Evaluation of Solid-Phase Microextraction for Analysis of Volatile Metabolites Produced by Staphylococci

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The evaluation of solid-phase microextraction (SPME) for analysis of flavor compounds produced by bacteria has been studied. First, it was necessary to determine the optimal conditions to extract the different volatile compounds in dilute aqueous solutions. For this, the effects of salt, headspace, and liquid samplings and two coating phases [poly(dimethylsiloxane) (PDMS) and poly(acrylate) (PA)] were tested. The addition of salt enhanced SPME absorption of all the compounds. Ethyl ester was better extracted in liquid sampling with PDMS phase, whereas all of the other compounds were better extracted in headspace sampling at 80 °C with PA phase. The SPME technique was applied to the analysis of bacterial metabolites. It has been shown that *Staphylococcus xylosus* (16) and *Staphylococcus carnosus* (833) were able to produce esters and to catabolize leucine and that *S. carnosus* (833) limited the oxidation of free fatty acids.

Keywords: *SPME; flavor compounds; bacterial metabolites*

INTRODUCTION

Staphylococci influenced the level of volatile compounds in dry sausages (Berdagué et al., 1993; Stahnke, 1994; Montel et al., 1996). These volatile compounds originated mainly from amino acid catabolism (leucine, phenylalanine), from free fatty acid degradation, and from reaction of ethanol with different acids (esters). They were influenced by the strains and contributed to the flavor of sausages. Therefore, for the development of dry sausages with typical dry cured aroma, it will be necessary to select starter cultures for their aromatic potential. To screen strains on their production of aromatic compounds in liquid aqueous media, it is crucial to have a simple, efficient, and inexpensive analytical method.

Solid-phase microextraction (SPME) is a simple solvent-free method developed by Pawliszyn and co-workers (Arthur and Pawliszyn, 1990; Arthur et al., 1992a,b; Louch et al., 1992; Potter and Pawliszyn, 1992). It uses a fine fused-silica fiber coated with a polymeric coating to extract compounds from their matrix and directly transfer them into the injector of a gas chromatograph for thermal desorption and analysis. The SPME can be used for gaseous and liquid samples (Zang and Pawliszyn, 1993). It has been used successfully for analyzing pollutants in environmental water samples (Arthur et al., 1992a,b; Potter and Pawliszyn, 1992) and flavor compounds of coffee, fruit juice beverages, and truffles (Hawthorne et al., 1992; Yang and Peppard, 1994; Pelusio et al., 1995; Steffen and Pawliszyn, 1996). It has also been used to collect volatile organic compounds emitted from *Penicillium* (Nilsson et al., 1996).

The purpose of this work was to evaluate SPME for the analysis of volatile metabolites produced by staphylococci in liquid media. Before the analysis of bacterial metabolites, it was necessary to determine the optimal conditions to extract the different volatile compounds in aqueous solutions.

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MATERIALS AND METHODS

Optimization of SPME. *Preparation of Test Solutions.* The following solutions were prepared: (1) ethyl butanoate; (2) 3-methylbutanol, 3-methylbutanal, and 3-methylbutanoic acid, compounds from leucine catabolism; (3) phenylacetaldehyde, 2-phenylethanol, and β-phenylpyruvic acid, compounds from phenylalanine catabolism; and (4) heptane, 2-pentanone, hexanal, hexanol, hexanoic acid, a mixture of five molecules representative of different chemical families of compounds resulting from lipid degradation. Solutions 1−3 were prepared in 0.1 M phosphate buffer at a concentration of 10 ppm, and solution 4 was prepared at a concentration of 50 ppm.

General SPME Procedures. The SPME device was purchased from Supelco Co. (Canada). Two coating phases were tested: poly(dimethylsiloxane) (PDMS, 100 μm) and poly(acrylate) (PA, 85 μm). All of the experiments were run in triplicate.

Two sampling methods were used: liquid and headspace. For liquid sampling, the SPME fiber was inserted into a 1.5 mL vial containing 1.3 mL of sample; the fiber remained in the liquid for 15 min under magnetic stirring. For headspace sampling, 0.7 mL of liquid sample was placed in a 1.5 mL vial and the fiber was exposed to the headspace of the solution. The sample equilibrated for 15 min, prior to 15 min of sampling at different temperatures under stirring. The sampling time of 15 min had been chosen because data in the literature indicate that for most analytes the equilibrium had been reached.

Once sampling was finished, the fiber was withdrawn into the needle and transferred to the injection port of the gas chromatograph.

Effect of Salt. The extraction of the compounds was done without NaCl or with NaCl at 36% (w/v, saturated solution). The effect of salt was studied with the two coating phases (PDMS, PA) and in liquid and headspace samplings for solutions 1 and 2.

Effect of Temperature. To extract the compounds in headspace, three temperatures, 25, 80, and 100 °C, were studied with the two coating phases (PDMS, PA) on solutions 1 and 2.

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Table 1. Effect of Sodium Chloride on the Extraction of Different Compounds in Liquid and Headspace Samplings and with the Two Coating Phases (PDMS, PA)

	liquid sampling				headspace sampling			
	PDMS		PA		PDMS		PA	
compd[a]	0% NaCl	36% NaCl	0% NaCl	36% NaCl	0% NaCl	36% NaCl	0% NaCl	36% NaCl
ethyl butanoate	15367	140562	4071	35983	1442	9161	845	2158
3-methylbutanal[b]	2636	7428	447	3126	2580	7607	980	4063
3-methylbutanol[b]	910	4602	716	5436	2900	8200	4100	12771
3-methylbutanoic acid[b]	ND[c]	876	504	1882	380	1293	1950	11648

[a] The results are expressed in arbitrary units (peak area, average of three analyses). [b] pH of the solution was acid (4.0 for PDMS, 3.0 for PA). [c] ND, not detected.

Table 2. Effect of Temperature on the Extraction of the Different Compounds in Headspace Sampling with the Two Coating Phases (PDMS, PA)

	PDMS			PA		
compd[a]	25 °C	80 °C	100 °C	25 °C	80 °C	100 °C
ethyl butanoate	9161	1730	1400	915	2158	1096
3-methylbutanal[b]	8156	8607	2230	1431	4063	2538
3-methylbutanol[b]	8150	11800	3870	2633	15400	7610
3-methylbutanoic acid[b]	404	1293	1340	3200	11648	9120

[a] The results are expressed in arbitrary units (peak area, average of three analyses). [b] pH of the solution was acid (4.0 for PDMS, 3.0 for PA).

Effect of Coating Phases, pH, and Samplings on the Extraction of the Different Compounds. To optimize the extraction of the compounds, an experimental design was set up with four factors on the separate solutions described above as follows: factor 1, coating phase, two levels (PDMS, PA); factor 2, pH, two levels (3.0 and 7.0 for PA phase, 4.0 and 7.0 for PDMS phase); factor 3, sampling, two levels (liquid, headspace); and factor 4, compounds, one for solution 1, three for solutions 2 and 3, and five for solution 4.

Production of Bacterial Catabolites. *Strains. Staphylococcus carnosus* (833) and *Staphylococcus xylosus* (16) were grown in PYS medium (Lechner et al., 1988) for 12 h at 25 °C under stirring (150 rpm). The cultures were then centrifugated at 10000*g* at 4 °C and the cells collected. The cells were washed and resuspended in phosphate buffer and used to study the catabolism of leucine and the production of esters.

Catabolism of Leucine. The resting cells (final optical density of 1.0, wavelength of spectrometer $\lambda = 600$ nm) were incubated in phosphate buffer (0.1 M, pH 5.8) with 10 mM leucine and pyridoxal phosphate (0.1 mM) during 24 h at 25 °C. The metabolites produced from leucine were then extracted by SPME with PA coating phase, headspace sampling (80 °C), acid pH (3.0), and saturated solution of NaCl during 15 min.

Production of Esters. The resting cells (final optical density of 1.0) were incubated in phosphate buffer (0.1 M, pH 7.0) with ethanol (11 μmol mL^{-1}) and different acids separately (5.55 μmol mL^{-1}) during 4 h at 25 °C. The esters were then extracted by SPME with PDMS coating phase, liquid sampling, neutral pH, and saturated solution of NaCl during 15 min.

Effect of S. carnosus (833) on Oxidation of Unsaturated Free Fatty Acids. The strain was grown in PYS media supplemented with a mixture of unsaturated free fatty acids: oleic (3.54 μmol mL^{-1}), linoleic (3.56 μmol mL^{-1}), and linolenic (3.59 μmol mL^{-1}) acids. After 0, 3, and 9 days of incubation at 22 °C, the volatiles of the inoculated sample and the sterile control were extracted by SPME with PA coating phase, headspace sampling (80 °C), acid pH (3.0), and saturated solution of NaCl during 15 min.

Gas Chromatography Analysis. A Delsi 700 gas chromatograph was equipped with a conventional split/splitless injector and a flame ionization detector (FID, 280 °C). The temperature of the injector (200 °C) was held constant during analysis. A 50 m, 0.32 mm i.d., 1 μm film thickness, DB-Wax column was used. The GC oven temperature was programmed as follows: 50 °C held for 1 min, increased to 220 °C at a rate of 3 °C min^{-1}. Helium was used as carrier gas.

For thermal desorption the SPME fiber remained in the injector for 5 min. Splitless injection mode was used.

Statistical Analysis. The effects of coating phases, pH, samplings, and chemical nature of compounds of solutions 1−4 were studied by four analyses of variance using a general linear model procedure (SAS, 1988). Statistical analyses were done on results expressed in arbitrary units (peak area). The mathematical models used in the analysis of variance were

solution 1

$$X = m + P_i + \mathrm{pH}_j + S_k + P{*}\mathrm{pH}_{i,j} + P{*}S_{i,k} + \mathrm{pH}{*}S_{j,k} + e_{i,j,k}$$

solutions 2−4

$$X = m + P_i + \mathrm{pH}_j + S_k + C_l + P{*}\mathrm{pH}_{i,j} + P{*}S_{i,k} + P{*}C_{i,l} + \mathrm{pH}{*}S_{j,k} + \mathrm{pH}{*}C_{j,l} + S{*}C_{k,l} + P{*}\mathrm{pH}{*}S_{i,j,k} + P{*}S{*}C_{i,k,l} + P{*}C{*}\mathrm{pH}_{i,l,j} + \mathrm{pH}{*}S{*}C_{j,k,l} + e_{i,j,k,l}$$

with X = peak area characteristics, m = constant term, P_i = mean effect of coating phase ($i = 2$), pH_j = mean effect of pH ($j = 2$), S_k = mean effect of sampling ($k = 2$), C_l = mean effect of compound ($l = 3$ for solutions 2 and 3; $l = 5$ for solution 4), P*pH, P*S, P*C, pH*S, pH*C, and S*C = mean effect of first-order interactions, P*pH*S, P*S*C, P*C*pH, and pH*S*C = mean effect of second-order interactions, and e = residual variations. For each model, the percentage of variance explained by experimental factors was calculated

% of variance = SSD of the factor/SSD of the model × 100

where SSD = sum of the squares of the differences.

RESULTS AND DISCUSSION

Optimization of SPME Conditions. *Effect of Salt.* The addition of salt is widely used to increase the sensitivity of analytical methods. Among the different salt concentrations investigated, Steffen and Pawliszyn (1996) had shown that saturated solution (36% w/v) enabled the highest amount of analyte to partition into the coating for most of the analytes. Therefore, only this concentration has been tested for the compounds listed in Table 1. We confirm that addition of salt to the sample matrix increased the absorption of the compounds in both liquid and headspace samplings and for the two coating phases (PDMS, PA). Therefore, for all of the compounds, salt at saturated concentration (36% w/v) was used throughout the study.

Table 3. Effect of Coating Phase, pH, and Sampling on the Extraction of Different Compounds

	compds from							
	leucine catabolism (solution 2)		phenylalanine catabolism (solution 3)		fatty acid degradation (solution 4)		ethyl butanoate (solution 1)	
factor								
compds	**[a]	19.0%[b]	**	17.4%	***	36.7%		–
phases	*	3.4%	**	16.9%	**	3.5%	***	93.0%
pH	NS	0.0%	NS	0.0%	**	4.3%	NS	0.0%
samplings	**	27.0%	***	21.6%	**	6.7%	*	4.2%
main interactions								
compds−phases	*	5.5%	**	11.5%	**	23.4%		–
compds−pH	*	8.4%	NS	1.8%	*	6.6%		–
compds−samplings	*	11.5%	*	9.3%	NS	3.6%		–
phases−pH	*	3.5%	NS	0.0%	NS	0.0%	NS	0.1%
phases−samplings	**	6.2%	**	13.0%	NS	0.0%	NS	2.8%
pH−samplings	NS	0.4%	NS	0.03%	NS	0.3%	NS	0.0%
compds−phases−pH	NS	1.4%	NS	0.2%	*	7.9%		–
compds−phases−samplings	NS	0.8%	NS	4.8%	NS	3.7%		–
compds−pH−samplings	NS	6.0%	NS	2.1%	NS	1.8%		–
phases−pH−samplings	*	3.7%	NS	1.1%	NS	0.2%		–
residual factor		0.7%		0.6%		1.4%		0.1%

[a] Results of the analysis of variance, $\alpha = 5\%$, significant at $p < 0.1$ (*), $p < 0.05$ (**), $p < 0.01$ (***); NS, not significant (NS); –, factor not tested. [b] Percent of variance explained by the factors: (SSD of factor/SSD of total variations) × 100; SSD, sum of the squares of the differences.

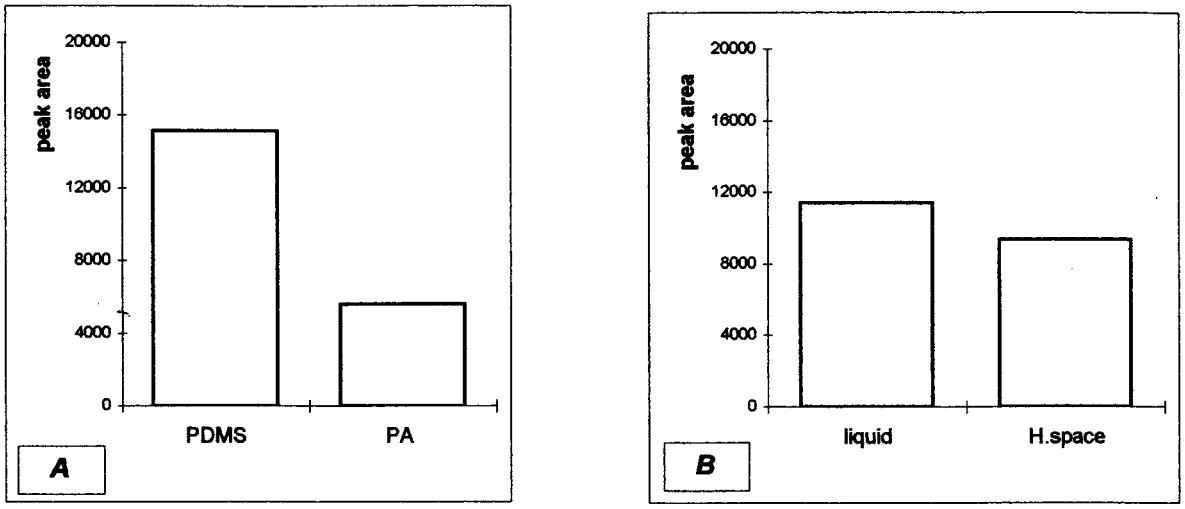

Figure 1. Effect of coating phases (PDMS, PA) (A) and samplings (liquid, headspace) (B) on the extraction of ethyl butanoate.

Effect of Temperature. Heating a sample to elevated temperature provides energy for molecules to overcome energy barriers that tie them to the matrix. However, the absorption of analytes by the fiber coating is an exothermic process, which means that while the high temperature is good for the release of analytes from their matrix, it can adversely affect the absorption by the coating due to the decrease of the partition coefficients (Zang and Pawliszyn, 1995). As a result, there is usually an optimum temperature for headspace SPME sampling (Table 2). Ethyl butanoate had optimal temperatures of extraction at 25 °C with PDMS phase and at 80 °C with PA phase. The other compounds were better extracted at 80 °C for both phases. Therefore, 80 °C was chosen for headspace extraction of the compounds of solutions 2−4.

Effect of Coating Phases, pH, and Samplings on the Extraction of the Different Compounds. The results of the statistical analysis are summarized in Table 3.

Ethyl Butanoate Extraction. The nature of the coating phase was primordial for the extraction of ethyl butanoate (Table 3). Extraction was better with the apolar phase PDMS than with PA phase and also in liquid sampling than in headspace (Figure 1).

The pH of the sample in the range 4−7.0 units had no significant impact on the extraction efficiency of the ethyl butanoate from water (Table 3).

Optimal conditions of ester extraction are as follows: PDMS phase, liquid sampling, solution saturated with salt, and extraction time of 15 min.

Extraction of the Other Metabolites. Solutions 2−4 were composed of a mixture of compounds with different chemical natures (acids, alcohols, aldehydes), so SPME extraction was greatly influenced by the factor compounds (19% of the variance for leucine, 17.4% for phenylalanine, and 36.7% for fatty acid metabolites) (Table 3). Whatever the extraction conditions, alcohols (3-methylbutanol, 2-phenylethanol, hexanol) were the compounds most easily extracted and acids (3-methylbutanoic, β-phenylpyruvic acid, hexanoic) less easily extracted (Figures 2−4).

Sampling also affected the extraction of the compounds (Table 3). Headspace sampling was better performed than liquid for all of the compounds (Figures 2−4) and for the two coating phases (Figures 2B and 3B).

The nature of the coating phases was important (Table 3). The polar PA phase extracted more analytes

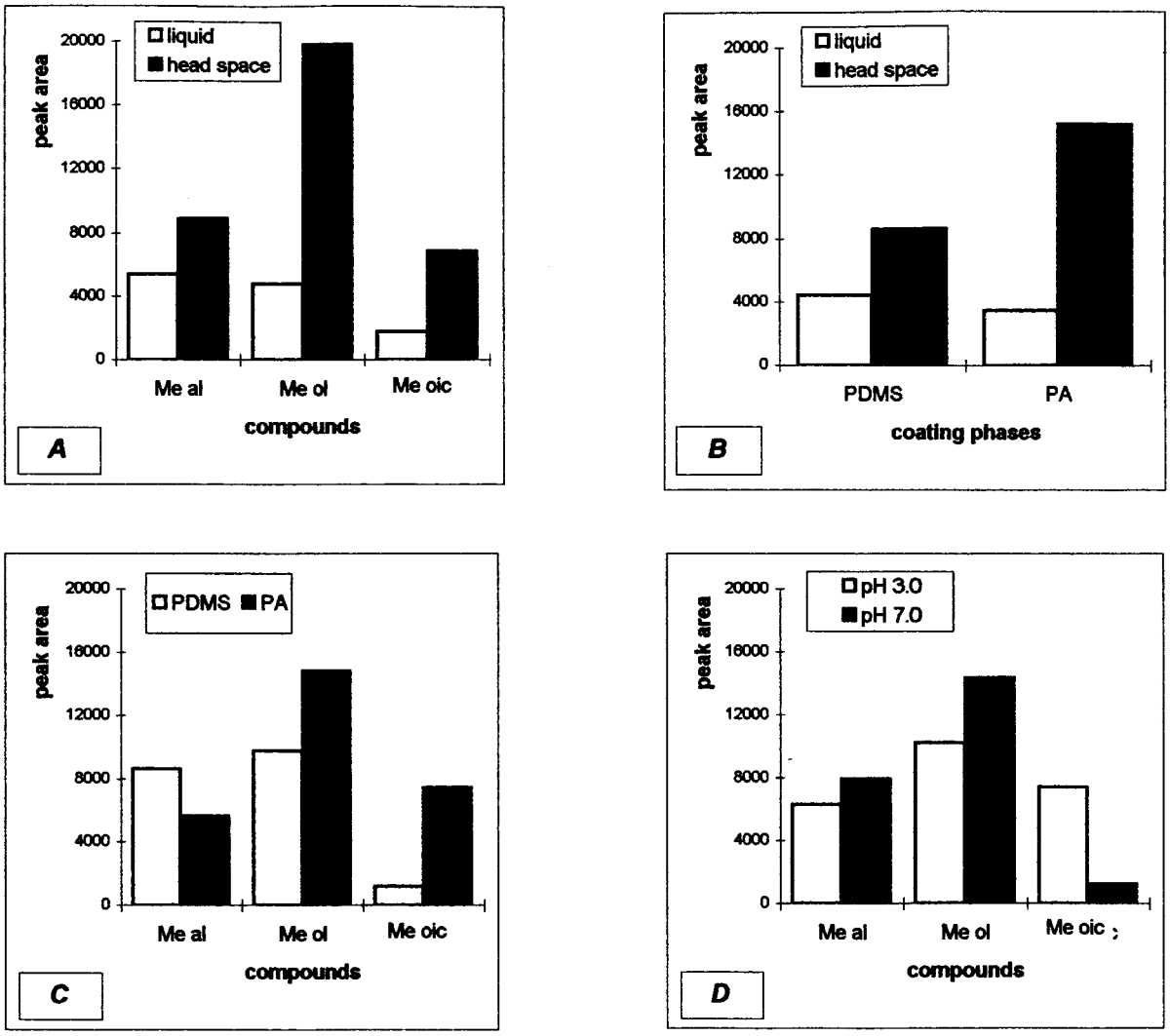

Figure 2. Effect of the main factors on the extraction of 3-methylbutanal (Me al), 3-methylbutanol (Me ol), and 3-methylbutanoic acid (Me oic). Significant factors or interactions were chosen from the variance analysis summarized in Table 3.

in comparison to the PDMS phase. With regard to the compounds, only 3-methylbutanal and heptane were better extracted with the apolar phase PDMS (Figures 2C and 4C), whereas all of the other compounds were more readily extracted with the polar PA phase (Figures 2−4). Also, Steffen and Pawliszyn (1996) had shown that the extraction of flavor volatiles from orange juice was favored by the PA fiber coating.

There was significant interaction between compounds and pH (Table 3), which was mainly due to the great improvement of extraction of 3-methylbutanoic acid and hexanoic acid at acid pH (Figures 2D and 4D). In acidic conditions, acids are not dissociated (R−COOH), their polarity is decreased, and therefore the extraction yield is improved. The positive effect of the acid pH has already be mentioned for the extraction of phenols with the PA phase (Shirey, 1994). However, for most of the compounds studied, the acid pH either decreased slightly the amount extracted or had no effect (Figures 2D and 4D); this was also noticed by Steffen and Pawliszyn (1996) during the extraction of flavor volatiles from orange juice.

In conclusion, the best conditions to extract the compounds of solutions 2−4 are as follows: PA coating phase, headspace sampling (80 °C), acid pH (3.0), solution saturated with NaCl, and extraction time of 15 min.

Table 4. Linear Range of the Compounds Studied

compd[a]	linear range (ppm)	compd[a]	linear range (ppm)
ethyl butanoate[b]	0.06[d]−200	heptane[c]	0.1−50
3-methylbutanal[c]	0.5−10	2-pentanone[c]	1.0−50
3-methylbutanol[c]	0.2−10	hexanal[c]	0.2−50
3-methylbutanoic acid[c]	0.2−10	hexanol[c]	0.2−50
phenylacetaldehyde[c]	0.5−10	hexanoic acid[c]	0.2−50
2-phenylethanol[c]	0.2−10		
β-phenylpyruvic acid[c]	0.2−10		

[a] The compounds were prepared in phosphate buffer. [b] Extraction of ethyl butanoate was done with PDMS phase in liquid sampling. [c] Extraction of all these compounds was done with PA phase in headspace sampling. [d] The lowest value is the limit of detection of the compounds.

To use SPME in quantitative analysis, calibration curves had been done for each of the compounds. There was a linear relationship between the amount sorbed into the stationary phase and the concentration in the sample. The linear range was quite large for ethyl butanoate: it was 0.06−200 ppm. For most of the compounds, we have checked the linearity between 0.2 and 10 or 50 ppm (Table 4). The limit of detection ranged from 0.06 to 1 ppm for the compounds studied (Table 4). Many data in the literature are on the extraction of apolar hydrocarbons from aqueous solu-

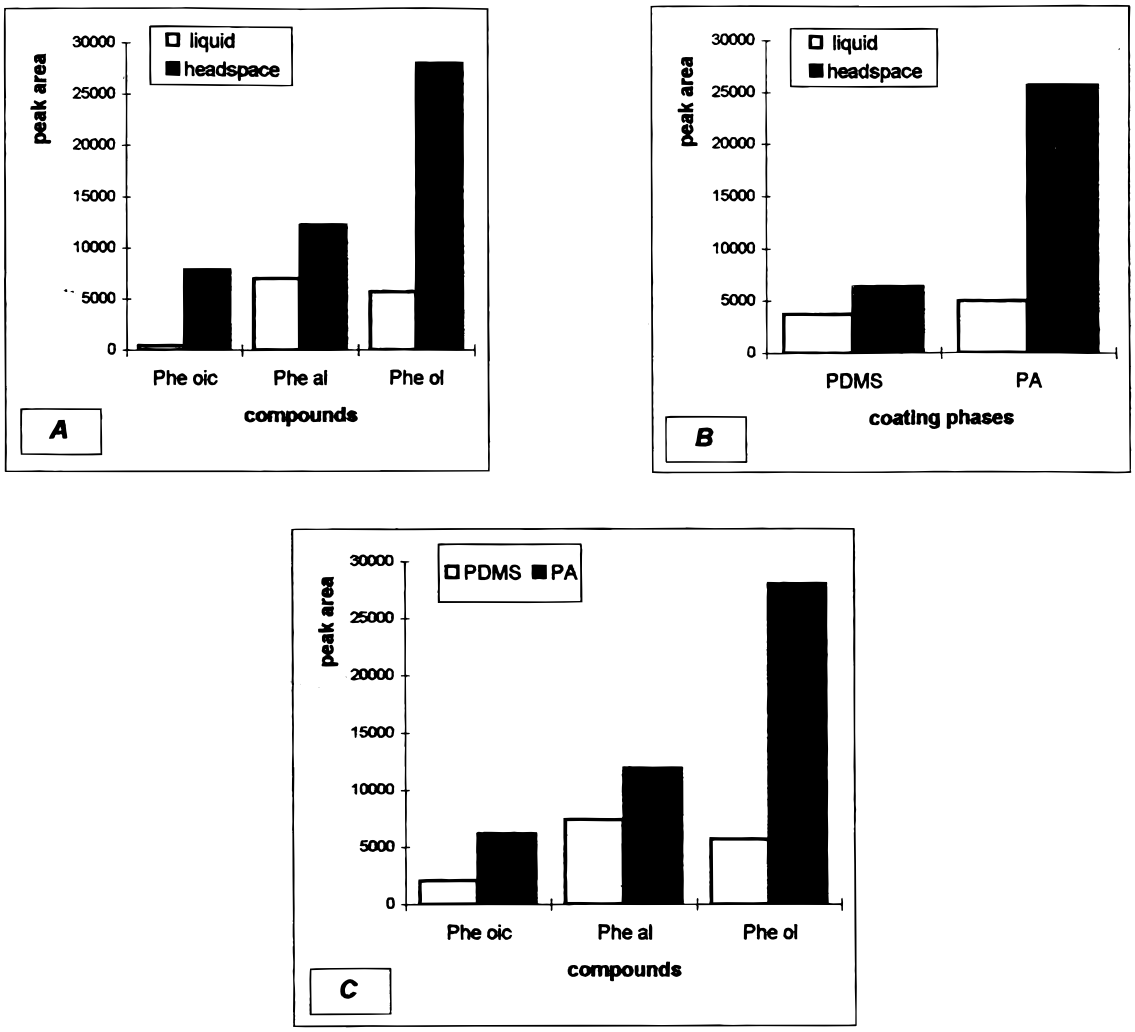

Figure 3. Effect of the main factors on the extraction of phenylpyruvic acid (Phe oic), phenylacetaldehyde (Phe al), and 2-phenylethanol (Phe ol). Significant factors or interactions were chosen from the variance analysis summarized in Table 3.

Table 5. Catabolism of Leucine by *S. xylosus* (16) and *S. carnosus* (833)

compd[a]	3-methyl-butanal	3-methyl-butanol	3-methylbutanoic acid
S. xylosus	0.86	1.39	52.14
S. carnosus	2.46	0.45	43.57

[a] Quantities are expressed in nmol mL^{-1} (optical density of bacterial cells =1.0, λ = 600 nm).

tion, so the detection limit was very low, on the order of 0.1 ppb to 1 ppt (Shirey, 1994). However, Yang and Peppard (1994) found same magnitude of detection limit as in our study for various flavor compounds.

Applications for Bacterial Metabolites. *Leucine Catabolism.* The leucine catabolites were extracted by SPME with PA coating phase, headspace sampling (80 °C), acid pH (3.0), solution saturated with NaCl, and extraction time of 15 min. Metabolites were quantified using external standard calibration done in the same matrix as the samples studied, i.e., phosphate buffer. With this procedure, we showed that both staphylococci metabolized leucine; after 24 h of incubation, 3-methylbutanoic acid was the main metabolite produced (Table 5). *S. carnosus* produced also a small amount of 3-methylbutanol.

Production of Esters. The extraction of esters was done with PDMS phase, liquid sampling, solution saturated with salt, and extraction time of 15 min. To measure ethyl esters produced by the two *Staphylococcus* species, an external calibration was done for all of the esters in phosphate buffer (the same buffer used for the samples). The species differed by their levels of production: *S. xylosus* produced a higher amount of esters than *S. carnosus* (Table 6). They also differed by their substrate specificity. *S. xylosus* esterified preferentially butanoic acid and the esterification decreased with the increasing or decreasing chain length of acid. Moreover, isoacids (2-methylbutanoic, 3-methylbutanoic) were poorly esterified. By comparison, *S. carnosus* did not exhibit a high acid specificity and it esterified at the same rate straight chains of four to six carbons and isoacids.

Oxidation of Unsaturated Free Fatty Acids. Volatile compounds were extracted with PA coating phase, headspace sampling (80 °C), acid pH (3.0), solution saturated with NaCl, and extraction time of 15 min. Hexanal was the only compound originating from free fatty acid oxidation identified in our sample analyzed by SPME-GC. Many other compounds were different in the control and in the sample inoculated with *S. carnosus*. However, they were not identified since GC was not coupled to mass spectrometry. To quantify hexanal, it was added in a sterile PYS medium at different concentrations and then extracted by SPME.

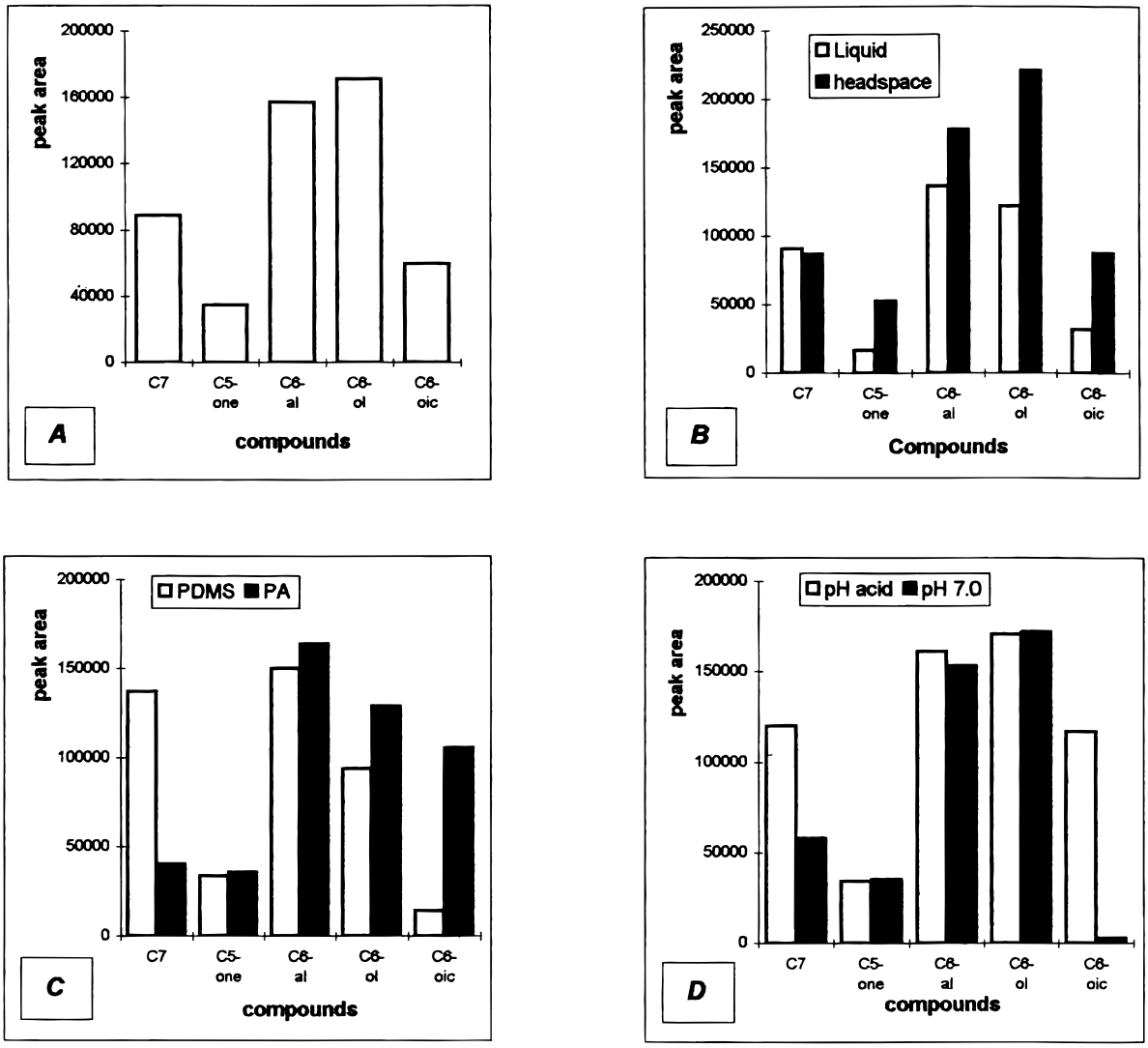

Figure 4. Effect of the main factors on the extraction of heptane (C7), 2-pentanone (C5-one), hexanal (C6-al), hexanol (C6-ol), and hexanoic acid (C6-oic). Significant factors or interactions were chosen from the variance analysis summarized in Table 3.

Table 6. Production[a] of Ethyl Esters by *S. xylosus* (16) and *S. carnosus* (833)

	ethyl acetate	ethyl butanoate	ethyl 2-methylbutanoate	ethyl 3-methylbutanoate	ethyl valerate	ethyl hexanoate	ethyl decanoate
S. xylosus	2.01	29.75	0.86	2.86	13.29	8.64	1.86
S. carnosus	0.00	0.44	0.33	0.10	0.22	0.28	0.00

[a] Quantities are expressed in nmol mL^{-1} (optical density of bacterial cells =1.0, λ = 600 nm).

Table 7. Effect of *S. carnosus* (833) on the Level of Hexanal[a]

	0 days	3 days	9 days
control	1.86	16.6	15.9
S. carnosus	1.86	1.62	1.10

[a] Quantities of hexanal are expressed in nmol mL^{-1} of medium.

It was clear that *S. carnosus* limited the oxidation of unsaturated free fatty acids as shown by the low level of hexanal in the inoculated samples compared to the sterile control after 3 and 9 days of incubation at 22 °C (Table 7).

In conclusion, SPME seemed to be a good tool to study the production of aromatic compounds by bacteria. As it is easy to use and does not need expensive equipement, it can be useful in screening new starter cultures on their production of aromatic compounds. Studies in our laboratory are in progress on the catabolism of leucine and on the production of esters by different species of staphylococci.

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