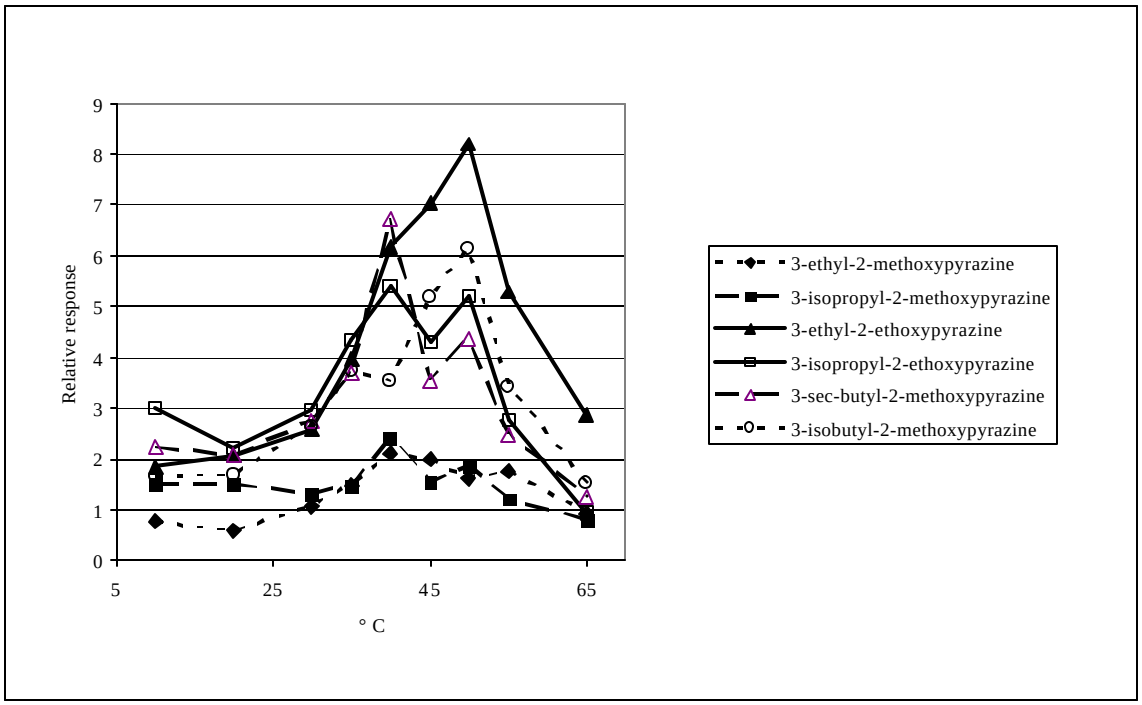


Figure 38 Effect of Extraction Temperature on Alkylalkoxypyrazine Recovery on DVB/CAR/PDMS

Sample Volume:

Increasing the sample volume is believed to improve the detection limit; this parameter was, therefore explored. PDMS fibers were used to extract the headspace of a range of volume spiked model solutions. The headspace volume was maintained at 20 mL while the sample size was increased incrementally from 10 to 100 mL,

Sensitivity was not increased substantially, even at large excess sample volume. The partitioning of analyte into the fiber through the headspace from the liquid sample is probably limited by the headspace concentration.

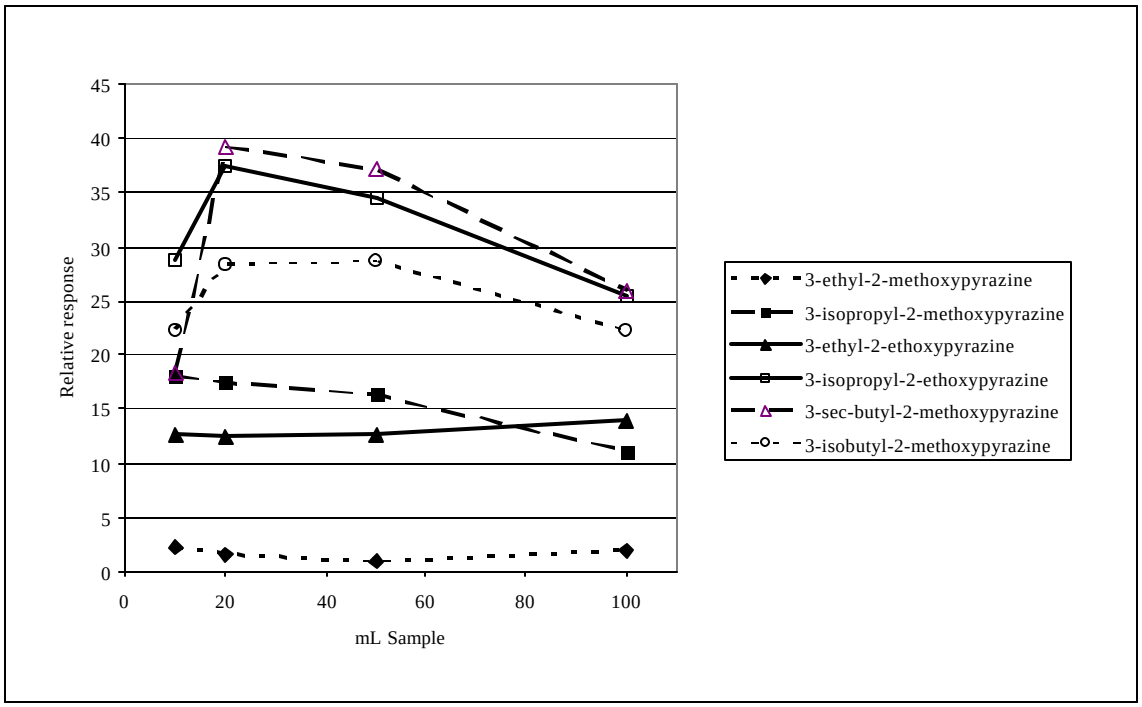


Figure 39 Effect of Sample Volume on HS SPME Recovery of Alkylalkoxypyrazines on PDMS

Analyte Depletion in the Headspace:

Repeated headspace extractions of the same sample of model solution were performed with DVB/Carboxen/PDMS fibers, to determine to what extent the analyte becomes depleted from the headspace and the system.

Analyte recoveries remained unchanged over five sequential extractions suggesting the fiber partitions a very small fraction of the overall analyte.

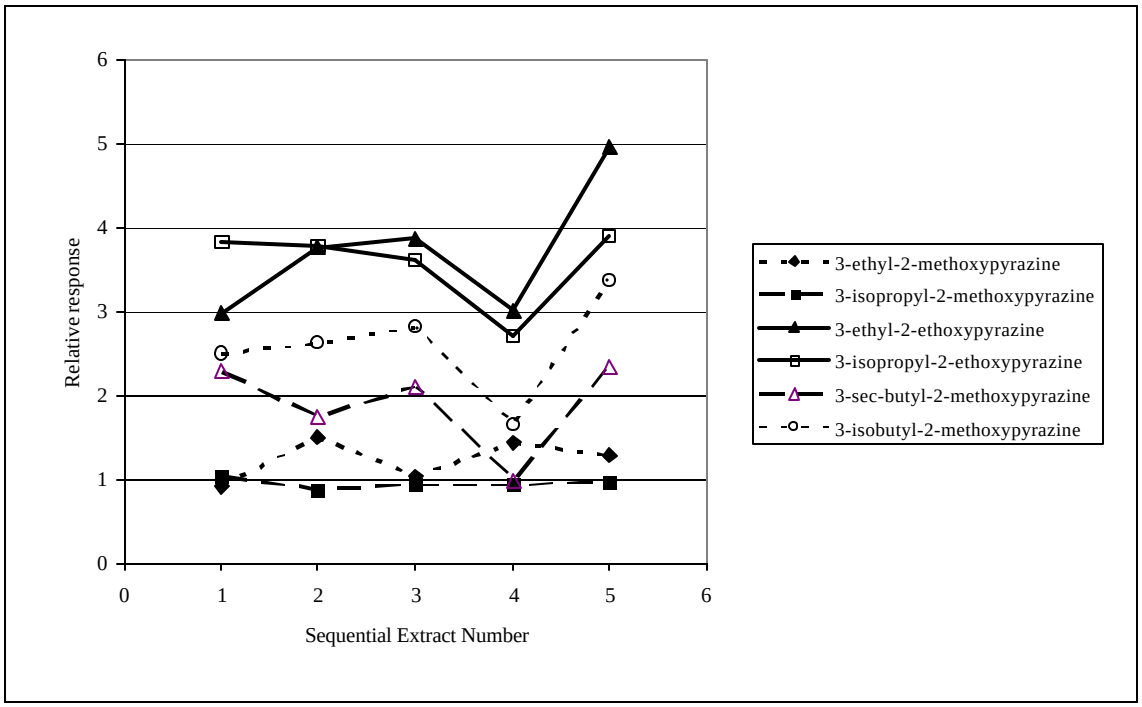


Figure 40 Effect of Repeated Extraction on Alkylalkoxypyrazine Recovery on DVB/CAR/PDMS

Desorption Temperature:

Fiber desoprtion temperatures in the GC inlet were studied by extracting the headspace of the model matrix with PDMS at standard conditions. The fibers were then desorbed for 5 minutes at temperatures ranging from 250° and 270 °C in the GC inlet. This variation in temperature showed minimal influence on the extraction efficiency.

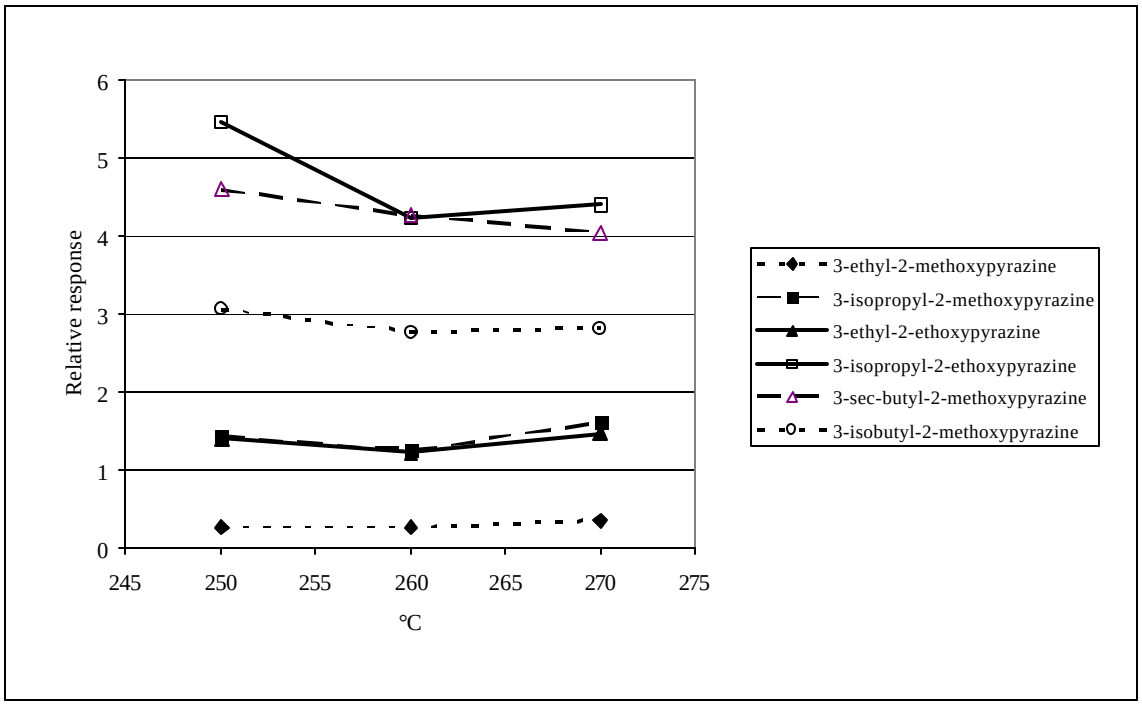


Figure 41 Effect of Desorption Temperature on HS SPME Alkylalkoxypyrazine Recovery on PDMS

Reproducibility:

Seven analyses of the spike model solutions performed on separate days with PDMS fibers resulted in analyses with relative standard deviations averaging 13.6%.

Table 21 Reproducibility of Spiked Model Solutions at Normal Conditions

Analyte	RT minutes	Averge Area	RDS %
ethylmethoxypyrazine	11.72	1.63	8.44
isopropylmethoxypyrazine	12.4	7.90	15.52
ethylethoxypyrazine	12.83	8.13	9.59
isopropylethoxypyrazine	13.38	37.54	15.77
secbutylethoxypyrazine	13.58	25.89	13.45
isobutylethoxypyrazine	13.69	18.32	18.73

Chapter VI: The Influence of Matrix Composition on HS SPME Analyte Recovery

Ethanol Concentration:

When SPME was performed on model solutions, an increase in ethanol concentrations from 0 to 20% (v/v), dramatically decreased analyte recovery, as illustrated in Figure 42. In most cases, ten times as much of the methoxypyrazines were recovered from non-alcoholic samples, as from samples with 20% (v/v) alcohol.

Ethanol increases the solubility of the pyrazine analytes in the aqueous phase shifting the equilibrium concentration away from the headspace. Ethanol is also a volatile compound; present at several million times the concentration of the analytes, which competes strongly for solubility in the half microliter of coating on the SPME fiber. These two effects combine to reduce the effectiveness of SPME for pyrazine analyte extraction from aqueous ethanol solutions.

Although the detection limit of pyrazines in alcoholic solutions is not low enough to measure the naturally occurring levels in wines, SPME may be more applicable to the measurement of non alcoholic matrices such as fruit and juice.

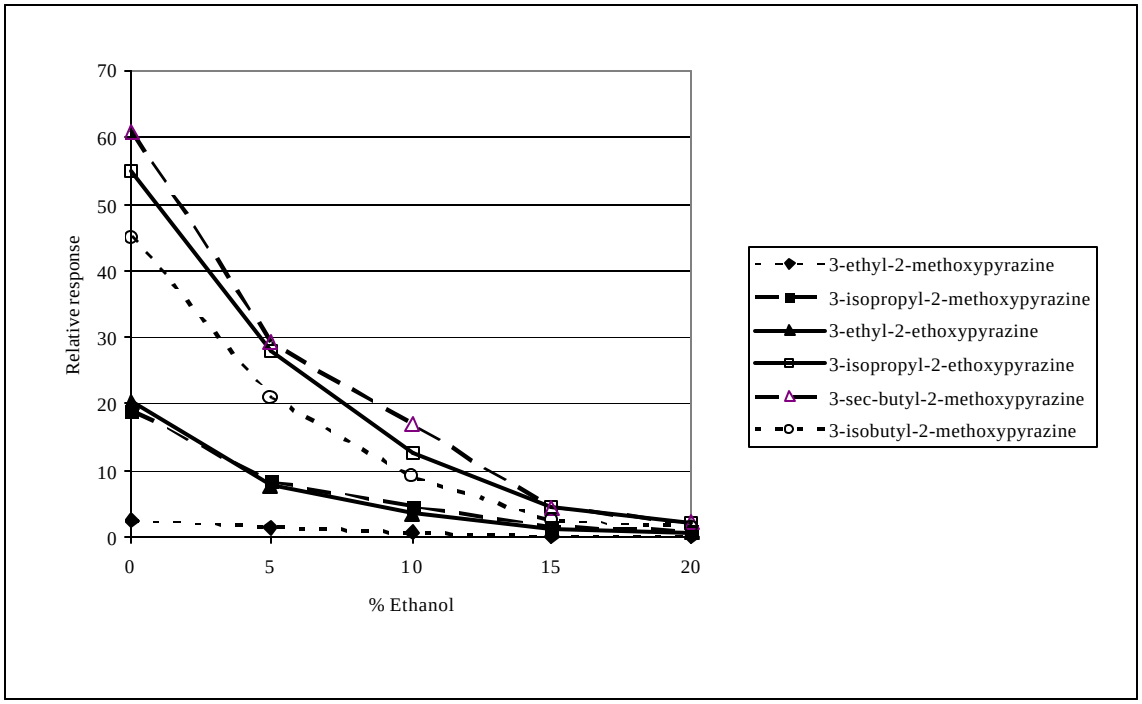


Figure 42 Effect of Ethanol Concentration of HS SPME Recovery of Alkylalkoxypyrazines on PDMS

pH:

pH may affect the perception of vegetative aromas in wine by influencing headspace concentration. The alkyl-methoxypyrazines are organic bases, which are protonated at low pH to form nonvolatile quaternary ammonium ions. Spiked model solutions were acidified, sealed, and subjected to SPME analyses with PDMS fibers at 35°C for 30 minutes. Solution pH was measured after the SPME extraction. As evident in Figure 43, analyte recovery dropped off significantly below pH 2.0, and was virtually eliminated below pH 1.0.

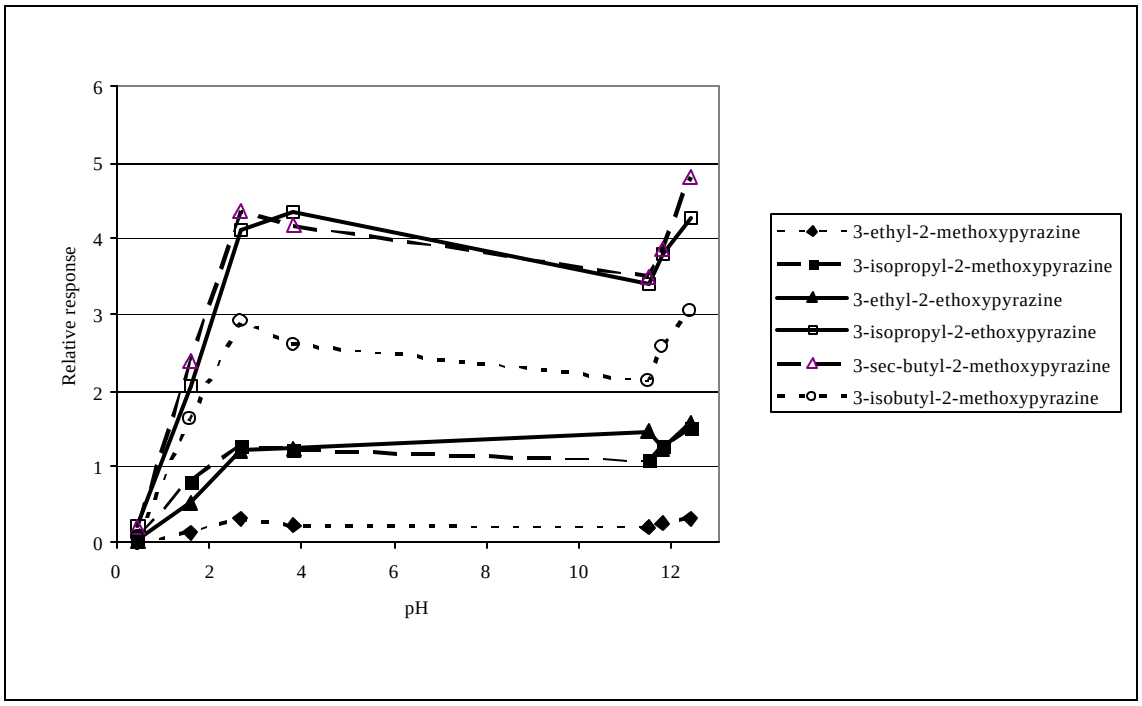
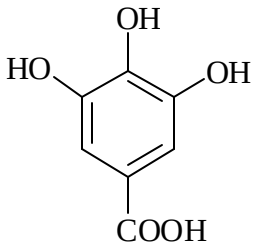


Figure 43 Effect of pH on HS SPME Alkylalkoxypyrazine Recovery on PDMS

Wine Phenols:

Wine phenolics, such as gallic acid and epicatechin, are aromatic compounds with multiple hydroxyl groups which could potentially bind pyrazines, reducing headspace concentrations. In the most extreme of several experiments, equal weights of gallic acid and epicatechin (combined concentrations up to 2.0 g/L), were added to the spiked model solutions and equilibrated for 20 days. Although not rigorously excluded, oxygen was not permitted into sealed vials during equilibration. These conditions, which exceed the typical phenolic levels in wine, did not show measurable effects on the pyrazine concentrations recoverable by SPME.

Gallic acid (3,4,5-trihydroxybenzoic acid) has three acidic hydroxyl groups potentially capable of acid base or hydrogen bonding activities. These could reduce the level of pyrazine analytes in the headspace by binding to the pyrazines in the aqueous phase. To establish whether alkylmethoxypyrazines react with gallic acid, synthetic matrices were spiked with gallic acid and analyzed under standard conditions within several hours of preparation.



Structure 3 Gallic Acid

Figure 44 shows the addition of gallic acid exerts no significant influence on the recovery of alkylmethoxypyrazines. Gallic acid containing solutions also exhibited a slightly lower pH of 2.4 compared to unspiked model solutions, which were 2.7.

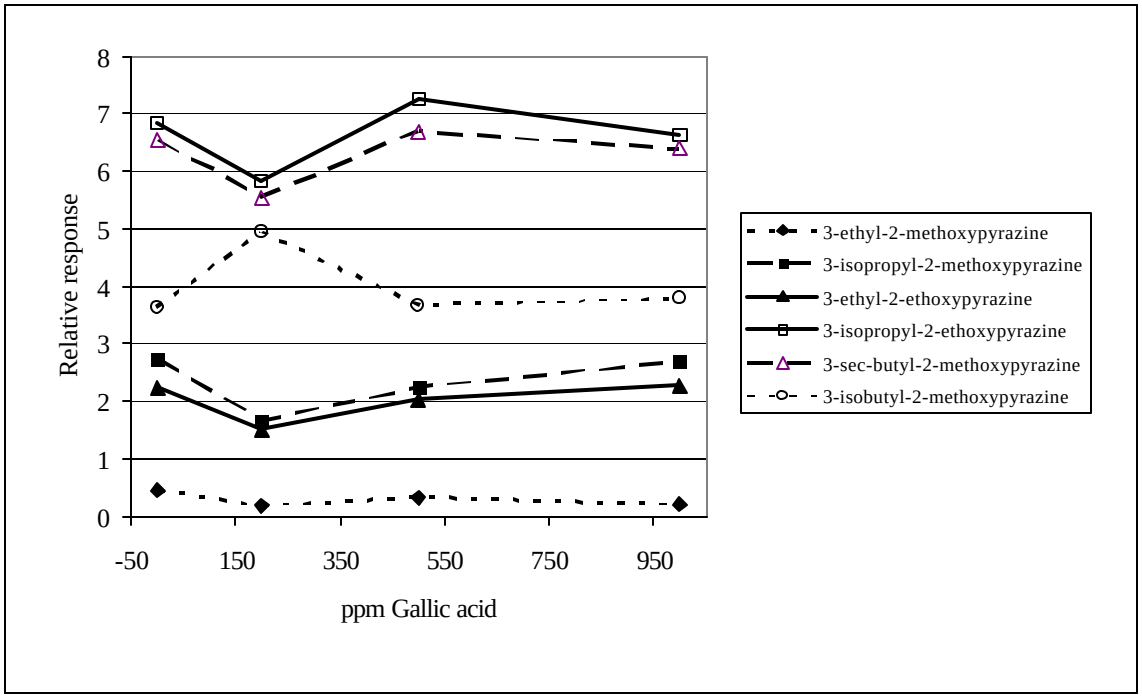
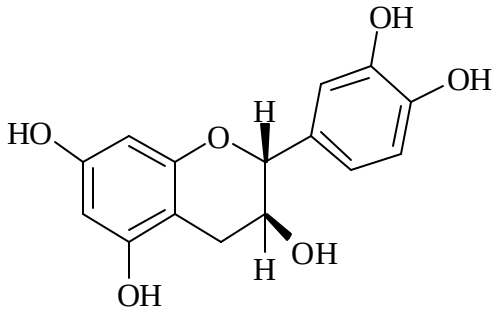


Figure 44 Effect of Gallic Acid on Recovery of Alkylalkoxypyrazines on PDMS

Catechin , illustrated below in Structure 4, is a monomer of common wine tannins. The headspace of synthetic matrices spiked with catechin were extracted to

establish whether it reacts with the target analytes. Samples were analyzed within several hours of preparation.



Structure 4 (+)-Catechin

HS SPME with PDMS fibers under the standard experimental conditions showed no evidence of reaction between the alkylmethoxyopyrazines and catechin. The specimen pH was 2.7 in the unspiked model solutions and 2.5 in solutions with catechin.

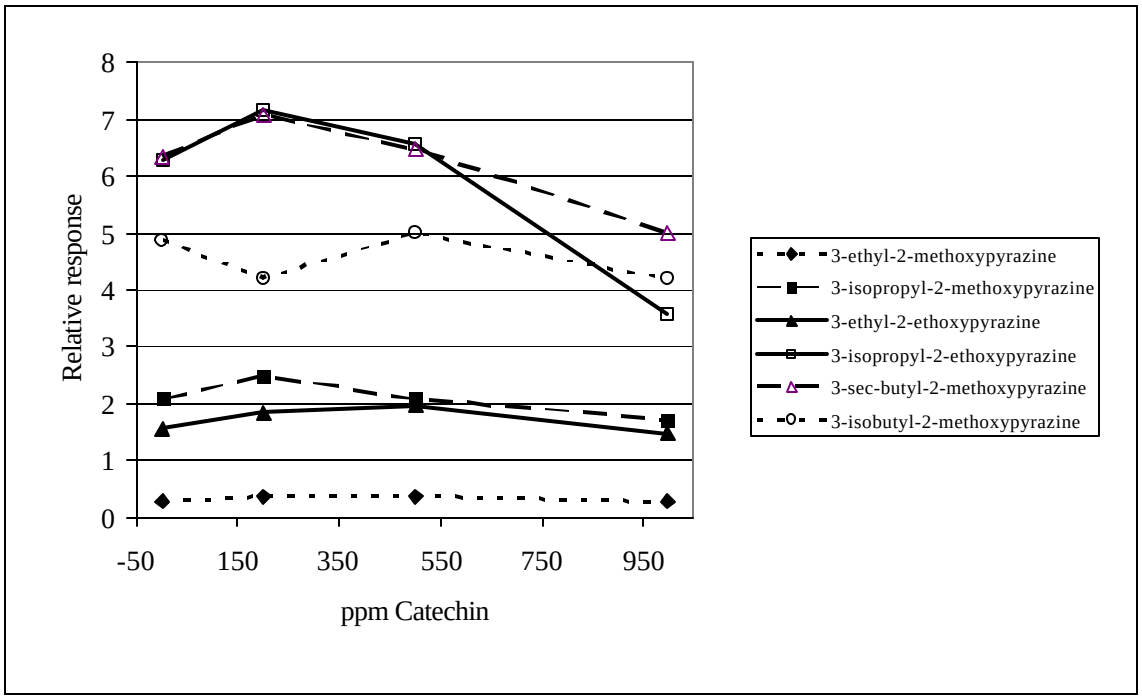


Figure 45 Effect of Catechin Concentration of HS SPME Recovery of Alkylalkoxypyrazines on PDMS

Both catechin and gallic acid were spiked into synthetic matrices containing 5ppb of the target analytes and allowed to equilibrate for 12 days in the headspace vial before analysis. The combination of the two phenolic compounds and extended reaction period was intended to drive any possible reaction with the target analytes. PDMS fibers were then used under the standard analytical conditions to determine how the headspace concentrations of alkylmethoxypyrazines were influenced.

There appears to be no appreciable reaction between the phenols or their reaction products, even after extend periods as illustrated in Figure 46. Model solution pH remained at 2.7 regardless of phenol concentration.

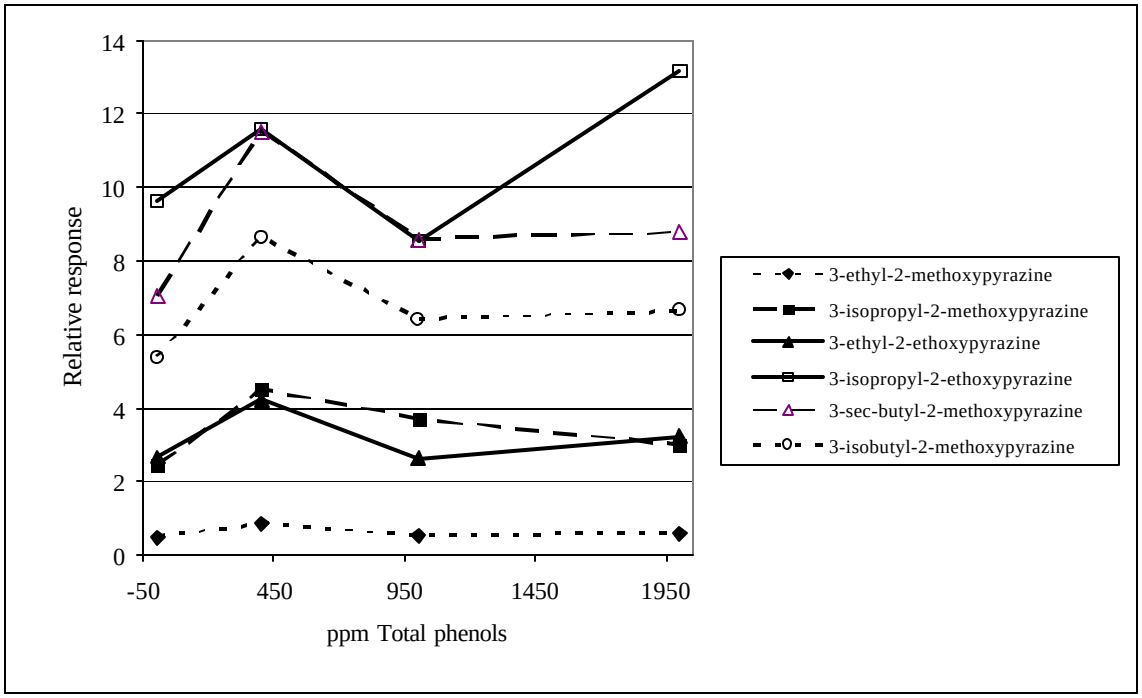


Figure 46 Effect of Gallic Acid and Catechin Recovery of Alkylalkoxypyrazines on PDMS

Oak Exposure:

Oak exposure is commonly employed in wine production during fermentation and aging. Oak aging causes wine phenolics and pigments to be stabilized by oxidation, introduces lactones and other components which contribute to the bouquet, and the concentrates the alcohol. It is not unreasonable to expect that chemical interactions could occur between alkylmethoxypyrazines and oak during aging.

To simulate the effect of wine exposure to oak during aging, oak sawdust was added and equilibrated with the spiked model solutions for 24 days. These solutions were then subjected to standard PDMS HS SPME extractions. The results are summarized in Figure 47. The level of recovered analytes were not substantially affected by the presence of oak shavings. The pH of the model solutions decreased from 3.0 to 2.4 for the 4% oak specimen.

The absorption of aroma compounds by oak has been reported.[24] This may be expected with wood, a high surface area substrate with polar hydroxyl groups and aromatic lignin structures. White oak sawdust was introduced into the spiked model solutions at 0, 0.25, 0.50, and 1% by weight, and equilibrated for 20 days. No measurable absorption was observed, suggesting oak does not have a strong affinity for alkylmethoxypyrazines.

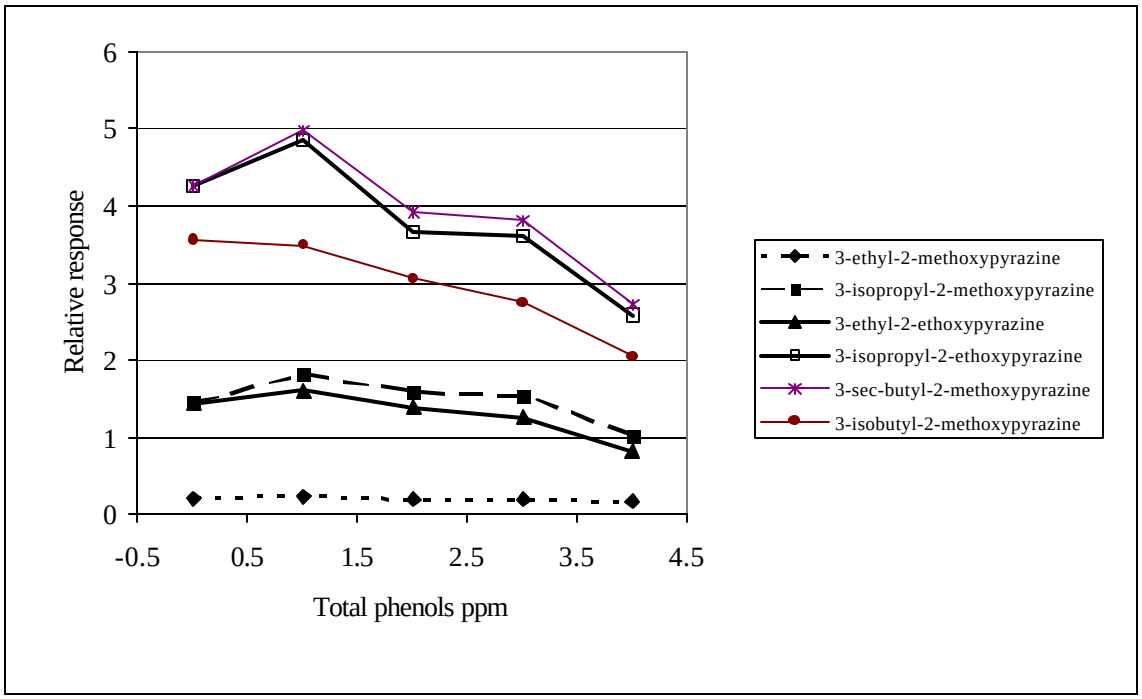


Figure 47 Effect of Oak Exposure on HS SPME Recovery of Alkylalkoxypyrazines on PDMS

Photodegradation:

Alkylmethoxypyrazines are reported to be sensitive to degradation by sunlight. A sample vial containing the spiked model solution was exposed to direct sunlight for 11

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24 Ramirez, G. R., S. Lubbers, C. Charpentier, M. Feuillat, A. Voilley, and D. Chassagne. "Aroma compound sorption by oak wood in a model wine." J. Agric. Food Chem. 49:3893-3897 (2001).

days to determine the impact photodegradation could have on analyses. Reductions in analyte recoveries were only marginally reduced. This demonstrates that ordinary laboratory conditions do not substantially degrade these compounds interfering with analyses. The vial and window glass practically eliminated exposure to ultraviolet light.

Chapter VII Conclusions

HS-SPME has proven to be a powerful and useful tool for probing the chemistry of complex matrices, including wine. The technique is very sensitive to experimental conditions, and is selective in its response to various analytes. Temperature, extraction time, pH, salt concentration and fiber selection were shown to influence the efficiency of analyte collection. Consequently, careful optimization of the analytical method is necessary.

Of the broad range of SPME fibers available PDMS and DVB/CAR/PDMS were the most durable and sensitive to the analytes.

Increasing sample temperature increased the concentrations of the analytes in the headspace and their recoveries up to a point. Optimum recoveries were achieved at 30°C on PDMS and 55°C on DVB/CAR/PDMD. At higher temperatures, recoveries decreased as fiber heating evaporated the absorbed pyrazines.

Longer extraction times produced better sensitivity. Exposure of the fiber to the headspace for more than 30 minutes resulted in negligible increases.

Adding 30% sodium chloride to the sample improves the sensitivity of the alkyl-methoxypyrazine extraction five fold by reducing their solubility in the aqueous sample. Sodium chloride resulted in higher recoveries than equal weights potassium chloride, calcium chloride, sodium iodide, sodium sulfate, and magnesium sulfate. Sodium chloride is the most soluble salt. It produces highest molar concentration of ions of the salts evaluated.

Acidity is a significant consideration for the alkyl-methoxypyrazines because they protonate below pH 2.0 producing highly soluble salt. At low pH's analytes are not sufficiently concentrated in the headspace to extract by SPME.

Sample analyses performed on a Hewlett Packard (Palo Alto, CA) HP6890 GC, equipped with a nitrogen phosphorous detector (NPD) with a 30 meter, 0.25 millimeter i.d., 0.25 micrometer film thickness HP-5MS (5% phenyl-polydimethylsiloxane) column separated the analytes in wine and related compounds very efficiently.

Using these optimized extraction conditions, the influence of wine matrix ingredients on the extraction of alkyl-methoxypyrazines added at 5 µg/L to model wines were studied. The following relationships were suggested.

Ethanol concentrations, at levels comparable to those in wine, significantly reduced the quantity recoverable by SPME fibers. This interference arises because ethanol increases analyte solubility in the aqueous samples and saturates the absorbent coating on the fiber.

While pHs below 2.0 depressed the volatility of these analytes; at wine pH levels, headspace 3-alkyl-2-methoxypyrazines are fairly stable. Monomeric phenolics, including gallic acid and (+)-catechin, and oak shavings did not interact with these analytes in model solutions.

Because relationships were determined at easily measurable µg/L levels in simple model solution, these trends may not be valid extrapolated to the ng/L alkyl-methoxypyrazines levels and more complex composition naturally occurring in wines.

Appendix A Volatile Analytes Reported in Wine

Table 22 Volatile Analytes Reported in Wine[25]

Compound	Class	Formula	MW	BP °C	CAS No	Comments
2-Methylpropanol	alcohol	$C_4H_{10}O$	74.1	107.9	78-83-1	Colorless liquid with a mild, non-residual odor
n-Butanol	alcohol	$C_4H_{10}O$	74.1	117.6	71-36-3	Colorless liquid with a strong, characteristic odor. In microscopy for preparing paraffin imbedding materials
3-Methylbutanol*	alcohol	$C_5H_{12}O$	88.1	130	123-51-3	Colorless liquid with an alcoholic odor which causes coughing. Artificial flavoring.
cis-3-Hexenol	alcohol	$C_6H_{12}O$	100.2	156	928-96-1	Colorless liquid
1-Octen-3-ol	alcohol	$C_8H_{16}O$	128.2	174	3391-86-4	One of the principal components of mushroom. Colorless liquid
2-Ethylhexanol	alcohol	$C_8H_{18}O$	130.2	183	104-76-7	Clear colorless liquid
Benzyl Alcohol	alcohol	C_7H_8O	108.1	205	100-51-6	AIR SENSITIVE; . Colorless oily liquid
Hexanol	alcohol	$C_6H_{14}O$	102.2	156 - 157	111-27-3	Colorless liquid
2-Phenylethanol*	alcohol	$C_8H_{10}O$	122.2	432 at 0 mm	60-12-8	Along with geraniol, comprises odor of roses. Colorless liquid
cis-9-Nonenol	alcohol					
Ethyl acetate*	ester	$C_4H_8O_2$	88.1	77.1	141-78-6	Colorless liquid with a pleasant, fruity odor detectable at 7 to 50 ppm. MOISTURE SENSITIVE

25 Vas, G., Koteleky, K.,Farkas, M., Dobo, A., and Vekey, K. "Fast screening method for wine headspace compounds using solid-phase microextraction and capillary GC technique". Am. J. Enol. Vitic. 49:100-104 (1998).

Table 22 Volatile Analytes Reported in Wine[25]

Compound	Class	Formula	MW	BP °C	CAS No	Comments
Isoamylacetate	ester	$C_7H_{14}O_2$	130.2	142	123-92-2	Clear colorless liquid with a banana-like odor. Present in fruit like pear and apples, more so with ripening. Artificial flavoring
Ethyl hexanoate*	ester	$C_8H_{16}O_2$	144.2	168	123-66-0	Colorless to pale yellow liquid
Benzaldehyde	ester	C_7H_6O	106.1	179	100-52-7	Colorless to yellow liquid, readily discernible almond-like odor. A component of the odor of cherries, almonds, apricot and peach pits. Light/air sensitive
Diethyl malonate	ester	$C_7H_{12}O_4$	160.2	199	105-53-3	Colorless liquid
Diethylsuccinate	ester	$C_8H_{14}O_4$	174.2	217	123-25-1	
Ethyl decanoate*	ester	$C_{12}H_{24}O_2$	200.3	245	110-38-3	Colorless liquid
Ethyldodecanoate	ester	$C_{14}H_{28}O_2$	228.4	269	106-33-2	Colorless oily liquid
Ethyllactate	ester	$C_5H_{10}O_3$	118.1	154 and 151	687-47-8 and 97-64-3	
Ethyl octanoate	ester	$C_{10}H_{20}O_2$	172.3	206 - 208	106-32-1	Colorless liquid
Isoamyloctanoate	ester	$C_{13}H_{26}O_2$	214.3	267 - 268	2035-99-6	Colorless liquid
Hexyl acetate*	ester	$C_8H_{16}O_2$	144.2	338 at 0 mm	142-92-7	Colorless oily liquid
Phenethylacetate	ester	$C_{10}H_{12}O_2$	164.2	450 at 0 mm	103-45-7	Colorless liquid
Ethyl-9-decenoate	ester					
Furfural	heterocycle	$C_5H_4O_2$	96.1	167	98-01-1	Colorless to light brown liquid which darkens in light and air, with an odor like almonds. LIGHT/AIR

Table 22 Volatile Analytes Reported in Wine[25]

Compound	Class	Formula	MW	BP °C	CAS No	Comments
						SENSITIVE. Synthesis of tetrahydrofuran and furfuryl alcohol, phenolic and furan polymers.
gamma-Butyrolactone	lactone	$C_4H_6O_2$	86.1	204 - 205	96-48-0	Colorless, oily liquid.
Acetic acid*	organic acid	$C_2H_4O_2$	60.1	117.9	64-19-7	Colorless liquid or solid with a strong vinegar-like odor detectable at 0.2 to 1.0 ppm.
3-Methylbutyric acid	organic acid	$C_5H_{10}O_2$	102.1	177	503-74-2	STENCH. Colorless liquid
Heptanoic acid	organic acid	$C_7H_{14}O_2$	130.2	223	111-14-8	Colorless, oily liquid
Octanoic Acid	organic acid	$C_8H_{16}O_2$	144.2	239.7	124-07-2	Oily liquid
trans-Cinnamic acid	organic acid	$C_9H_8O_2$	148.2	300	140-10-3	Pale amber powder
Hexanoic Acid	organic acid	$C_6H_{12}O_2$	116.2	202 - 203	142-62-1	Oily liquid with characteristic goat-like odor. Present in milk fats, coconut and palm oil. Colorless or slightly yellow oily liquid. STENCH.
Decanoic Acid	organic acid	$C_{10}H_{20}O_2$	172.3	268 - 270	334-48-5	White, crystalline solid
Syringic acid	organic acid	$C_9H_{10}O_5$	198.2		530-57-4	
Methionol	other	$C_4H_{10}OS$	106.2	195	505-10-2	STENCH. Pale yellow liquid
beta-Ionone*	other	$C_{13}H_{20}O$	192.3	229	14901-07-6	flowers (violets), dry grass - hay. Pale yellow liquid
Vanillin	other	$C_8H_8O_3$	152.1	285	121-33-5	vanilla seeds, flavoring in many foods. MOISTURE and LIGHT SENSITIVE. White to off white crystals
p-Cymene	other	$C_{10}H_{14}$	134.2	176 - 178	99-87-6	

Table 22 Volatile Analytes Reported in Wine[25]

Compound	Class	Formula	MW	BP °C	CAS No	Comments
Acetovanillone	other	$C_9H_{10}O_3$	166.2	295 - 300	498-02-2	
2-Acetylfuran	other	$C_6H_6O_2$	110.1	67 at 10 mm Hg	1192-62-7	Colorless crystals
beta-damascenone	other		190.1		23726-93-4	
2,6-di(t-bu)-4-OH-4-me-cyclohexadienone	other					
BHT-Aldehyde	other					
2,4,6-trichloroanisole	other	$C_7H_5Cl_3O$	211.5		87-40-1	
4-ethylphenol*	phenol	$C_8H_{10}O$	122.2	218	123-07-9	
4-ethylguaiacol*	phenol	$C_9H_{12}O_2$	152.2	234 - 236	2785-89-9	Colorless liquid
Linalool	terpene	$C_{10}H_{18}O$	154.3	199	78-70-6	One of the principal components of bergamot or french lavender. Colorless liquid
Citronellol	terpene	$C_{10}H_{20}O$	156.3	222	106-22-9	Colorless to pale yellow liquid

* Investigated by Whiton and Zoecklein[45]

Appendix B Nitrogen Containing Volatiles and Semivolatiles Reported in Wines

Table 23 Nitrogen Containing Volatiles and Semivolatiles Reported in Wines[26]

Compound	Class	Formula	MW	BP °C	CAS No.	Comments	Synonyms
n-butylacetamide	amide	$C_6H_{13}NO$	115.2	117 at 9 mm Hg	1119-49-9		
N,N-Dibutylformamide	amide	$C_9H_{19}NO$	157.3	120 at 15 mm Hg	761-65-9		N,N-Di-n-butylformamide; Di-n-butylformamide
N-isobutylacetamide	amide	$C_6H_{13}NO$	115.17		1540-94-9		
N-isopentylacetamide	amide	$C_7H_{15}NO$	129.2		13434-12-3		N-(3-methylbutyl) acetamide
N-[3-(methylthio)Propyl]-acetamide	amide	$C_6H_{13}NOS$	147.2		141-98-0		Carbamothioic acid, ethyl-, O-(1-methylethyl) ester
n-(2-phenylethyl)-acetamide	amide	$C_{10}H_{13}NO$	163.2		877-95-2		
N-methyl-N-butyl-acetamide	amide	$C_7H_{15}NO$					
Ethylamine	amine	C_2H_7N	45.1	16.6	75-04-7	Colorless liquid or gas with a strong ammonia-like odor	
Isopropylamine	amine	C_3H_9N	59.1	32.4	75-31-0	Colorless liquid with a pungent, ammonia-like odor.	
Methylamine	amine	CH_5N	31.057	48	74-89-5	colorless gas with an ammonia-like odor, at low concentrations fishy odor, liquid under pressure	
Propylamine	amine	C_3H_9N	59.1	48	107-10-8	colorless liquid with a pungent odor similar to ammonia	
sec-butylamine	amine	$C_4H_{11}N$	73.1	63.5	13952-84-6		
Isobutylamine	amine	$C_4H_{11}N$	73.1	66	78-81-9	Clear, colorless liquid	
n-Butylamine	amine	$C_4H_{11}N$	73.1	77	109-73-9	Colorless liquid with and odor like ammonia or fish	
Isopentyl amine	amine	$C_5H_{13}N$	87.164	97	107-85-7		
n-Pentylamine	amine	$C_5H_{13}N$	87.164	104.4	110-58-7	colorless, volatile liquid with ammonia like odor.	
Pentylamine	amine	$C_5H_{13}N$	87.164	104.4	110-58-7		
Ethylenediamine	amine	C_2H_8N	60.1	116.5	107-15-3	Colorless liquid or solid with an ammonia-like odor. LACHRYMATOR/	

26 Jia, M.,Zhang, Q.H., and D.B. Min. "Optimization of solid-phase microextraction analysis for headspace flavor compounds of orange juice". J. Agric. Food Chem. 46:2744-2747 (1998).

Table 23 Nitrogen Containing Volatiles and Semivolatiles Reported in Wines[26]

Compound	Class	Formula	MW	BP °C	CAS No.	Comments	Synonyms
1,3-Propanediamine	amine	$C_3H_{10}N_2$	74.1	140	109-76-2	WATER-WHITE MOBILE LIQUID.	
Nitrosodimetnylamine	amine	$C_2H_6N_2O$	74.1	149	62-75-9	Yellow liquid of low viscosity, no appreciable odor	
1,4-butanediamine	amine	$C_7H_{19}N_3$	88.152	158	110-60-1	animals, rotting flesh	Putrescine; Tetramethylenediamine; 1,4-Butanediamine; 1,4-Butadiamine;
2-aminoethanol	amine	C_2H_7NO	61.1	171	141-43-5	Colorless liquid with a mild ammonia-like odor; solid below 51 degrees F.	
N-methylbenzamine	amine	C_7H_9N	107.15	196.3	100-61-8	Yellow to light-brown liquid with a weak, ammonia-like odor.	
phenethylamine	amine	$C_8H_{11}N$	121.18	200	64-04-0	AIR SENSITIVE	2-Phenylethylamine; Beta-phenylethylamine; Benzeneethanamine;
N-methylethylphenethylamine	amine	$C_9H_{13}N$	135.21	203	589-08-2		
Hexylamine	amine	$C_6H_{15}N$	101.19	131 - 132	111-26-2		
p-(2-aminoethyl)phenol	amine	$C_8H_{11}NO$	137.18	175 - 181 at 8 mm Hg	51-67-2		tyrosamine; 4-(2-Aminoethyl)phenol; 2-(p-Hydroxyphenyl)ethylamine; L-Tyramine; 4-Hydroxyphenylethylamine
Butanediamine	amine	$C_4H_{12}N_2$	88.152		4426-48-6 and 590-88-5		
2-amino-4-octadecen-1,3-diol	amine	$C_{18}H_{37}NO_2$	299.5				D-erythro-Sphingosine
1,5-Pentyldiamine	amine	$C_5N_{14}N_2$					
Pyrrolidine	heterocycle	C_4H_9N	71.122	87	123-75-1	liquid. LACHRYMATOR.	tetrahydropyrrole; 1-Pyrrolidine; 2-pyrrolidine
Piperidine	heterocycle	$C_5H_{11}N$	85.149	106.3	110-89-4	Clear colorless liquid, amine-like odor, strongly basic	Hexahydropyridine; Pentamethyleneimine; Azacyclohexane; cyclopentimine; cypentil; hexazane
Indole-3-ethanol	heterocycle	$C_{10}H_{11}NO$	161.2	174 at 2.66 mm Hg	526-55-6	3-Hydroxyethyl indole; beta-3-Indolylethanol; 3-(2-Hydroxyethyl)-indole; Indole-3-ethanol; 3-Indoleethanol	
2-acetyl-1-pyrroline	heterocycle	C_6H_9NO	111.1		99583-29-6		
Histamine	heterocycle	$C_5H_9N_3$	111.15		51-45-6		1H-Imidazole-4-ethanamine; 4-(2-Aminoethyl)-1H-imidazole; 2-(4-Imidazolyl)ethylamin; Histamine Base

Table 23 Nitrogen Containing Volatiles and Semivolatiles Reported in Wines[26]

Compound	Class	Formula	MW	BP °C	CAS No.	Comments	Synonyms
2-methoxy-3-ethyl pyrazine	heterocycle	$C_7H_{10}N_2O$	138.2		25680-58-4		2-Methoxy-3-ethylpyrazine
2-benzothiazole	heterocycle	C_7H_5NS	151.19		934-34-9		
2-methoxy-3-isopropyl pyrazine	heterocycle	$C_8H_{12}N_2O$	152.2		25773-40-4		2-Methoxy-3-isopropyl-pyrazine; 2-Isopropyl-3,5 or 6-methoxypyrazine; 2-Methoxy-3,5(6)-isopropyl pyrazine;
2-methoxy-3-isobutyl pyrazine	heterocycle	$C_9H_{14}N_2O$	166.2		24683-00-9	Flavor constituent of green bell pepper	Pyrazine, 2-methoxy-3-(2-methylpropyl)-; 3-Isobutyl-2-methoxypyrazine; 2-Methoxy-3-isobutylpyrazine; 2-Methoxy-3(6)-isobutyl-pyrazine; 2-Isobutyl-3-, 5 or 6-methoxy-pyrazine; 2-Isobutyl-3-methoxypyrazine, cont. 20% 2-isobutyl-6-methoxypyrazine
3-indolylacetic acid	heterocycle	$C_{10}H_9NO_3$	175.19		87-51-4		
3-(3-indolyl)proprionic acid	heterocycle	$C_{11}H_9NO_2$	189.21		830-96-6	3-Indolepropionic Acid; 1H-Indole-3-propanoic acid; beta-Indole-3-propionic acid;	
5-methoxy-2-indolylcarboxylic acid	heterocycle	$C_{10}H_9NO_3$	191.19		4382-54-1	5-Methoxyindole-2-carboxylic acid	
2-acetyl-1,4,5,6-tetrahydropyridine	heterocycle						
2-acetyl-3,4,5,6-tetrahydropyridine	heterocycle						
ethyltetrahydropyridine	heterocycle						
Histidine	heterocycle	$C_6H_9N_3O_2$			71-00-1		Glyoxaline-5-alanine; His; H; Histidine; Alpha-amino-4-imidazolepropionic acid; L-Histidine, Free Base; L-(-)-histidine; 2-Amino-3-(1H-imidazol-4-yl)-propanoic acid; L-2-Amino-3-(4-imidazolyl)propionic acid; Alpha-amino-1H-imidazole-4-propionic acid;
Sec-butymethoxypyrazine	heterocycle						
Thymine	heterocycle	$C_5H_6N_2O_2$			65-71-4		2,4-Dihydroxy-5-methylpyrimidine; 5-Methyluracil; T; Thy; 2,4-dioxy-5-methyl pyrimidine; 2,4(1H,3H)-

Table 23 Nitrogen Containing Volatiles and Semivolatiles Reported in Wines[26]

Compound	Class	Formula	MW	BP °C	CAS No.	Comments	Synonyms
							Pyrimidinedione, 5-methyl
Uracil	heterocycle	$C_4H_4N_2O_2$			66-22-8		2,4-Dihydroxypyrimidine; U; 2,4-dioxy pyrimidine; 2,4(1H,3H)-Pyrimidinedione
3-Amino-1-propanol	nitrogen compoound	C_3H_9NO	75.1	187 - 188	156-87-6		
Methyl isothiocyanate	nitrogen compound	C_2H_3NS	73.112	117	556-61-6		Isothiocyanatomethane; Methyl mustard oil; Trapex; Degussa methyl isothiocyanate; MTC; Biomet 33; MENCS
Benzothiazole	nitrogen compound	C_7H_5NS	135.2	231	95-16-9		
Methyl anthranilate	nitrogen compound	$C_8H_9NO_2$	151.16	237	134-20-3	Colorless to pale yellow liquid with light blue fluorescence. LIGHT SENSITIVE	o-aminobenzoic acid methyl ester; o-carbomethoxyaniline; 2-(methoxycarbonyl)aniline; Carbomethoxyaniline; methyl o-Aminobenzoate; Methyl amino benzoate; methyl anthranilate fcc; ANTRANILATO DE METILO
N-methyl benzamide	nitrogen compound	C_8H_9NO	135.2	291	613-93-4		
4-methylthiazole	nitrogen compound	C_4H_5NS	99.2	133 - 134	693-95-8		
ethyl nicotinate	nitrogen compound	$C_8H_9NO_2$	151.2	223 - 224	614-18-6		Ethyl 3-pyridinecarboxylate
Ethyl carbamate	nitrogen compound	$C_3H_7NO_2$	87.1	91 at 8 mm Hg	625-50-3		urethane [51-79-6]
2-(diacetylamino)ethanol	nitrogen compound	$C_6H_{11}NO_3$					
dihydroactiniolide	nitrogen compound						

Appendix C Alkylmethoxypyrazine Analyisis per Sala et.al.

Table 24 Alkylmethoxypyrazine Analyisis per Sala et.al. .[21]

Fiber				
Phase	PDMD-DVB	Carboxen-PDMS	PDMS	CW-DVB
Diameter um	65	75	100	65
Precondition				
Temperature °C	250			
Time minutes	1			
Extraction				
Contact	Headspace			
Vial ml	20			
Sample ml	10			
NaCl g	3			
Time hours	4			
Temperature °C	30			
Agitation	continuous			
Comments	dark			
Desorption				
Temperature °C	250			
Time minutes	1			
Instrument				
Make	HP			
Model	5890			
Inlet				
Type	Splitless			
Purge minutes	1			
Inlet diameter mm	0.75			
Temperature °C	250			
Helim ml/min	0.8			
Split ml/min	52			
Purge ml/min	2			
Column				
Phase	CP Wax 57CB Bonded poly(35%diphenyl-65%dimethyl)siloxane	SPB-35		
Length meters	50	30		
Diameter mm	0.25	0.25		
Film thickness um	0.2	0.25		
Oven				
Initial Temperature °C	30			
Hold Minutes	1			
Rate °/min.	25			
Temperature °C	100			
Hold Minutes	20			
Rate °/min.	25			
Rate	180			

Hold Minutes	20
Detector	NPD
Temperature °C	250
Hydrogen ml/min	4
Air ml/min	100
Make-up ml/min	24

Analyte Retention Times (minutes)

Analyte	**Sala, et.al.**
3-sec-butyl-2-methoxypyrazine	17
3-isobutyl-2-methoxypyrazine	18
3-isopropyl-2-methoxypyrazine	13.5
3-ethyl-2-methoxypyrazine	13.7

[27]

[27] Sala, C., M. Mestres, M.P. Marti, O. Busto, and J. Guasch. "Headspace solid-phase microextraction method for 3-alkyl-2-methoxypyrazines in musts by means of polydimethylsiloxane-divinylbenzene fibers." J. Chromatogr. A. 880:93-99(2000).

Appendix D Published Research

Measurement of 3-Alkyl-2-Methoxypyrazine by Headspace Solid-Phase Microextraction in Spiked Model Wines

Peter J. Hartmann,[1] Harold M. McNair,[2] and Bruce W. Zoecklein[3]*

The effect of wine matrix ingredients and conditions on the headspace sampling of 3-alkyl-2-methoxypyrazines was investigated with solid-phase microextraction (SPME) and capillary gas chromatography, using a nitrogen phosphorus detector. Changes in the recovery of 3-ethyl-, isopropyl-, *sec*-butyl-, and isobutyl-2-methoxypyrazines from the static headspace of synthetic wine matrices spiked with 5 µg/L of each analyte were investigated and reported as a function of SPME fiber type, extraction time, and temperature. The influence of pH, ethanol, phenolics, and oak was studied. Divinylbenzene/carboxen/polydimethylsiloxane (PDMS) SPME fibers at an extraction temperature of 50°C for 30 minutes with 30% (w/v) added sodium chloride resulted in the highest analyte recoveries. Although PDMS (100 µm) SPME fibers at an extraction temperature of 35°C for 30 minutes with 30% (w/v) added sodium chloride resulted in lower analyte recoveries, the fiber remained functional for 50 to 75 analyses after other coatings deteriorated. Changing the sample ethanol concentration from 0 to 20% (v/v) resulted in an exponential decrease in the recovered analytes. Below pH 2, there was extensive loss of the analytes in the headspace. No measurable impact on alkyl-methoxypyrazine headspace concentrations was observed with exposures to selected phenolics and to oak.

Key words: Gas chromatography, solid-phase microextraction, alkyl-methoxypyrazines, pyrazines

Premium wine quality can be achieved only through a favorable balance between fruit, fermentation, and processing-derived aromas and flavors. The 3-alkyl-2-methoxypyrazines produce vegetative odors characteristic of green bell peppers and peas. The olfactory threshold at which these compounds are sensed is extraordinarily low: 1 to 10 ng/L in water. These compounds, 3-isobutyl-2-methoxypyrazine, 3-*sec*-butyl-2-methoxypyrazine, 3-isopropyl-2-methoxypyrazine, and 3-ethyl-2-methoxypyrazine, are shown in Figure 1 in decreasing order of natural abundance [2]. As reported by Allen et al. [3,4], methoxypyrazine concentrations in grapes are influenced by variety, fruit maturity, season, climate, and solar exposure of the fruit. These plant metabolites are found in grape varieties including Cabernet Sauvignon and Sauvignon blanc and may detract from quality and varietal character. Measurement of these compounds is, therefore, of significant interest to the winemaker.

Quantitation of these potent aroma compounds in wine at ng/L levels has been achieved by solvent extraction and concentration, followed by isotope dilution gas chromatography/mass spectrometry (GC/MS) [1]. Liquid-liquid extractions can be time-consuming and labor intensive. Solid-phase microextration (SPME) has been investigated as a simple and rapid way to isolate and concentrate wine aroma components [8,13].

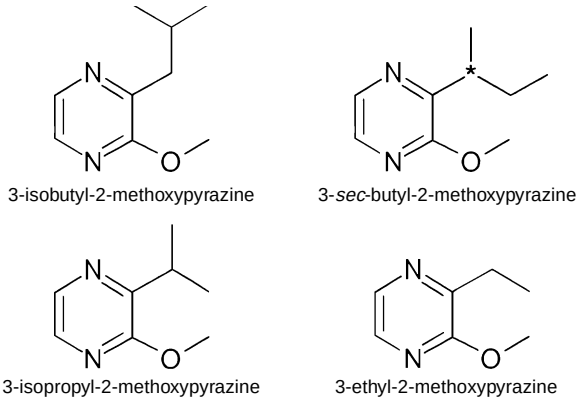

Figure 1 3-Alkyl-2-methoxypyrazine compounds with vegetative character present in grapes and wines.

Headspace (HS)-SPME has been applied to characterize and measure the aromas from a variety of food and beverage products, including coffee [12], orange juice [5], and peanut butter [9]. HS-SPME has been used for analysis of a variety of desirable and off odors in wines, including trichloroanisole and ethylphenols [6,7]. Sala et al. [11] reported the measurement of alkyl-methoxypyrazines at the ng/L level in grape musts.

Materials and Methods

Model solutions. Pyrazine standards (3-ethyl-, 3-isopropyl, 3-*sec*-butyl-, and 3-isobutyl-2-methoxypyrazine) and internal standards (3-ethyl- and 3-isopropyl-2-ethoxypyrazine) were purchased from Pyrazine Specialties (Atlanta, GA). SPME studies were performed on model solutions prepared with high-

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performance liquid chromatography (HPLC)-grade water with 12% (v/v) ethanol and 2 g/L potassium bitartrate, spiked with 5 µg/L of each target analyte. The solution pH averaged 3.3.

Sampling. A 20-mL aliquot of the model solution, containing 100 ng of each analyte, was added to a 40-mL headspace vial with a magnetic stirring bar and 6.0 g sodium chloride. Sodium chloride was used to increase the partitioning of the organic analytes from the aqueous sample into the headspace. The temperature of the sample vial was maintained inside a jacketed beaker, filled with water to the level of the liquid inside the vial, and connected to a temperature controlled circulating bath. During extraction, the sample was stirred as rapidly as possible without splashing. SPME fibers (Supelco, Bellefonte, PA) were introduced into the sample headspace for selected time periods, then withdrawn and introduced into the heated inlet of the gas chromatograph for 10 min.

Gas chromatography. Sample analyses were performed on a Hewlett Packard (Palo Alto, CA) HP6890 GC, equipped with a nitrogen phosphorous detector (NPD). The carrier gas was helium, flowing at 1.2 mL/min. The splitless GC inlet was maintained at 250°C. The purge and temperature programming were activated to commence the analysis immediately after the fiber was introduced into the inlet. SPME fibers were kept in the heated inlet for a total of 10 min: 5 min splitless and an additional 5 min with a purge flow of 50 mL/min to clean the fiber. The analytes were separated on a 30 m, 0.25 mm i.d., 0.25 µm film thickness HP-5MS (5% phenyl-polydimethylsiloxane) column. The oven was initially held at 40°C for 5 min during fiber desorption, heated at 10°C/min to 80°C to separate the pyrazines, then ramped at 20°C/min to 230°C, and finally held for 5 min at 230°C to elute retained high-boiling-point compounds.

Results and Discussion

Typical HS-SPME chromatographic results for the target analytes and related 3-alkyl-2-ethoxypyrazines are tabulated in Table 1. The analytes eluted in order of increasing molecular weight. Linear calibrations between 100 and 1000 ng/L, corresponding to 2 to 20 ng in each 20 mL sample, were obtainable. Detection limits in spiked model solutions and Cabernet Sauvignon were approximately 100 ng/L, compared to the 5 ng/L naturally present in wines. The sensitivity of the nitrogen phosphorus detector to directly injected alkyl-methoxypyrazines was measured at approximately 1 picogram, which suggests that only 0.05% of the analytes from the aqueous sample ultimately partition into the 1 µL of absorbent coating on the SPME fiber. This is not atypical of HS-SPME, considering the relatively low volatility of the analytes. The variability, expressed as relative standard deviation, of seven replicated 5 µg/L spiked model solutions analyzed over several weeks with several polydimethylsiloxane (PDMS) fibers ranged between 8 and 18%.

Table 1 Gas chromatographic 3-alkyl-2-methoxypyrazine analysis extracted on PDMS SPME fibers.

Analyte	Molecular wt	Retention time (min)	Kovats Retention Index
3-Ethyl-2-methoxypyrazine	138.17	11.72	1063
3-Isopropyl-2-methoxypyrazine	152.20	12.40	1099
3-Ethyl-2-ethoxypyrazine	152.20	12.83	1127
2-Isopropyl-3-ethoxypyrazine	166.22	13.38	1165
3-*sec*-Butyl-2-methoxypyrazine	166.22	13.58	1180
3-Isobutyl-2-methoxypyrazine	166.22	13.69	1187

SPME fiber selection. SPME fibers are composed of fused silica or polymer, coated with layers of materials with an affinity for organic compounds. Several types of SPME fibers, with a range of polarities and mechanisms, are commercially available. Six SPME fibers were evaluated to establish the best choice for trace analysis of pyrazines. The results expressed in terms of normalized analyte recovery are summarized in Figure 2.

Two types of coating were evaluated. The first type extracts the analytes into the film on the fiber and is termed "absorptive." This includes PDMS and polyacrylate (PA). Of the absorptive fibers, PDMS resulted in the best overall performance in chromatography, desorption characteristics, and analyte recovery. It did not recover low molecular weight 3-ethyl-2-methoxypyrazine as efficiently as the 3-butyl-2-methoxypyrazines.

The second type of coating contains porous particles of divinylbenzene (DVB) or carbon (Carboxen™), which adsorb organics on the surface of their pores. The adsorptive coatings evaluated included carbowax/divinylbenzene (CW/DVB), polydimethylsiloxane/divinylbenzene (PDMS/DVB), and divinylbenzene/Carboxen™/polydimethylsiloxane (DVB/CAR/PDMS) in standard 1- and 2-cm lengths. The 2-cm DVB/CAR/PDMS resulted in the best recoveries, as well as acceptable chromatography. The larger 3-butyl-2-methoxypyrazine molecules were collected much more effectively than 3-ethyl-2-methoxypyrazine, just as with the PDMS-coated fibers.

Extraction time. The optimum headspace sampling time period is that required for the analytes in the fiber to achieve equilibrium with the headspace. Shorter periods may compro-

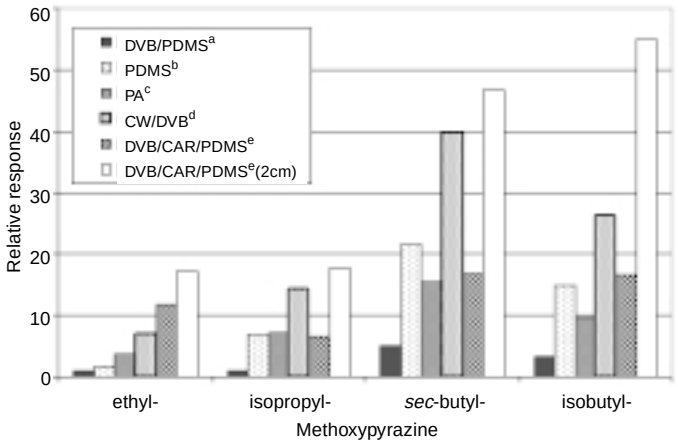

Figure 2 Performance of selected SPME fiber types on the relative response of 3-alkyl-2-methoxypyrazines extracted from the headspace of model solutions: ([a]divinylbenzene/PDMS, [b]PDMS, [c]polyacrylate, [d]carbowax/divinylbenzene, [e]divinylbenzene/Carboxen™/PDMS).

mise sensitivity and risk variability, while longer periods may reduce the efficiency of the analyses. SPME fibers were exposed to the headspace of the model solutions for 0.25 to 4.5 hr to establish the optimum extraction time. PDMS and DVB/CAR/PDMS were saturated in the first 30 min, with little change thereafter. The typical response leveling off after 30 min for 2 cm divinylbenzene/Carboxen™/PDMS SPME fibers at an extraction temperature of 45°C is shown in Figure 3.

Extraction temperature. Sample temperature controls the kinetics and equilibrium between the aqueous sample and the headspace. As temperature increases, the liquid and gas phases equilibrate faster. Increasing the temperature drives more of the high-boiling-point compounds, such as the pyrazine analytes, into the vapor phase. Increasing fiber temperature causes a competitive effect, the evaporation of volatile analytes from the absorbent layer on the SPME fiber, ultimately limiting its analytical sensitivity.

Model solutions were extracted at temperatures ranging from 10 to 65°C with both PDMS and DVB/CAR/PDMS fibers for 30 min. The optimum temperature for most analytes was 30°C with PDMS and 40°C to 50°C with DVB/CAR/PDMS (Figures 4 and 5).

Sample pH. pH may also affect the perception of vegetative aromas in wine by influencing headspace concentration. The alkyl-methoxypyrazines are organic bases, which are potentially protonated at low pH to form nonvolatile quaternary ammonium ions. Spiked model solutions were quickly acidified, sealed, and subjected to SPME analyses with PDMS fibers at 35°C for 30 min. Solution pH was measured after the SPME extraction. Analyte recovery dropped off significantly below pH 2.0 and was virtually eliminated below pH 1.0, as evident in Figure 6.

Ethanol content. When SPME was performed on model solutions, an increase in ethanol concentrations from 0 to 20% (v/v) dramatically decreased analyte recovery, as illustrated in Figure 7. In most cases, 10 times the methoxypyrazines were recovered from nonalcoholic samples as from samples with 20% (v/v) alcohol.

Ethanol increases the solubility of the pyrazine analytes in the aqueous phase, shifting the equilibrium concentration away from the headspace. Ethanol is also a volatile compound, present at several million times the concentration of the analytes, that competes strongly for solubility in the 1 µL of coating on the

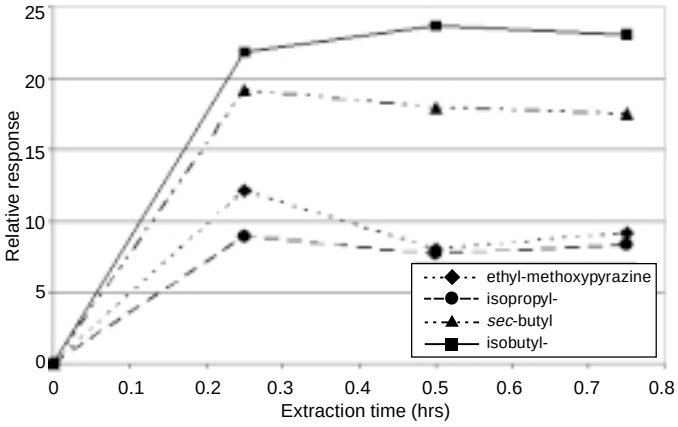

Figure 3 Effect of extraction time on the relative response of 3-alkyl-2-methoxypyrazines extracted on 2-cm divinylbenzene/Carboxen™/PDMS SPME fibers.

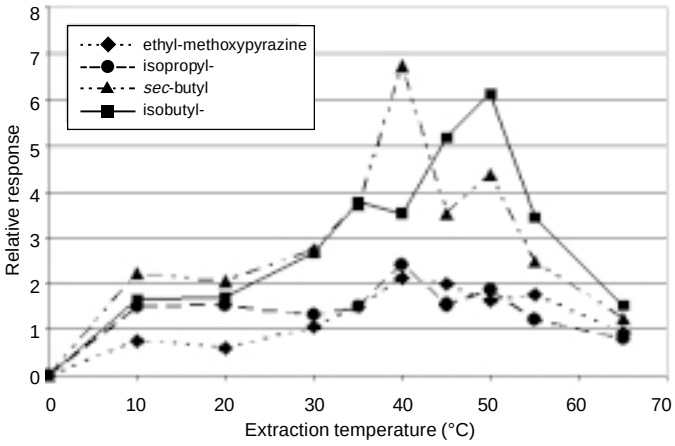

Figure 5 Effect of sample temperature on the relative response of 3-alkyl-2-methoxypyrazines extracted on 2-cm divinylbenzene/Carboxen™/PDMS SPME fibers.

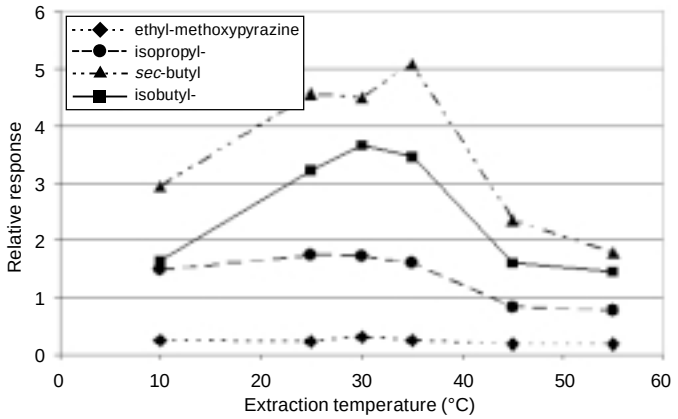

Figure 4 Effect of sample temperature on the relative response of 3-alkyl-2-methoxypyrazines extracted on PDMS SPME fibers.

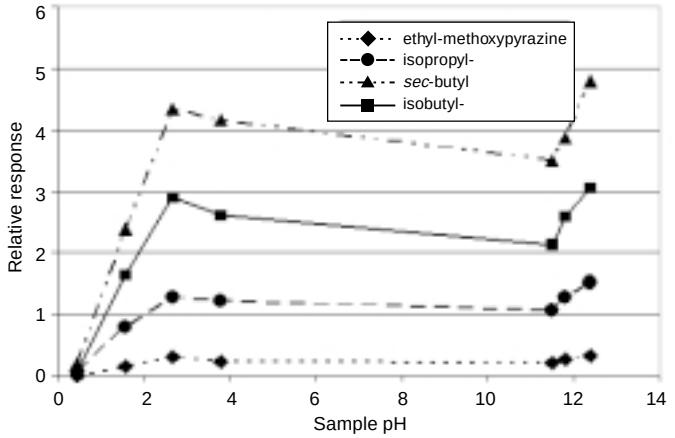

Figure 6 Effect of sample pH on the relative response of 3-alkyl-2-methoxypyrazines extracted on PDMS SPME fibers.

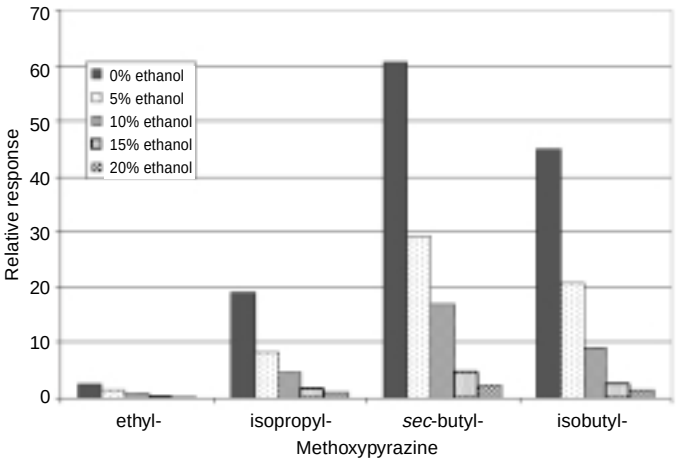

Figure 7 Effect of ethanol concentration on the relative response of 3-alkyl-2-methoxypyrazines extracted on 2-cm divinylbenzene/ Carboxen™/ PDMS SPME fibers.

SPME fiber. These two effects combine to reduce the effectiveness of SPME for pyrazine analyte extraction from aqueous ethanol solutions.

The detection limit of methoxypyrazines in alcoholic solutions may not be low enough to measure the naturally occurring levels in wines. SPME may be more applicable to the measurement of nonalcoholic matrices such as fruit and juice as previously demonstrated by Sala et al. [11].

Phenolics. Wine phenolics, such as gallic acid and epicatechin, are aromatic compounds with multiple hydroxyl groups that could potentially bind pyrazines, reducing headspace concentrations. In the most extreme of several experiments, equal weights of gallic acid and epicatechin (combined concentrations up to 2.0 g/L) were added to the spiked model solutions and equilibrated for 20 days. Although not rigorously excluded, oxygen was not permitted into sealed vials during equilibration. These conditions, which exceed the typical phenolic levels in wine, did not show measurable effects on the pyrazine concentrations recoverable by SPME (data not shown).

Oak exposure. The absorption of aroma compounds by oak has been reported [10]. This may be expected with wood, a high surface area substrate with polar hydroxyl groups and aromatic lignin structures. White oak sawdust was introduced into the spiked model solutions at 0, 0.25, 0.50, and 1% by weight and equilibrated for 20 days. No measurable absorption was observed, suggesting oak does not have a strong affinity for alkylmethoxypyrazines (data not shown).

Summary

HS-SPME has proven to be a powerful and useful tool for probing the chemistry of complex matrices, including wine. The technique is sensitive to experimental conditions and is selective in its response to various analytes. Temperature, extraction time, and fiber selection were shown to influence the efficiency of analyte collection. Consequently, careful optimization of the analytical method is necessary.

This work has allowed study of the influence of experimental conditions on the extraction of methoxypyrazines when added at 5 mg/L to model wine. Ethanol concentrations, at levels comparable to those in wine, significantly reduced the quantity recoverable by SPME fibers. Acidic pH levels below 2.0 depressed the volatility of these analytes; at wine pH levels, headspace 3-alkyl-2-methoxypyrazines are fairly stable. Monomeric phenolics and oak did not interact with these analytes in model solutions. Because relationships were determined at easily measurable mg/L levels in a simple model solution, these trends may not be valid when extrapolated to the ng/L alkylmethoxypyrazine levels and more complex composition naturally occurring in wines.

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Vita

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