Main Factors Affecting Headspace Analysis of Some Pyrazines Produced by Microorganisms

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Pyrazines produced by microorganisms can be found in low quantities in culture media. The best way to concentrate these molecules is to trap them on porous polymers during headspace sampling. Because of the very high solubility of pyrazines in water, trapped quantities are generally low. To determine the main factors affecting headspace analysis of four pyrazines, a fractional factorial design involving four parameters has been carried out: extraction time, salt quantity added, pH, and vapor-phase temperature. The multivariate analysis showed that for 2-methylpyrazine, 2,5-dimethylpyrazine, and 2,3,5-trimethylpyrazine the most important factors are sampling time and salt quantity added. There was no change in the percentage of the recovery for the 3-methyl-2-methoxypyrazine for these parameters.

INTRODUCTION

Many methods are used for extraction of volatiles. Among these methods, headspace technique has been developed for flavor analysis. An inert gas sweeps volatile compounds out of the solution which are then adsorbed on a trap. Adsorption on a porous polymer has been used to extract microbiological samples, for example, volatiles produced by *Pseudomonas taetrolens* (Miller et al., 1973) and *Tricholoma matsutake* (Yajima et al., 1981).

Polymers present many advantages as adsorbents: (1) efficient concentration of volatiles with minimum interference from most other constituents; (2) hydrophobic character; (3) reverse adsorption at moderate temperature; (4) no (or low) chemical or catalytic activity at operating temperature.

The porous polymers mainly employed are Tenax GC, Chromosorb 105, and Porapak Q. Adsorbents with a large surface area tended to have a high sampling capacity, but a single polymer was not suitable for all sampling applications. Withycombe et al. (1978) studied various polymers to analyze volatile compounds from hydrolyzed vegetable protein and found that Tenax GC produced the best correlation with sensory evaluation and Porapak Q was the best adsorbent for many compounds, especially pyrazines. Previous work carried out in our laboratories with extraction, concentration, and analysis, using the technique of Butler and Burke (1976), confirmed Lin's (1976) conclusions that Tenax GC had a higher retention capacity for high boiling point compounds than Porapak Q. Because of this feature, washed Porapak Q acetone (Hayesep Q) was selected for the analysis of the main factors affecting headspace sampling of culture media containing pyrazines.

Pyrazines are heterocycles with two nitrogen atoms which give different sensory responses. Four pyrazines have been reported to have a "nutty" character: 2-methylpyrazine, 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, and 3-methyl-2-methoxypyrazine. Table I lists microorganisms that produce pyrazines. However, as far as we known, pyrazines have never previously been analyzed at the low levels found in culture media.

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MATERIALS AND METHODS

(1) Headspace Sampling of a Model Medium Containing Pyrazines. *(1.1) Composition of Culture Medium.* For 1 L of medium: Bacto Yeast carbon base (Difco), 11.7 g; DL-threonine, 2.203 g; monoammonium phosphate, 1.846 g; L-alanine, 1.435 g; sodium acetate, 1.322 g; sodium nitrate, 1.369 g; glycerol, 0.742 g; pyruvic acid, 0.769 g; pH 5.3.

Four pyrazines (2-methylpyrazine, 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, and 3-methyl-2-methoxypyrazine) were added together to the medium at 10 ppm (v/v).

(1.2) Sampling. Ten milliliters of culture medium containing pyrazines was put into a 30-mL closed flask, connected to a column (2 mm i.d. × 80 mm) packed with 69 mg of Hayesep Q 80–100 mesh (Porapak Q acetone washed) as shown in Figure 1. The temperature of the flask was maintained at 60 °C in a thermostatic water bath. To avoid water condensation in the flask, the vapor phase was passed through an oven prior to the entrainment on the column of Haseyep Q. Then, the column was isolated and the trapped water on the porous polymer was exhausted by flushing with nitrogen (60 mL min^{-1}) for 10 min.

(1.3) Gas Chromatographic Procedure. For desorption, the trap loaded with adsorbed volatile was inserted directly into the injection port of a Packard 427 chromatograph fitted with a stainless steel column (3 m × 2.2 mm i.d.) containing Chromosorb W AW 100–120 mesh coated with 10% Carbowax 20 M. The flame ionization detector temperature was 250 °C, and injector temperature was 180 °C. The column temperature was programmed from 70 to 170 °C at 2 °C min^{-1}. The flow rates were nitrogen (carrier gas) 20 mL min^{-1}, hydrogen 20 mL min^{-1}, air 200 mL min^{-1}. Integrator was a Shimadzu CR3A.

(2) Methodology. The first step was to establish which factors affect the recovery of pyrazines from a model medium. The importance of experimental variables must be known to extend the use of headspace analysis.

Our preliminary experimental results coupled with published works led us to the investigation of the four following factors: (1) sampling time; (2) addition of salts to the medium [Several authors (Van Praag et al., 1988; Voilley et al., 1977) added various salts to the medium to increase the activity coefficient and improve the extraction of volatile constituents; ammonium sulfate was used as the salt in this study.]; (3) pH [Since pyrazines are alkaline compounds, they are found in the basic fraction of volatile extracts (Liardon et al., 1982). An increase of the pH decreases their solubility in an aqueous medium. During the study of the tetramethylpyrazine production by *Bacillus subtilis* (Zak et al., 1972), pH was adjusted at 8.3. At this value, tetramethylpyrazine was insoluble in water.]; (4) vapor-phase temperature (Since pyrazines are very soluble in water, the gas phase was heated prior to the trapping stage to prevent water condensation. This led to a decrease of the variability of the results.)

0021-8561/91/1439-2176$02.50/0

Table I. Biosynthesis of Alkylpyrazine and Alkoxymethoxypyrazine by Microorganisms

microorganism	alkylpyrazine[a]	alkylmethoxypyrazine[a]	ref
Aspergillus oryzae	+		Kosuge et al. (1971)
Aspergillus sojae	+		Kosuge et al. (1971)
Bacillus natto	+		Kosuge et al. (1971)
Acetobacter aceti	+		Kosuge et al. (1971)
Saccharomyces cerevisiae	+		Kosuge et al. (1971)
Pseudomonas taetrolens	+	[a]+	Morgan (1976)
Aspergillus oryzae	+	+	Liardon and Ledermann (1980)
Cedecea davisae	+	+	Gallois and Grimont (1985)
Serratia rubidae	+	+	Gallois and Grimont (1985)
Serratia odorifera	+	+	Gallois and Grimont (1985)
Serratia ficaria	+	+	Gallois and Grimont (1985)
Penicillium caseicolum		+	Karahadian et al. (1985)
Penicillium camemberti		+	Karahadian et al. (1985)

[a] +, mean present.

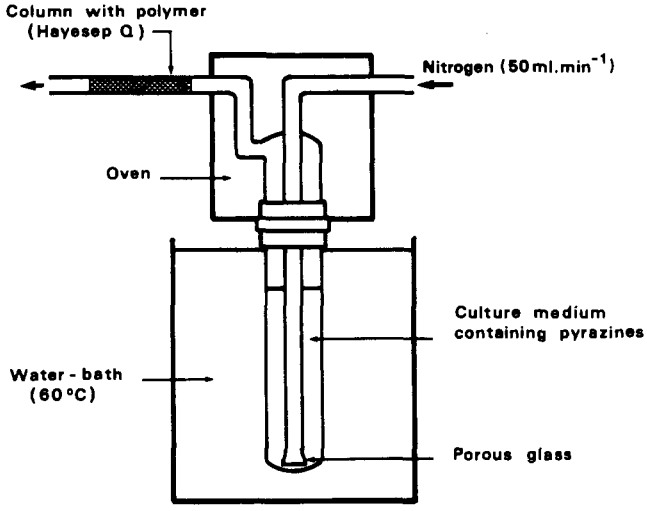

Figure 1. Headspace extraction system.

Table II. Experimental Factor Levels

factor	level (−)	level (+)
X_1 = sampling time, h	1.5	3
X_2 = ammonium sulfate, g L^{-1}	100	150
X_3 = pH	5.3	8.3
X_4 = headspace temperature, °C	85	105

A fractional factorial design was used because it measures the magnitude of the main effects (De Meo et al., 1985). Two levels were used for each variable (see Table II).

A complete factorial design using all possible combinations of two levels of each factor would have required $2^4 = 16$ experiments. Levels were coded: the symbol − represented the lower level and the symbol + the higher level (Table II). From a X^4 complete factorial matrix (16 experiments), 16 effects (coefficients) could be calculated, but only 8 effects were considered: b_0, arithmetical means of responses; b_i, four main effects; b_{ij}, three first-order interactions (sampling time–salt quantity, sampling time–pH, salt quantity–pH). If a 2^{4-1} fractional factorial matrix is used, eight coefficients can be calculated.

We assumed that the vapor-phase temperature had no interaction with the three other factors because the only role of the factor X_4 was to avoid water condensation before the polymer trap.

(2.1) 2^{4-1} Fractional Factorial Matrix Construction (Table III). The columns 1, 2, and 3 of the 2^3 complete factorial design represent the three factors X_1, X_2, and X_3 in standard order. The multiplication of the individual elements X_1, X_2, and X_3 gives columns $X_4 = X_1X_2X_3$. This cross-product column was used to define the levels of the factor X_4 and was added to the 2^3 factorial matrix. X_1, X_2, X_3, and X_4 together form the experimental matrix.

For each pyrazine, both the percentage of recovery and the variation were calculated. Then, also for each pyrazine, the main effect of each factor and first-order interactions were determined using the NEMROD program.

Table III. Experiment Design

expt	X_1	X_2	X_3	$X_4 = X_1X_2X_3$
1	−	−	−	−
2	+	−	−	+
3	−	+	−	+
4	+	+	−	−
5	−	−	+	+
6	+	−	+	−
7	−	+	+	−
8	+	+	+	+

Table IV. Recovery Percentage for Each Pyrazine and Each Experiment of the Design[a]

expt	2-MeP	2,5-DiMeP	2,3,5-TriMeP	3-Me-Mo-P
1	43.7 (2.8)	41.4 (2.3)	30.6 (3.7)	65.1 (2.5)
2	47.2 (6.3)	54.7 (1.7)	39.5 (1.5)	62.8 (1.2)
3	51.6 (1.9)	47.3 (1.4)	40.1 (2.3)	69.5 (3.3)
4	61.2 (1.9)	62.1 (0.2)	42.3 (5.4)	64.5 (2.9)
5	43.0 (2.1)	41.1 (5.4)	31.3 (2.6)	61.8 (2.2)
6	51.9 (5.8)	50.9 (4.8)	41.5 (3.6)	66.1 (2.0)
7	49.1 (0.7)	44.3 (3.9)	37.8 (3.4)	64.2 (1.0)
8	47.6 (3.7)	56.1 (0.9)	42.9 (3.2)	63.1 (1.6)

[a] Me, methyl; Mo, methoxy; P, pyrazine. Results are the average of three trials. Percentage of variation is shown in parentheses.

RESULTS AND DISCUSSION

The experimental results are given in Table IV. The best recoveries obtained were 61.2% for 2-methylpyrazine in experiment 4 (sampling time, 3 h; ammonium sulfate, 150 g L^{-1}; pH 5.3; vapor-phase temperature, 85 °C); 62.1% for 2,5-dimethylpyrazine in experiment 4 (same conditions as for 2-methylpyrazine); 42.9% for 2,3,5-trimethylpyrazine in experiment 8 (sampling time, 3 h; ammonium sulfate, 150 g L^{-1}; pH 5.3; vapor-phase temperature, 105 °C); and 69.5% for 3-methyl-2-methoxypyrazine in experiment 3 (sampling time, 1 h, 30 min; ammonium sulfate, 150 g L^{-1}; pH 5.3; vapor-phase temperature, 105 °C).

The fractional factorial design allowed us to calculate the coefficient b_i and b_{ij} for every pyrazine, representing the effect of every variable $X_i(b_i)$ and their interaction with $X_j(b_{ij})$. For each coefficient, a Student test was carried out. The results for all pyrazines are summarized in Table V.

(1) 2-Methylpyrazine. $b_1 = 2.56$ and $b_2 = 2.97$ were significant at the 0.1% level. The amount of ammonium sulfate and sampling time were the factors affecting recovery. Their positive values showed that maximum recovery is obtained with 3 h of sampling time and 150 g L^{-1} ammonium sulfate. Vapor-phase temperature was the third factor affecting recovery, there was a significant effect

Table V. Results Obtained for Each Pyrazine Headspace Sampling, b_i, b_{ij} Significant Threshold, and Optimum Level

factor	coefficient	Student test[a]	optimum level
(1) 2-Methylpyrazine			
sampling time (X_1)	$b_1 = 2.56$	+++	+ (3 h)
ammonium sulfate (X_2)	$b_2 = 2.97$	+++	+ (150 g L^{-1})
pH (X_3)	$b_3 = -1.49$	+	- (5.3)
headspace T (X_4)	$b_4 = -2.06$	++	- (85 °C)
interaction X_1, X_2	$b_{12} = -0.59$	>10%	
interaction X_1, X_3	$b_{13} = -0.71$	>10%	
interaction X_2, X_3	$b_{23} = -2.52$	+++	+ (X_2), - (X_3)
	$b_0 = 49.42$		
(2) 2,5-Dimethylpyrazine			
sampling time (X_1)	$b_1 = 6.23$	+++	+ (3 h)
ammonium sulfate (X_2)	$b_2 = 2.70$	+++	+ (150 g L^{-1})
pH (X_3)	$b_3 = -1.66$	++	- (5.3)
headspace T (X_4)	$b_4 = 0.09$	>10%	
interaction X_1, X_2	$b_{12} = 0.42$	>10%	
interaction X_1, X_3	$b_{13} = -0.84$	>10%	
interaction X_2, X_3	$b_{23} = -0.60$	>10%	
	$b_0 = 49.78$		
(3) 2,3,5-Trimethylpyrazine			
sampling time (X_1)	$b_1 = 3.30$	+++	+ (3 h)
ammonium sulfate (X_2)	$b_2 = 2.52$	+++	+ (150 g L^{-1})
pH (X_3)	$b_3 = 0.12$	>10%	
headspace T (X_4)	$b_4 = 0.20$	>10%	
interaction X_1, X_2	$b_{12} = -1.46$	++	+ (X_1), + (X_2)
interaction X_1, X_3	$b_{13} = 0.53$	>10%	
interaction X_2, X_3	$b_{23} = -0.56$	>10%	
	$b_0 = 38.25$		
(4) 3-Methyl-2-methoxypyrazine			
sampling time (X_1)	$b_1 = -0.47$	>10%	
ammonium sulfate (X_2)	$b_2 = 0.70$	>10%	
pH (X_3)	$b_3 = 0.83$	>10%	
headspace T (X_4)	$b_4 = -0.79$	>10%	
interaction X_1, X_2	$b_{12} = -0.98$	<10%	
interaction X_1, X_3	$b_{13} = 1.32$	+	
interaction X_2, X_3	$b_{23} = 0.80$	>10%	
	$b_0 = 64.68$		

[a] +++, 0.1% significant. ++, 1% significant.

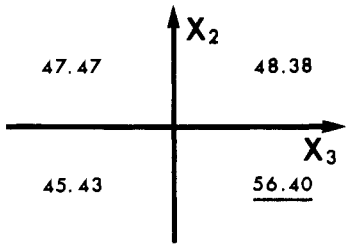

Figure 2. Diagram of interaction X_2X_3.

at the 1% level. The fourth was pH. The best recovery was obtained with the lower levels of vapor-phase temperature and pH. For first-order interaction, only the interaction between the ammonium sulfate level (X_1) and pH (X_3) was significant. The interaction term $b_{23} = -2.52$ means that the effect of salt quantity is different for each pH value. The interaction X_2X_3 (see Figure 2) shows that ammonium sulfate had the greatest effect at pH 5.3 (optimum level in our conditions).

(2) 2,5-Dimethylpyrazine. Classification of factors in decreasing order was as follows: (1) sampling time; (2) ammonium sulfate quantity; (3) pH. The vapor-phase temperature had no significant effect. There was no first-order interaction in the field studied. The values $b_1 = 6.23$ and $b_2 = 2.70$ mean that the recovery increased when both sampling time and ammonium sulfate quantity increased. The optimum values for pH and vapor-phase temperature were 5.3 and 85 °C, respectively.

(3) 2,3,5-Trimethylpyrazine. The two main factors

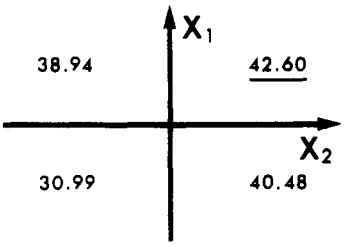

Figure 3. Diagram of interaction X_1X_2.

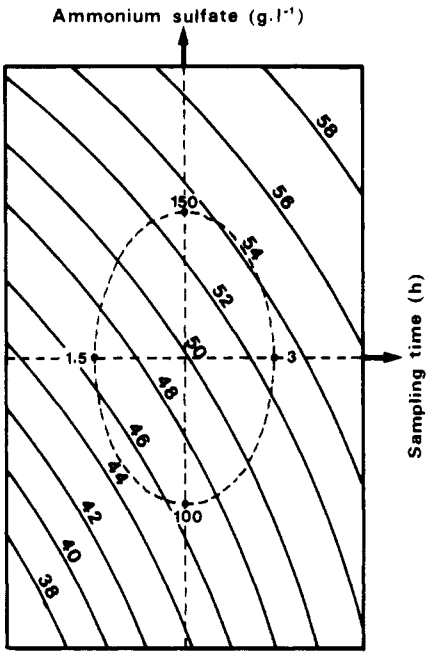

Figure 4. Effects of sampling time and ammonium sulfate on recovery percentage of 2-methylpyrazine. Headspace temperature was 85 °C and pH 5.3.

were sampling time and ammonium sulfate quantity. pH and vapor-phase temperature did not affect 2,3,5-trimethylpyrazine headspace sampling. The first-order interaction between sampling time (X_1) and salt quantity (X_2) was significant at the 1% level ($b_{12} = -1.46$) (Figure 3). The sampling time variation had the greatest effect when the ammonium sulfate quantity was 150 g L^{-1} (optimum level in our conditions).

(4) 3-Methyl-2-methoxypyrazine. No factor has any influence on headspace sampling at the 10% level.

(5) Alkylpyrazine Headspace Sampling. *(5.1) Effect of Sampling Time.* For each pyrazine, the greatest percentage of recovery was obtained after 3 h of sampling. It was one of the most important factors (significant at the 0.1% level). Its associated coefficients always had a positive value. This indicated that alkylpyrazines were very soluble in water and needed a long time to be released into the vapor phase. To increase the recovery, trapping could be extended to 4 h or more.

(5.2) Effect of Ammonium Sulfate. Voilley et al. (1977) found that the presence of salt in the solution generally increases the activity coefficient. Ammonium sulfate added affects headspace sampling for each pyrazine at the 0.1% level. Increasing the salt in the medium increased the recovery.

(5.3) Effect of pH. For alkylpyrazines pH had no (for 2,3,5-trimethylpyrazine) or low (2-methylpyrazine and 2,5-dimethylpyrazine) influence on the recovery. For 2-methylpyrazine and 2,5-dimethylpyrazine the optimum pH was 5.3 (negative value of b_3). This indicated that in the range 5.3–8.3 the alkaline character did not appear; however,

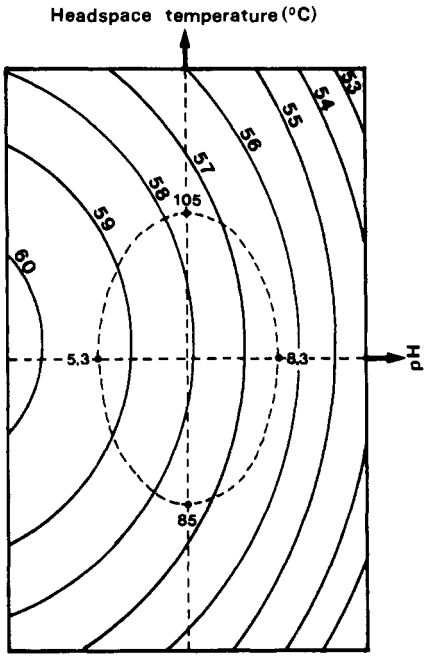

Figure 5. Effects of headspace temperature and pH on recovery percentage of 2,5-dimethylpyrazine. Sampling time was 3 h and ammonium sulfate concentration 150 g L^{-1}.

Figure 2 shows that the increase of pH had a positive action when salt quantity is at the level 100 g L^{-1} and a negative one when salt quantity is 150 g L^{-1}. Ammonium sulfate has such a strong effect that it masks the alkaline character of pyrazines, and the maximum recovery was obtained for ammonium sulfate 150 g L^{-1} and pH 5.3.

(5.4) Effect of Vapor-Phase Temperature. The vapor-phase temperature had no effect on 2,5-dimethylpyrazine and 2,3,5-trimethylpyrazine recoveries (at the 10% level). It had a negative effect on 2-methylpyrazine, because this pyrazine is more volatile (bp 135 °C) compared to 2,5-dimethylpyrazine and 2,3,5-trimethylpyrazine (bp 155 and 171–172 °C, respectively).

(6) 3-Methyl-2-methoxypyrazine. No factor had any influence on headspace sampling in the field studied (at the 10% level). Preliminary trials carried out in our laboratories gave 80% recovery from water; there is a chemical interaction between 3-methyl-2-methoxypyrazine and the solutes. To increase the percentage of recovery, another experimental field should be tested with other factors (for example, porous polymer column temperature, subambient and ambient temperatures).

The representation of the isoresponse diagrams shows that the time samplings and the salt concentrations have the main effect and must be increased to optimize the extraction for all four pyrazines (Figure 4). On the other hand, a change in the vapor-phase temperature does not affect the extraction and must be fixed at an intermediate level, between 85 and 105 °C; the pH of the solution has a great influence on the extraction yields, the most efficient being an acidic solution (Figure 5).

CONCLUSION

The maximum alkylpyrazine recovery was obtained under the following experimental conditions: sampling time of 3 h; ammonium sulfate concentration of 150 g L^{-1}; pH of 5.3; and vapor-phase temperature of 85 °C.

The fractional factorial design allowed us to determine the main factors affecting headspace analysis of pyrazines. Future experiments will study the two factors of greatest importance (sampling time and salt level) to further optimize extraction efficiency. An experimental methodology like Simplex will lead to the optimization of these factors for the highest recovery.

ACKNOWLEDGMENT

We are grateful to Professor Malcome C. Bourne (Cornell University, Geneva, NY) for his help.

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Received for review July 18, 1991. Accepted August 2, 1991.

Registry No. 2-Methylpyrazine, 109-08-0; 2,5-dimethylpyrazine, 123-32-0; 2,3,5-trimethylpyrazine, 14667-55-1; 3-methyl-2-methoxypyrazine, 28473-30-5.