Determination of Volatile Compounds in Whiting (*Merlangius merlangus*) Using Headspace–Solid-Phase Microextraction–Gas Chromatography–Mass Spectrometry

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Abstract

A method using headspace–solid-phase microextraction–gas chromatography–mass spectrometry to extract volatile compounds from whiting is developed. Several parameters such as sorption time, desorption time, fiber type, and matrix form are optimized to achieve better sensitivity in minimal analysis time. The efficiency of the method is determined by the linear range and repeatability; a mean relative standard deviation of approximately 7% is measured. It was possible to identify and quantitate 30 volatile compounds of interest present in spoiled whiting.

Introduction

Freshness is a key element in the assessment of fish quality (1). Identification and quantitation of volatile compounds is one of the freshness evaluation methods described by Olafsdottir et al. (2). Spoilage of fish during storage generates volatile compounds that may be indicators of freshness (3). In 1986, Josephson et al. (4) defined two classes of compounds characterizing freshness of whitefish and its spoilage. Long-chain alcohols and carbonyls (C6–C9) characterize the freshness of short-chain alcohols, sulfur compounds, amines, sweet esters, aromatics, and dienals, and their spoilage. Jensen et al. (5) inventoried and described different methods of determining volatile compounds—static or dynamic headspace (HS), simultaneous distillation and extraction, vacuum distillation, supercritical fluid extraction and solid-phase microextraction (SPME)—and presented static HS and SPME as the most suitable because they limit exogenous thermal and chemical changes in the sample. To date, four SPME studies have been devoted to fish: determination of volatile compounds compared with overall sensory changes during storage of sardine (1); determination of volatile compounds of fish muscle, skin, and prawn muscle of some Japanese sea fish and prawn species (6); determination of volatile components of raw and smoked black bream and rainbow trout (7); and development of a direct SPME method to quantitate total volatile basic nitrogen in salmon and whiting (8). SPME has also been applied to other foodstuffs, including: cheese (9–11), wine (12–14), coffee (15), banana (16), apple (17), orange juice (18), olive oils (19), ham (20), and tobacco (21).

SPME was developed in 1990 by Arthur and Pawliszyn (22). Compounds are extracted as a function of their affinity for a fiber and are then desorbed thermally in a classic injector and determined by gas chromatography (GC)–mass spectrometry (MS) (5,23,24). Using SPME to evaluate the freshness of sardines, Triqui and Bouchriti (1) obtained 14 compounds. They attributed this poor yield to the small sample and large vial volume, which shows why parameter optimization is essential for good sensitivity of SPME. Optimization is first applied to sorption (extraction) and then to desorption.

Extraction efficiency depends principally on the compound's affinity for the SPME fiber (9,10,13–15,19,21,25–27), but also on other parameters (25) that numerous authors have optimized for different matrices, such as pH (13,21), mixing (12,14), salt concentration (14,27), extraction temperature (13,14,20), equilibrium time (10), and sorption time (9,10,13,14,17,19,20,27,28). Mixing accelerates transfer of compounds from the sample matrix to the fiber, and supersaturation of the sample with salts makes the extraction of compounds by the salting-out effect more efficient (25). The sample is often heated in order to increase the concentration of compounds in the gas phase. HS–SPME is the most suitable sampling method in (GC) and GC–MS analyses of volatile compounds in a complex matrix (25). Although SPME sensitivity is maximal at equilibrium, complete equilibrium is not necessary for precise analyses because of the linear relation between the quantity of a compound sorbed by the SPME fiber and its initial concentration in the sample matrix under nonequilibrium conditions (29). Vial size and sample quantity

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are critical and must be held constant throughout SPME (25). Other factors affect extraction; for instance, if the proportion of extracted compounds to compounds present in the sample is not proportional (i.e., not in the linear range) (15) and competition between molecules, which can generate quantitation bias (30). Such competition can be countered using isotopes of the molecules to be quantitated as internal standard [isotope dilution assays (IDAs)] (15). However, this method is costly in a determination of a large range of volatile compounds of very different chemical natures.

According to Kataoka et al. (25), the desorption of compounds from the fiber or the capillary layer depends on temperature and desorption time in the injector (12,26,27). Fiber position in the injector is also important according to De la Calle Garcia et al. (12) and Okeyo and Snow (26): the penetration depth must be adjusted to position the fiber in the center of the hottest zone of the injector. Split/splitless injectors must operate in splitless mode and, in general, the optimal desorption temperature is approximately equal to the boiling point of the least volatile compound (25). To prevent peak broadening, the initial temperature of the GC column should be kept low, or the column should even be cooled (25). However, use of a GC injector insert of small diameter improves resolution and offsets the need for a cryogenic system for thermal desorption (25,31). The present study seeks to optimize these various SPME parameters in order to characterize the volatile compounds present in spoiled whiting.

Experimental

Chemicals

Carboxen (CAR)–polydimethylsiloxane (PDMS) stableflex 65 µm, CAR–PDMS 75 µm, divinyl benzene (DVB)–PDMS 65 µm, carbowax (CW)–DVB 65 µm, polyacrylate (PA) 85 µm, PDMS 75 µm, and PDMS 100 µm fibers were from Supelco (Bellefonte, PA). Before first use, each SPME fiber was conditioned as recommended by the manufacturer. Sodium chloride was from Oxoid Ltd. (Basingstoke, Hampshire, U.K.), and Milli-Q water (high-performance liquid chromatographic water) was from Fisher Scientific Labosi (Elancourt, France).

Table I. Optimization Parameters and Validation Tests

		Extraction temperature	Matrix	Fiber	Penetration depth	Experimental design	Equilibrium time	Adsorption time	Desorption time	Sample volume	Linear range	Repeatability
SPME mode	manual (450 rpm, 40 mL vial)	* (without mixing)	*	*								
	automatic (500 rpm, 20 mL vial)				*	*	*	*	*	*	*	*
Matrix	27 g of filet without water	*										
	9 g of filet with water saturated with NaCl			*								
	preparation as described in M and M				*	*	*	*	*	*	*	*
Days of spoilage	1 day				*							
	9 days					*	*	*	*	*		
	10 days	*	*	*								
	14 days											*
Sample volume	9 mL				*	*	*	*	*			
	11 mL										*	*
Fiber	CAR–PDMS 85	*	*									
	CAR–PDMS 75			*	*	*	*	*	*	*	*	
Equilibrium time	10 min									*	*	*
	1 h	*	*	*	*			*	*			
Adsorption time	1 min										*	
	40 min									*	*	*
	1 h	*	*	*	*		*		*			
Desorption time	10 s									*	*	*
	1 min	*	*	*								
	3 min				*		*	*				

* Parameter used.

Sample preparation

Whiting (*Merlangius merlangus*) was caught the night before the start of the study, filleted, and stored on ice by the Cooperative Maritime Etaploise (CME, Boulogne-sur-Mer, France) until dispatch to the laboratory. It was cut into 1-cm cubes, mixed, and stored at 4°C in freezer bags during a given time of spoilage, after which the spoiled cubes were held under vacuum in 50-g sachets at –21°C until analysis.

Before analysis, the bags were thawed in water at room temperature for 1 h. The contents of each bag were placed in a Stomacher bag and 100 mL of ultrapure water saturated in NaCl was added. The contents were then homogenized for 2 min in a Stomacher Lab-Blender 400 (Seward, Norfolk, U.K.).

The aqueous phase was removed and centrifuged in two tubes (each containing 25 mL for each bag) for 10 min at 4°C and 12,000 *g* (Multifuge 3 S-R Heraeus, Kendro Laboratory Products, Courtaboeuf, France). The aqueous phase supernatant was recovered.

SPME procedure

Pending analysis, the aqueous phase was stored (for less than 24 h) at 4°C in completely filled (to reduce HS) 15-mL vials. On analysis, the aqueous phase, plus 80 ppm of 3-buten-1-ol, 3-methyl (internal standard), was introduced into a hermetically sealed 20-mL vial. The vial was placed in the sample tray of the Combi-Pal (CTC Analytics, Zwingen, Switzerland) and then transferred to the mixer, where it was heated and mixed at 500 rpm.

After the equilibrium time, the SPME fiber was inserted in the vial for sorption and then in the Merlin Microseal injector (250°C) of the GC 17A equipped with an MS QP5000 (Shimadzu, Kyoto, Japan) for desorption in the splitless mode. The injector was equipped with an SPME-specific insert (95- × 5- × 0.75-mm i.d.) (Supelco, Bellefonte, PA), without glass wool.

GC–MS analysis conditions

The GC was equipped with a BPX5 capillary column (60 m × 0.25 mm × 0.25 µm) (SGE, Courtaboeuf, France). The carrier gas was helium at 1.8 mL/min and linear velocity was 34 cm/s at 40°C. The column was kept at 40°C for 5 min and the temperature increased at a rate of 5°C/min to 100°C, then by 20°C/min to 280°C, and maintained for 5 min at 280°C. The pressure program was started at 182 kPa, held for 5 min, then increased by 2.7 kPa/min to 216.1 kPa, then increased by 11 kPa/min to 313.6 kPa, and held for 5 min.

Electron-impact mass spectra conditions were as follows: capillary direct interface, 260°C; ionization voltage, 1.5 eV; and mass range, *m/z* 33–150. After each injection, the fiber was heated to 300°C for 5 min in the SPME fiber conditioning station.

Mass spectral matches were made by comparisons with National Institute of Standards and Technology (NIST) 21 and

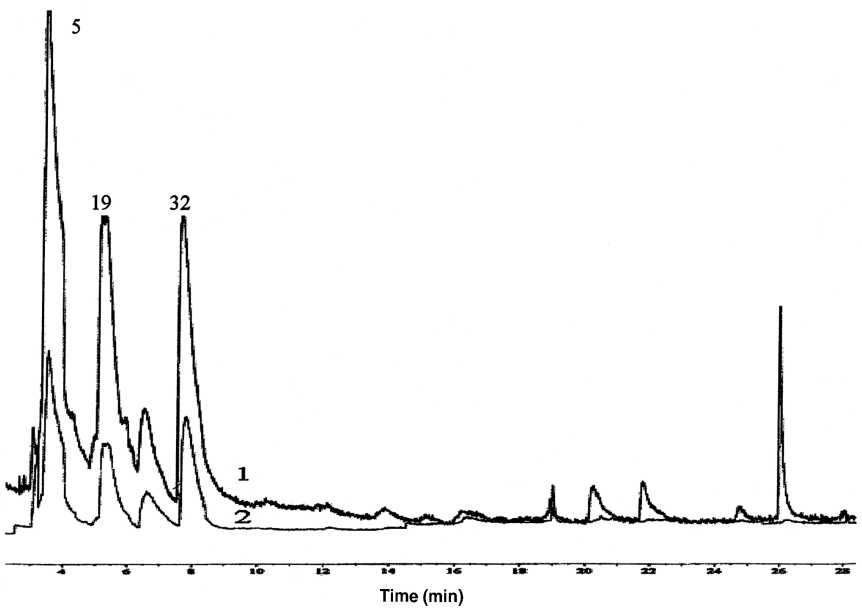

Figure 1. Optimization of extraction temperature: 50°C (1) and 20°C (2). Peak numbering is as listed in Table III.

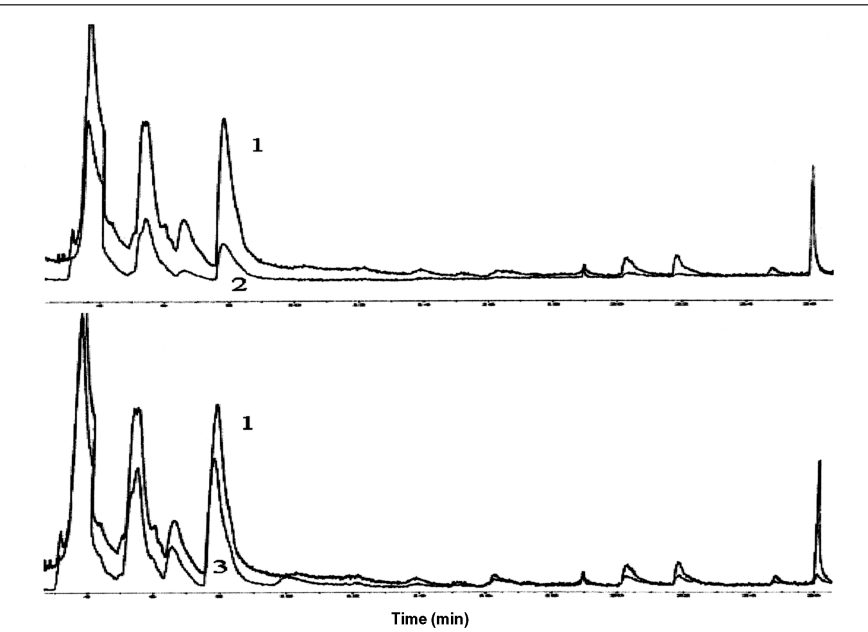

Figure 2. Optimization of matrix: 9 g of flesh with 18 mL deionized and saturated water with salt (1), 9 g of flesh with 18 mL deionized water (2), and 9 g of flesh (3).

107 mass spectra libraries (developed for Shimadzu by NIST, July 2002). The parameters of the different optimization and validation tests are shown in Table I.

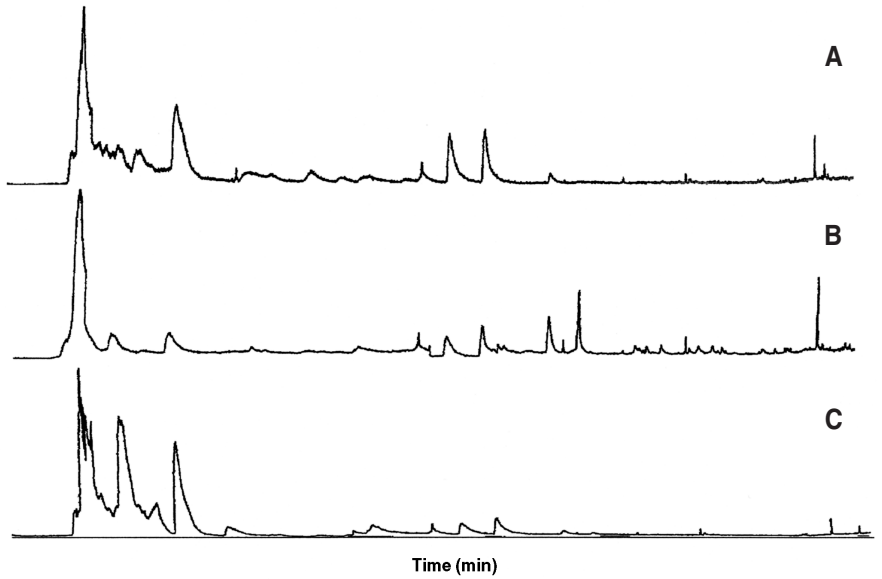

Figure 3. Optimization of fiber type: CAR–PDMS 85 µm (A), DVB–PDMS 65 µm (B), and CAR–PDMS 75 µm (C).

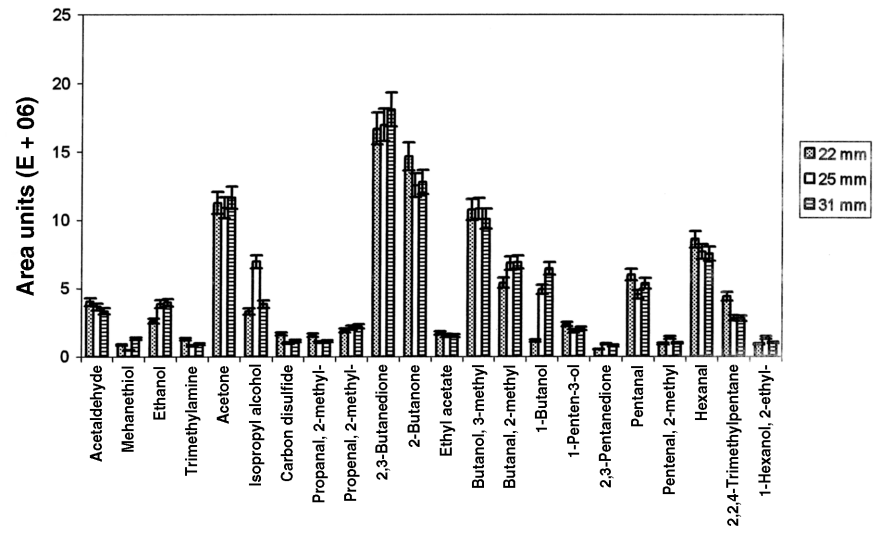

Figure 4. Optimization of fiber penetration depth in the SPME vial.

Experimental design

A 2^3 design was utilized; eight experiments were performed by combining two extreme levels (positive and negative) for three parameters (equilibrium time, 0 s and 1 h; sorption time, 10 s and 1 h; and desorption time, 10 s and 5 min). The values correspond to the addition or subtraction (for positive and negative effects, respectively) of the peak areas and numbers of compounds for the eight experiments.

Results and Discussion

Influence of extraction temperature

Two extraction temperatures were tested: room temperature (20°C) and 50°C. Raising the temperature increased sensitivity while allowing extraction of more compounds, notably the least volatile of which were not extracted at all at 20°C (Figure 1). These results agree with those of other authors who have optimized a method of HS–SPME determination of volatile compounds (13,14): temperature has a positive effect on the method's sensitivity. Liu and Yang (16) explained this by the fact that temperature is one of the factors most affecting the vapor pressure and equilibrium between volatile compounds, which in turn greatly influence the method's sensitivity. The temperature herein was set at 50°C as a good compromise between sensitivity gain and matrix spoilage caused by heating of protein residues in the suspension (20).

Comparison of matrix forms

It is important to optimize the form of the matrix; it can either be untreated or transformed so as to improve extraction of volatile compounds. Several methods have been

Table II. Experimental Design for the Optimization of Desorption (Factor a), Equilibrium (Factor b), and Sorption Time (Factor c)

	Factor a	Factor b	Factor c	Interaction factors a/b	Interaction factors a/c	Interaction factors b/c	Interaction factors a/b/c
Peak area (in 10 million counts)	8.44	0.78	13.95	0.77	7.45	0.6	–0.21
Number of compounds	–5	–0.5	11	3	–4.5	–3.5	–1

developed for foodstuff analysis but few can directly analyze the matrix as sample. In general, the analytical methods require sampling, sample preparation, separation, detection, and data analysis, and over 80% of the analysis time is used for sample preparation, which includes extraction, concentration, fractionation, and isolation of the compounds (25).

Three matrices were tested. These included 27 g of fish flesh, 9 g of fish flesh plus 18 mL of ultrapure water, and 9 g of fish flesh plus 18 mL of ultrapure water saturated in NaCl. The best sensitivity was achieved with the last of these (Figure 2), thus confirming the opinion of various authors (16,25,31) that in sorption analysis: "salting out" caused by the addition of electrolytes enhances volatilization of HS compounds by lowering the solubility of hydrophobic compounds in the aqueous phase and, therefore, increasing sensitivity. Kataoka et al. (25) even speak of supersaturating samples by adding salts.

Comparison of fibers

Seven fibers were tested and three were selected visually (visual evaluation of the intensity of the peaks) for their sensitivity: CAR–PDMS 75 µm and 85 µm and PDMS–DVB 65 µm (Figure 3). CAR–PDMS 75 µm was chosen because it combines the best signal-to-noise ratio (i.e., the best sensitivity) with maximum extraction of compounds.

Roberts et al. (15), Shirey et al. (32) and Mansur et al. (6) considered that the CAR–PDMS is well suited to the extraction of highly volatile compounds, such as those in this study. In addition, Mansur et al. (6) used it for determination of the volatile compounds of different species of fish.

Fiber penetration depth in the SPME vial

The two extreme values of the apparatus and an intermediate value were tested (i.e., 22, 25, and 31 mm). The results varied between the compounds (Figure 4) and, therefore, the total peak area (102 E+06 for 22 mm, 105 E+06 for 25 mm, and 106 E+06 for 31 mm) was used to choose the best fiber penetration depth (31 mm).

Evaluation of the combined effects of the equilibrium, sorption, and desorption times

The sensitivity of HS–SPME analysis is influenced considerably by the vapor pressure of the volatile compounds in the vial, and vapor pressure and equilibrium are in turn affected by time. Hitherto, factor-by-factor optimizations were done, but these ignore between-factor interactions (33).

Equilibrium, sorption, and desorption times were tested simultaneously, with their extreme values, using a complete experimental plan with three factors and two levels. The results are presented in Table II .

In terms of the mass of compounds, sorption time had a large positive effect, desorption time had a moderate positive effect, and equilibrium time had no effect. Only the interaction between the desorption and sorption times had an effect, which was positive. Because desorption time influences the mass of molecules desorbed, if it is short it will result in less baseline increase, thus enabling better integration of peaks.

The quantity of compounds desorbed was greatly affected by sorption time, whereas equilibrium time had no effect and desorption time had a small negative effect. Desorption time and equilibrium time interacted positively, whereas desorption time/sorption time and equilibrium time/sorption time

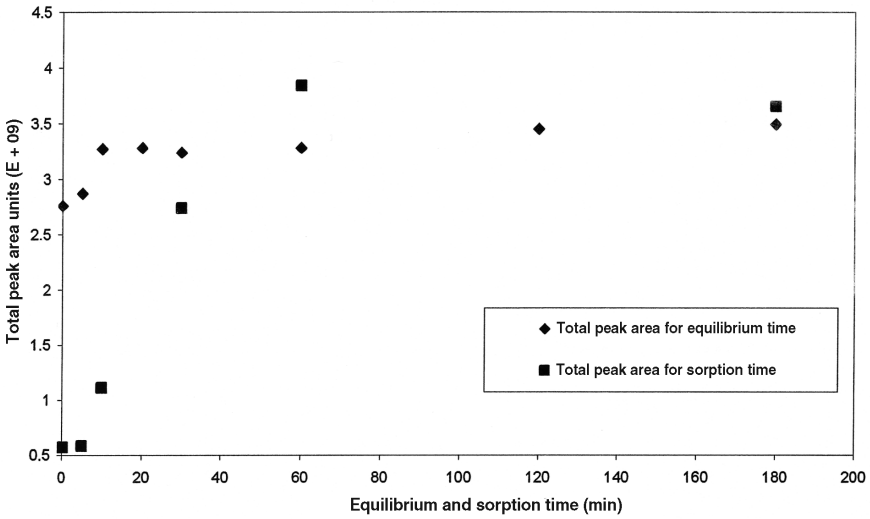

Figure 5. Optimization of equilibrium and sorption times.

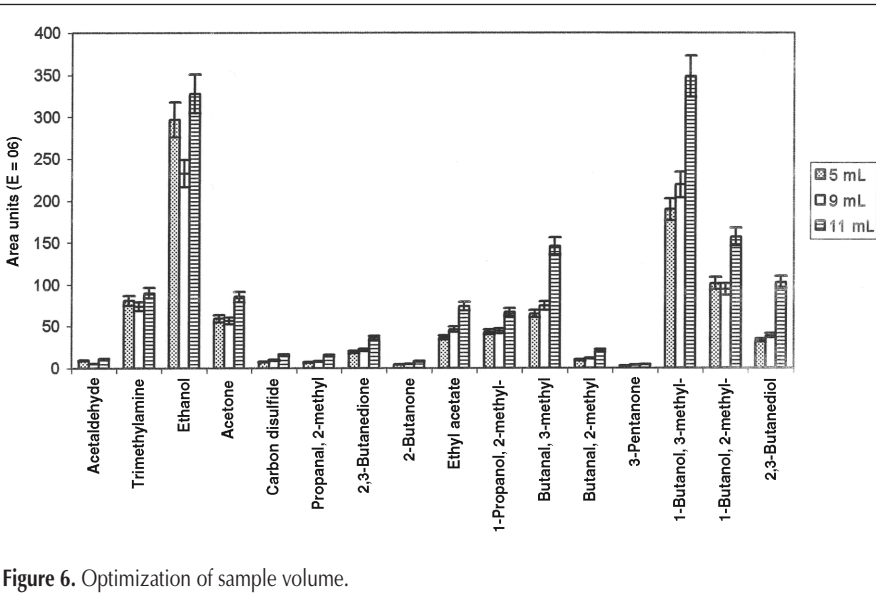

Figure 6. Optimization of sample volume.

interactions were negative. The interaction of the three times was nil. In conclusion, the effects on the mass and quantity of compounds were similar for sorption time and equilibrium time, but not for desorption time.

This experimental plan shows that equilibrium time must be optimized but will have little effect on the mass of compounds recovered. Sorption time, on the other hand, is very important. With desorption time, a compromise must be found between maximum recovery of compounds in both quantity and mass. The interaction between equilibrium time and sorption time is positive for the mass of compounds but negative for the quantity of compounds.

Equilibrium time

Equilibrium times of 0 to 180 min were tested. Under the test conditions, and notably with a sorption time of 1 h, an equilibrium time of 10 min was a good compromise between the quantity of compounds adsorbed and analysis time. The results are presented in Figure 5.

Sorption time

Sorption time was tested from 10 s (minimum value possible with the apparatus) to 180 min (Figure 5). The maximum quantity of compounds adsorbed was reached after 1 h. A sorption time of 40 min was chosen as a compromise between quantity of compounds sorbed and analysis time because, provided the various parameters of the method can be controlled, which is the case with the automated sample carousel, it is not necessary to wait for equilibrium to determine the volatile compounds. According to Ai (29), a quantitative SPME analysis is possible in a nonequilibrium situation if mixing is used and the sorption time is reached precisely. This is possible but to the detriment of the method's sensitivity.

Jung and Ebeler (28) report that SPME analyses are influenced by the partition coefficient of each compound between the HS and matrix and between the fiber and HS. First, an equilibrium is established for a compound between the HS and matrix. The SPME fiber inserted in the HS then extracts HS compounds, thus perturbing the equilibrium and resulting in a re-equilibration between the three phases. However, the extraction time will affect the re-equilibration time. For example, if HS–SPME is utilized to quantitate compounds, the fiber is held in the HS until the equilibrium is reached between the three phases and a maximum of compounds are sorbed on the fiber (i.e., extracted HS) (15). On the other hand, Roberts and coauthors (15) propose that HS–SPME with a short extraction time (< 1 min) can determine the true HS concentration at equilibrium between the HS and water, thus diminishing the perturbations caused by the fiber/HS partition. The true HS described by Roberts (15) reflects the volatile compounds in the HS when the equilibrium between the HS and the liquid sample is achieved. But in this study the "long sorption time" theory was chosen because, in a preliminary test using a "short sorption time", low quantities of compounds were obtained.

Desorption time

Three desorption times were chosen: 10 s, 30 s, and 1 min. The quantity of compounds increased with desorption time

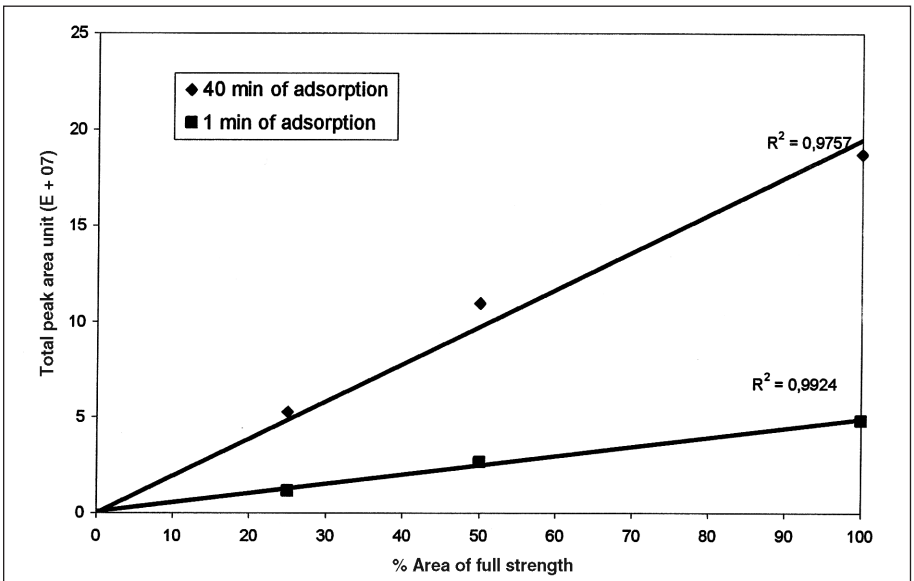

Figure 7. Linearity determination with dilution of full strength for 1 min and 40 min of adsorption time.

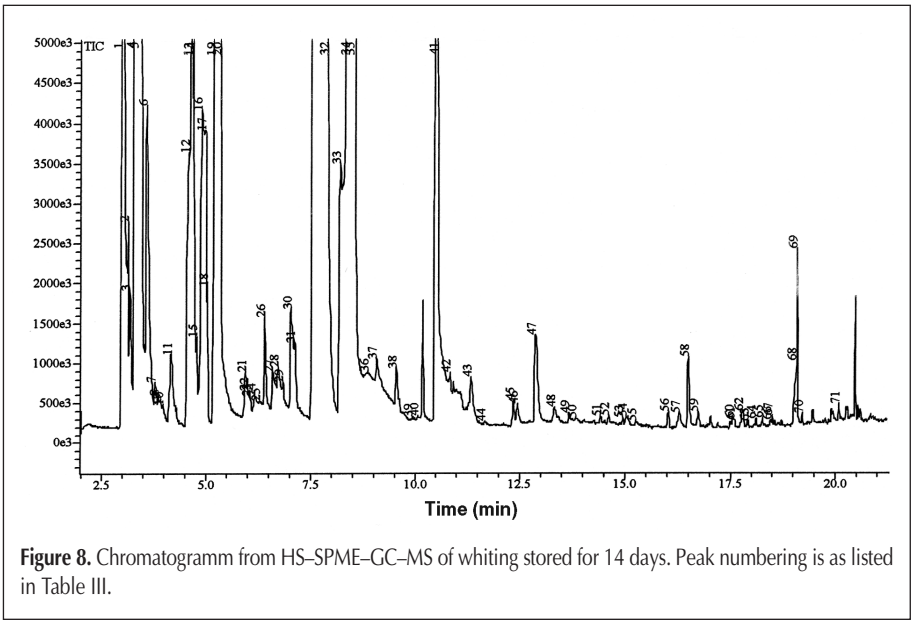

Figure 8. Chromatogramm from HS–SPME–GC–MS of whiting stored for 14 days. Peak numbering is as listed in Table III.

(data not show), but this resulted in difficulties and errors in peak integration through accentuation of coelutions. The desorption time of 10 s was selected because it allowed desorption of a large number of compounds and still enabled good peak integration.

Optimization of sample volume

Three volumes were tested (5, 9, and 11 mL). The manufacturer of the Combi-Pal recommends a volume of 15 mL for 20-mL vials, but a maximum volume of 11 mL was chosen to avoid contamination of the fiber by the matrix during mixing. The results are presented in Figure 6. There were few differences between the volumes of 5 and 9 mL, but 11 mL allowed recovery of more compounds. This volume was, therefore, chosen for the present study.

Other parameters

The position of the fiber in the injector influences the sensitivity of the method (12,26). No test was done, but the value used (49 mm) was optimized by the manufacturer of the chromatograph (Shimadzu). De la Calle Garcia (12) placed the fiber as deep as possible, but Okeyo (26) considered the middle of the injection chamber, and therefore of the insert, as the hottest point. Like De la Calle Garcia (12), we did not use glass wool in the insert in order to avoid breaking the fiber. Okeyo (26) reported that a maximum desorption temperature of 250°C is enough to desorb the least volatile compounds completely from the SPME fiber, which is why this temperature was chosen.

Determination of the linear range

In monitoring the quantity of volatile compounds during spoilage, it is necessary to work within the linear phase (i.e., when the fiber is not saturated). Roberts et al. (15) tested the method they developed to check that they were working in the linear range. They considered that not all compounds can be in the linear range in analysis of natural products, such as coffee in their study and fish in ours, because the compounds have different affinities for the fiber and are present at different concentrations. They therefore suggested diluting the matrix and observing whether the decrease in concentration (evaluated using the relative area under the peaks) corresponds to the dilution used.

The method was compared using sorption times of 1 and 40 min. The analytical conditions were the same as before: spoilage of fish for 14 days. The two dilutions (25% and 50%) were done in ultrapure water saturated in NaCl. There was good linearity for both sorption times, the regression line having a coefficient of determination of 0.97 for the sorption time of 40 min. The results are presented in Figure 7.

Table III. Evaluation of Repetability with Whiting Stored for 14 Days*

Compounds (number of peak, Figures 1/8)	RI	Mean	SD	RSD
Acetaldehyde (3)	-	0.266	0.037	14.95%
Trimethylamine (4)	-	5.699	0.303	5.31%
Ethanol (5)	-	6.255	0.274	4.29%
Acetone (6)	501	0.943	0.071	7.67%
Dimethyl sulfide (7)	521	0.119	0.011	8.57%
Propanal, 2-methyl- (11)	561	0.288	0.023	7.89%
2,3-Butanedione (14)	602	0.623	0.008	1.22%
2-Butanone (15)	604	1.001	0.048	4.91%
Ethyl acetate (17)	615	0.821	0.073	8.73%
Acetic acid (18)	618	0.696	0.076	11.29%
1-Propanol, 2-methyl- (19)	629	10.432	0.485	4.68%
Butanal, 3-methyl- (21)	660	0.134	0.023	15.69%
Butanal, 2-methyl- (24)	671	0.085	0.008	8.96%
1-Penten-3-ol (27)	689	0.158	0.010	6.82%
2-Butanone, 3-methyl- (28)	695	0.182	0.012	6.55%
3-Pentanone (30)	705	0.350	0.012	3.44%
2-Butanone, 3-hydroxy- (32)	728	17.529	1.099	6.26%
3-Buten-1-ol, 3-methyl- (i e) (33)	737	1.000	0.000	0.00%
1-Butanol, 3-methyl- (34)	742	8.957	0.369	4.06%
1-Butanol, 2-methyl- (35)	745	3.009	0.173	5.77%
Acetonitrile (37)	761	0.338	0.030	9.17%
2,3-Butanediol (41)	800	3.177	0.073	2.27%
Ethanol,2-metoxy acetate (45)	845	0.046	0.002	3.60%
1,3-Butanediol (47)	859	0.279	0.009	3.33%
1-Hexanol (50)	883	0.011	0.001	9.17%
2-Heptanone (51)	897	0.013	0.000	2.81%
Butanoic acid, 3-hydroxy ethyl ester (57)	949	0.043	0.005	11.01%
2-Hexene, 3,5,5-trimethyl- (61)	985	0.013	0.001	8.38%
Pyrazine, trimethyl- (67)	1017	0.013	0.001	4.32%
1-Hexanol, 2-ethyl- (68)	1043	0.090	0.003	3.10%
Pyrazine, 3-ethyl-2,5-dimethyl- (71)	1089	0.017	0.002	10.30%
Average RSD for the 30 compounds				6.82%

* RI = retention index; SD = standard deviation; and RSD = relative standard deviation. Mean are relative areas calculated using an internal standard [i.e., (3-buten-1-ol, 3-methyl-)] for three consecutive repetitions.

Volatile compounds in a whiting stored 14 days at 4°C and repeatability of the method

The volatile compounds were identified on the first repetition of the method performed three times for the measure of repeatability. Seventy-one compounds were identified (Figure 8) and 30 were selected for their value in evaluation of freshness. Many of these 30 compounds are known (34–40), and so it is not surprising that they should be found after 14 days of storage. Six of these compounds (trimethylamine, 3-methyl-butanal, 2-methyl-butanal, 3-hydroxy-2-butanone, 3-methyl-1-butanol, and 2-methyl-1-butanol) show a great evolution in comparison with fresh fish analyzed.

The repeatability of the method was estimated as 6.8% by calculating the

mean relative standard deviation of 30 integrated peaks for three repetitions (Table III). The relative areas under the peaks of the compounds were calculated by determining the ratio of the peak area for the compound over the peak area for the internal standard (3-buten-1-ol, 3-methyl). Repeatability was comparable with that obtained for other matrices, such as parmesan, 2% (11); beer, 4.7% (41); and coffee, 7% (15). Yang and Peppard (31) considered that an average relative standard deviation (RSD) of 7% is generally acceptable in trace organic determination.

Conclusion

A chemical method of determining volatile compounds for the assessment of freshness, a key component of fish quality, has been developed. This method can be used during spoilage to identify and quantitate volatile compounds present in the sample HS. It is nonselective by the nature of the fiber utilized (CAR–PDMS 75 µm), thereby enabling extraction of a large range of compounds, repeatable (RSD of approximately 7%), rapid (analysis takes 1 h and 20 min), and sensitive. This method has optimized the nature of the matrix (fish flesh plus water saturated in NaCl), equilibrium time (10 min), sorption time (40 min), sample volume (11 mL), and penetration depth of the fiber in the flask (31 mm). The ability of this method to follow an increase in the quantity of compounds during storage was achieved by limiting desorption time to 10 s and validated by identifying the linear range. This method could be used to track changes in volatile compounds and, hence, in the freshness of whiting and other fish species, perhaps by monitoring trimethylamine, 3-methyl-butanal, 2-methyl-butanal, 3-hydroxy-2-butanone, 3-methyl-1-butanol, and 2-methyl-1-butanol changes, which have been found to be informative.

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