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

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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].

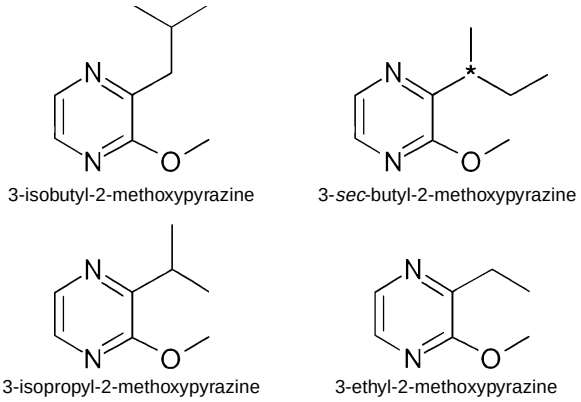

Figure 1 3-Alkyl-2-methoxypyrazine compounds with vegetative character present in grapes and wines.

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-

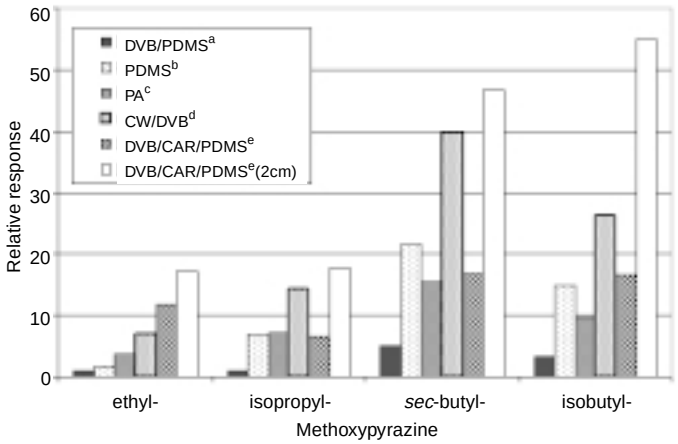

Figure 2 Performance of selected SPME fiber types on the relative response of 3-alkyl-2-methoxypyrazines extracted from the headspace of model solutions: ([a]divinylbenzene/PDMS, [b]PDMS, [c]polyacrylate, [d]carbowax/divinylbenzene, [e]divinylbenzene/Carboxen™/PDMS).

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

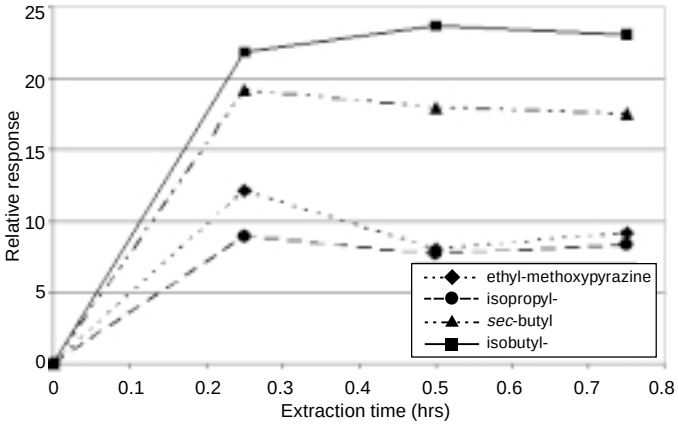

Figure 3 Effect of extraction time on the relative response of 3-alkyl-2-methoxypyrazines extracted on 2-cm divinylbenzene/Carboxen™/PDMS SPME fibers.

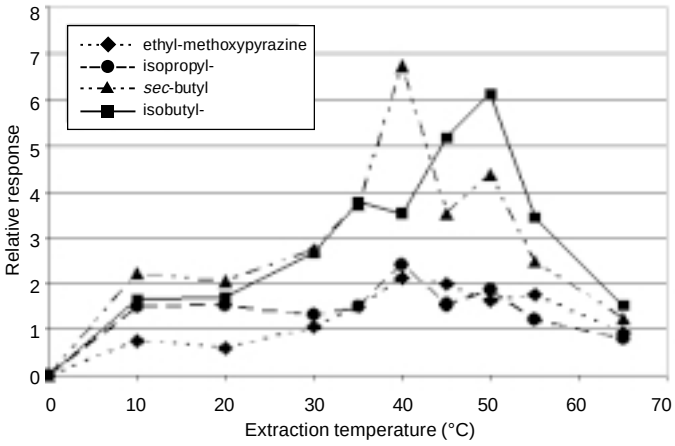

Figure 5 Effect of sample temperature on the relative response of 3-alkyl-2-methoxypyrazines extracted on 2-cm divinylbenzene/Carboxen™/PDMS SPME fibers.

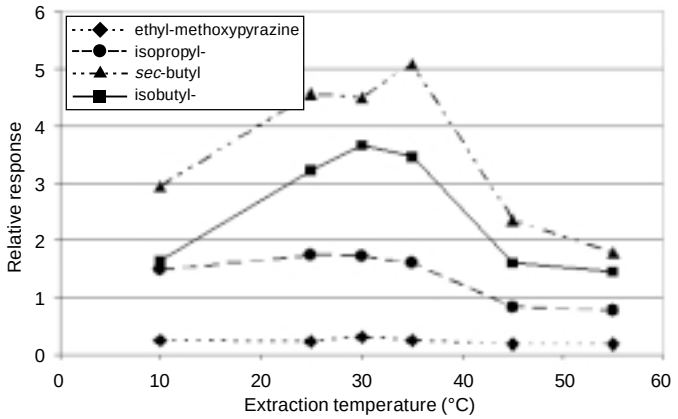

Figure 4 Effect of sample temperature on the relative response of 3-alkyl-2-methoxypyrazines extracted on PDMS SPME fibers.

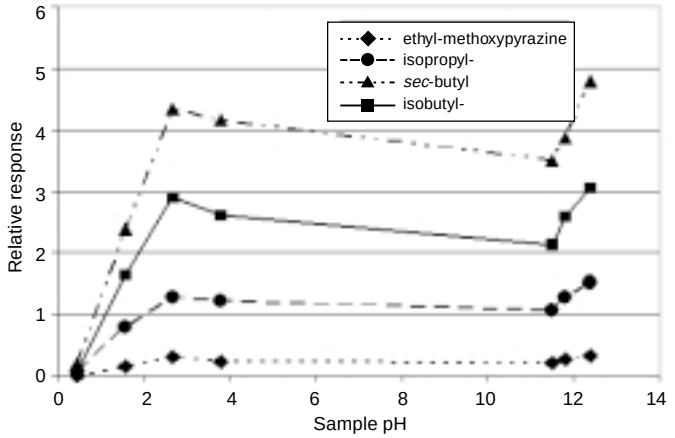

Figure 6 Effect of sample pH on the relative response of 3-alkyl-2-methoxypyrazines extracted on PDMS SPME fibers.

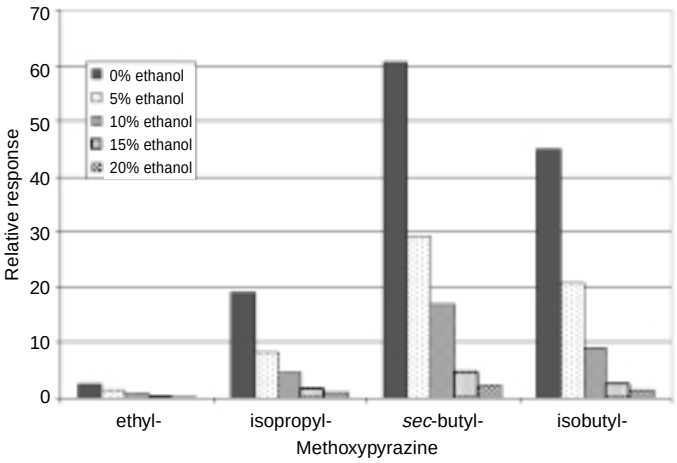

Figure 7 Effect of ethanol concentration on the relative response of 3-alkyl-2-methoxypyrazines extracted on 2-cm divinylbenzene/ Carboxen™/ PDMS SPME fibers.

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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