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Optimization of Multiple Headspace Solid-Phase Microextraction for the Quantification of Volatile Compounds in Dry Fermented Sausages

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Multiple headspace solid-phase microextraction (HS-SPME) is a stepwise method that eliminates the influence of the matrix sample on the quantitative analysis of solid samples. The process was optimized for the analysis of volatile compounds in dry fermented sausages by gas chromatography and mass spectrometry. Different amounts of fermented sausages and different vial volumes were studied to obtain the theoretical exponential decay of the peak area of the four successive extractions in order to calculate the total area in the sausage. The highest number of volatile compounds analyzed by multiple HS-SPME in dry fermented sausages was obtained in a 10 mL headspace vial with 0.07 g of sample in the presence of water, 0.75 mg butylated hydroxytoluene, and 0.5 g sodium chloride. Finally, the method was characterized in terms of linearity and detection limits and applied to analyze the volatile compounds present in fermented sausages manufactured with either nitrate or nitrite.

KEYWORDS: Multiple headspace solid phase microextraction; dry fermented sausage; cured aroma; flavor

INTRODUCTION

Thousands of volatile compounds have been identified in dry fermented sausages although only a few of them have been identified as the main odorants responsible for the dry-cured aroma (*1–3*). Furthermore, the quantitative analysis of these aroma active compounds is difficult due to their low concentrations and the effect of the matrix in the extraction technique used.

In the last years, the use of solid-phase microextraction (SPME) (*4*) has allowed the isolation and identification of a high number of volatile compounds in fermented sausages (*5*) and the study of the effect of several factors on their generation (*6*). However, the volatile compounds were not quantified while an estimated proportion was indicated. The reason for this fact was that SPME is not an exhaustive extraction technique (*7, 8*). The concentration of the compounds can be calculated using standard solutions. However, in complex matrix samples, the slope of the calibration curve differs from that obtained for standard solutions because the partition coefficients depend on composition and polarity of samples and solutions. Also, an internal standard can be used but the standard and the analyte may behave differently (*9*).

In this sense, the quantitative analysis of volatile compounds in a complex matrix was solved by Kolb et al. (*10*) with a procedure called multiple headspace extraction. The method is based on a stepwise gas extraction at equal time intervals allowing the total area for the compound to be calculated and eliminating the influence of the sample matrix. Multiple HS-SPME (headspace solid-phase microextraction) has the same aim as multiple headspace extraction, with the benefit of being a solvent free technique which provides more sensitivity and selectivity than direct headspace sampling (*11, 9*). The amount of analyte extracted by the fiber is proportional to the initial amount, and it is assumed that the analyte concentration in the sample will decay exponentially with the number of extractions. This method has already been applied in the field of food science for the quantitative determination of volatile compounds in multilayer packaging (*11, 12*) and red wine (*13*).

The result obtained by multiple HS-SPME is an area value which corresponds to the total amount in the sample vial independent from its distribution between both phases (*10*). In this way, after successive extractions, the concentration of analyte decays exponentially, and the total peak area (A_t) corresponding to an exhaustive extraction of the analyte can be calculated from the following (*11*):

$$A_t = \frac{A_1}{1 - e^k} \tag{1}$$

Where, A_1 is the peak area in the first extraction and k is the slope obtained when representing the natural logarithm of the peak area versus the number of extractions minus one.

The initial mass of compound in the sample can be calculated with the value of A_t by using a calibration curve obtained using standard solutions. The determination will be free of matrix-effect errors although sample and solution matrices are very different (*9*). However, the amount of compound in the sample is crucial because it is necessary to

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observe an exponential decay in peak area with the number of extractions. If the amount is too low, the amount extracted by the fiber could be under the limit of detection (LOD), but if it is too large, no decay would be observed. All factors affecting the SPME analysis would affect the multiple HS-SPME. Therefore, it is necessary to optimize not only the sample quantity but also all the experimental conditions that affect the volatile concentration in the headspace such as sample oxidation, ionic strength, sample quantity, ratio sample/headspace volume, and extraction time.

On the other hand, multiple HS-SPME has certain drawbacks such as increased analysis time, shorter linear range, small amount of sample used affecting reproducibility, and lack of depletion linearity (*9*). There is a way to reduce analysis time, that is, to perform multiple HS-SPME under a nonequilibrium situation (*9*). In this case, the variables affecting the extraction rate, such as extraction time and agitation, must be kept constant. On the other hand, multiple HS-SPME can be used to avoid matrix-effect errors in the quantitative determination of volatile compounds especially in solid samples. In this sense, the aim of the present work was to optimize the multiple HS-SPME for the quantification of the volatile compounds present in a meat products such as dry fermented sausage.

MATERIALS AND METHODS

Reagents and Standards. The chemical compounds used for the identification and to prepare the standard solutions were all obtained from Fluka Chemie AG (Buchs, Switzerland) except hexanal, (*E,E*)-hepta-2,4-dienal, phenyl acetaldehyde, (*E*)-octen-2-al, nonanal, and (*E*)-nonen-2-al that were obtained from Aldrich (Saint Louis, MO).

Dry Fermented Sausages. Two batches of sausages, one containing sodium nitrite and another containing potassium nitrate, were used (*14*). Dry fermented sausages were made with lean pork (80%) and pork back fat (20%). The following additives were added in gram per kilogram quantities to the meat mixture: sodium chloride (27), lactose (20), dextrin (20) (food grade quality), sodium caseinate (20), glucose (7), sodium ascorbate (0.5), and either sodium nitrite (0.15) or potassium nitrate (0.3). The sausages were submitted to a rapid fermentation process with a total fermentation and drying time of 44 days (*14*). The sausages were then vacuum packed and stored at 4 °C for approximately 2 months, and the total processing time was 111 days. At the end of the storage stage, three sausages chosen randomly from each batch were used to perform the analyses. From each sausage, slices were taken, vacuum packaged, and stored frozen at −20 °C until analysis.

Extraction of Volatile Compounds. Extraction of headspace volatile compounds was performed using a solid-phase microextraction (SPME) device (Supelco, Bellefonte, PA) with an 85 μm carboxen/polydimethylsiloxane StableFlex fibre (CAR/PDMS stb).

Gas Chromatography-Mass Spectrometry (GC-MS). For the identification and confirmation of the volatile compounds, a gas chromatograph HP 5890 series II equipped with an HP 5972 mass selective detector (Hewlett Packard, Palo Alto, CA) was used with the conditions described by Flores et al. (*15*). The compounds adsorbed by the fiber were desorbed in the injection port of the GC-MS for 6 min at 240 °C with the purge valve off (splitless mode). The compounds were separated on a DB-624 capillary column (J&W Scientific (Agilent Technologies, USA), 30 m, 0.25 mm i.d., film thickness 1.4 μm). The GC oven temperature program began at 38 °C, held for 13 min, ramped to 110 °C at 3 °C·min^{-1}, then to 150 °C at 4 °C·min^{-1} and to 210 °C at 10 °C·min^{-1}, and, finally, held at 210 °C for 5 min. Mass spectra were obtained by electron impact at 70 eV, and data were acquired across the range 29–400 amu (scan mode). The compounds were identified by comparison with mass spectra from the library database (Nist 98), kovats retention index (*16*), and by comparison with authentic standards. The quantification of the volatile compounds was done by SCAN mode using the total ion current (TIC) area or in selected ion monitoring (SIM) mode using the area of an specific ion for each compound (**Table 1**).

Table 1. Volatile Compounds Analyzed by Multiple HS-SPME and Ions Used for Quantification in the SIM Method

number	compound	KI [a]	ion (m/z)	stock solution (ng/μl) [b]
1	metanethiol	473	47	
2	ethanol	505	47	
3	propanal	520	58	333.3
4	acetone	526	43	
5	butanal	603	72	312.5
6	butan-2-one	628	72	350.0
7	ethyl acetate	635	43	383.3
8	3-methylbutanal	686	58	333.3
9	acetic acid	717	60	
10	pentan-2-one	727	43	337.5
11	pentanal	735	58	325.0
12	pentane-2,3-dione	741	57	429.2
13	ethyl butanoate	830	43	354.2
14	hexanal	840	56	366.7
15	2-methylpropanoic acid	854	43	
16	butanoic acid	878	60	
17	(*E*)-hexen-2-al	902	41	345.8
18	heptan-2-one	936	43	341.7
19	heptanal	941	70	337.5
20	methional	970	48	
21	(*E*)-hepten-2-al	1016	41	350.0
22	oct-1-en-3-ol	1025	57	345.8
23	octanal	1035	41	387.5
24	(*E,E*)-hepta-2,4-dienal	1057	81	391.7
25	hepta-2,4-dienal	1073	81	
26	hexanoic acid	1074	60	
27	phenyl acetaldehyde	1114	91	125.0
28	(*E*)-octen-2-al	1116	41	354.2
29	octan-1-ol	1128	41	333.3
30	nonan-2-one	1140	58	345.8
31	nonanal	1151	98	341.7
32	(*E*)-nonen-2-al	1226	41	362.5
33	ethyl octanoate	1227	88	366.7
34	octanoic acid	1289	60	

[a] KI: Kovacs index calculated for DB-624 capillary column (J&W Scientific: 30 m, 0.25 mm i.d., 1.4 μm film thickness) installed on a gas chromatograph equipped with a mass selective detector. [b] Stock solution used for the preparation of the successive dilutions for external calibration.

Selection of Volatile Compounds. The selection of the volatile compounds to quantify by multiple HS-SPME was done through the study of the aroma active compounds present in the headspace of fermented sausages (*3*). In order to confirm the retention time of each compound, 3 g of dry fermented sausage were added to a 10 mL headspace (HS) vial and sealed with a PTFE faced silicone septum (Supelco, Bellefonte, PA). The vial was left for 1 h in a thermoblock (J.P. Selecta, Barcelona, Spain) at 30 °C for equilibration. The 85 μm CAR/PDMS was then exposed to the headspace for 3 h while maintaining the sample at 30 °C. Then, the fiber was desorbed in the injection port of the GC-MS using the same conditions described above but using the MS detector in the scan mode.

Multiple HS-SPME Optimization. *Sausage Preparation.* A slice of fermented sausage was cut into cubes and frozen with liquid nitrogen. Then, the cubes were finely minced in a blender (Waring Commercial 8010, CT). The minced sausage was prepared prior to analysis.

Multiple HS-SPME Procedure. The extractions were done using the 85 μm CAR/PDMS fiber, maintaining the vial (10 mL HS) and fiber at 30 °C in a thermoblock (J.P. Selecta, Barcelona, Spain). In the case of the 100 mL HS vial, it was maintained in a water bath at 30 °C and agitated at 600 rpm during extraction. Previous to the initial extraction, the vial was equilibrated for 1 h at 30 °C. The multiple HS-SPME procedure consisted of four consecutive extractions on the same sample. Then, the fiber was desorbed in the injection port of the GC-MS using the same conditions described above. The results were expressed as the area of the TIC of each volatile compound when the SCAN mode of the MS was used for quantification. However, the results were expressed as the area of the selected ion for each volatile compound when data was acquired in SIM mode. In each multiple HS-SPME, the natural logarithm of the peak area versus the number of extractions

minus one was represented to calculate the slope and the regression coefficient. Each extraction by multiple HS-SPME was done in triplicate.

Inhibition of Sausage Lipid Oxidation. A 1 g portion of the minced sausage was homogenized with 15 mL of milli-Q water containing different quantities of butylated hydroxytoluene (BHT) 5% (w/v) in methanol. The mixture was homogenized at 26 000 rpm for 1 min in 20 s pulses, using a Polytron PT 1200 (Kinematica AG, Littau-Luzern, Switzerland). Then, 1 mL of the homogenized sample was added to a 10 mL HS vial, which was sealed with a PTFE faced silicone septum, containing 0.5 g NaCl. The vial was submitted to multiple HS-SPME by four consecutive extractions of 60 min each using the conditions indicated above. The compounds were identified and quantified by SCAN mode.

Effect of Ionic Strength. A 1 g portion of the minced sausage was homogenized as indicated previously with 15 mL of milli-Q water containing 0.75 mg BHT. Then, 1 mL of the homogenized sample was added to a 10 mL HS vial, containing different quantities of NaCl: 0.1, 0.25, 0.35, and 0.5 g. The vial was submitted to multiple HS-SPME by four consecutive extractions of 60 min each using the conditions indicated above. The compounds were identified and quantified by SCAN mode.

Selection of the Optimum Extraction Time. The selection of the extraction time was done in the two different vials: 10 mL HS and 100 mL HS vial. In the 10 mL HS vial, the sample was prepared using 1 g of minced sausage homogenized with 15 mL of milli-Q water containing 0.75 mg BHT. Then, 1 mL of the homogenized sample was added to a 10 mL HS vial, containing 0.5 g NaCl.

In the 100 mL HS vial, the sample was prepared using 0.2 g of minced sausage homogenized with 15 mL of milli-Q water containing 0.75 mg BHT. The whole homogenized sample was added to the 100 mL HS vial containing 7.5 g NaCl.

The vials were submitted to multiple HS-SPME by four consecutive extractions varying the extraction time (30, 60, 90, and 120 min) using the conditions indicated above. The compounds were identified and quantified by SCAN mode.

Selection of the Optimum Sample Quantity. Three different conditions were studied: sausage homogenized in water in the 10 mL HS vial and in the 100 mL HS vial and, finally, sausage without dilution in water.

In the 10 mL HS vial, the sample was prepared using different quantities of minced sausage (0.5, 1, and 2 g) that were homogenized with 15 mL of milli-Q water containing 0.75 mg BHT. Then, 1 mL of the homogenized sample was added to a 10 mL HS vial, containing 0.5 g NaCl.

In the 100 mL HS vial, the sample was prepared using different quantities of minced sausage (0.07, 0.1, and 0.15 g) that were homogenized with 15 mL of milli-Q water containing 0.75 mg BHT. The whole homogenized sample was added to the 100 mL HS vial containing 7.5 g NaCl.

The minced sausage was also analyzed without dilution in water. Different quantities of the minced sausage (0.03, 0.07, and 0.13 g) were added to a 10 mL HS vial, containing 0.75 mg BHT.

In each assay, the vials were submitted to multiple HS-SPME by 4 consecutive extractions of 90 min each using the conditions indicated above. Each assay was done in triplicate. The data was acquired in SIM mode.

Calibration by External Standard. The compounds that showed a linear decrease in the optimized multiple HS-SPME analysis were used to prepare a stock standard solution in methanol at the concentration shown in **Table 1**. The stock solution was diluted 1/50, 1/75, 1/100, 1/200, 1/800, 1/1600, and 1/3200 in methanol, and all the dilutions were analyzed by multiple HS-SPME.

The multiple HS-SPME procedure was applied using similar conditions as for the sausage. In this case, 5 μL of the standard dilution were added to 1 mL (1.4 g) of glass beads present in the 10 mL HS vial. The glass beads were used to compensate in the calibration vial for the missing sample volume (*10*). Since no sample matrix must be present, the vial contains the gas phase only, and consequently, the volatiles are analyzed as a homogeneous gas mixture in the vial (*10*).

The vials containing the standard dilution were submitted to multiple HS-SPME by four consecutive extractions of 90 min each using the conditions indicated above. Each dilution was analyzed in triplicate. The data was acquired in SIM mode. The slope obtained in the multiple HS-SPME was used to calculate the total area present in the vial using eq 1. Then, a linear calibration was obtained by representing the total area against the standard concentration added to the vial. Limits of detection (LOD) and quantification (LOQ) were calculated from the first extraction of a blank plus three and ten times the standard deviation of four blank replicates, respectively.

Application of the Optimized Multiple HS-SPME to Dry Fermented Sausage Quantification. The quantification of the volatile compounds present in dry fermented sausages was done by the optimized multiple HS-SPME. The dry fermented sausages manufactured containing nitrite and nitrate as described above were analyzed. A 1 g portion of each minced sausage (minced frozen with liquid nitrogen) was homogenized with 15 mL of milli-Q water containing 0.75 mg BHT. Then, 1 mL of the homogenized sample was added to a 10 mL HS vial, containing 0.5 g NaCl. The vial was submitted to multiple HS-SPME by 4 consecutive extractions of 90 min each using the conditions indicated above. Each assay was done in triplicate. The data was acquired in SIM mode. The total area present in the vial was calculated using eq 1. Then, the external standard calibrations were used to calculate the concentration of the volatile compounds in the sausage.

Statistical Analysis. The effect of BHT and NaCl addition on the total area value of each volatile compound extracted by multiple HS-SPME was determined by analysis of variance (ANOVA). Also, the effect of the different curing agents in the quantified volatile compounds was tested by ANOVA analysis using the statistic software Statgraphics plus (version 5.1).

RESULTS AND DISCUSSION

Optimization of Multiple HS-SPME for the Quantitative Determination of Volatile Compounds in Fermented Sausages. The amount of compound used in multiple HS-SPME should be small enough to be able to observe an exponential decay in peak area with the number of extractions. Due to the heterogeneity of the dry fermented sausage, it is difficult to weigh a small amount of it. This problem was solved by using a fine pulverized mixture of the fermented sausage obtained by mincing with liquid nitrogen.

For the previous analysis, a sample weight of 1 g was chosen, diluted with 15 mL milli-Q water and then homogenized. Then, 1 mL of the homogenized sample, that contained 0.07 g sausage, was added to the 10 mL HS vial for multiple HS-SPME. The selection of the volatile compounds present in fermented sausages was based on a previous study where GC-olfactometry was done and 55 odor active zones were described (*3*). However, only 34 aroma active compounds were found by multiple HS-SPME due to the small quantity of sample used and the lower extraction time applied. In this situation, those compounds present in very low quantities were not extracted and it was not possible to detect them. The confirmation of the retention times of the compounds analyzed was done with authentic standards, and the compounds studied are shown in **Table 1**.

The high proportion of fat in dry fermented sausages, in this case a 20% pork back fat used in their manufacture, makes this solid food very susceptible to oxidation during analysis by multiple HS-SPME. The presence of oxygen in the vial and the temperature applied during sampling encourage the oxidation phenomenon. Therefore, the first attempt during the optimization process was to control the sample oxidation by adding different quantities of BHT. Then, the samples were submitted to four successive extractions and the results are shown in **Figure 1**. In the absence of BHT, the compounds that come from the lipid oxidation did not follow a decay with the four extractions. Also, the compounds

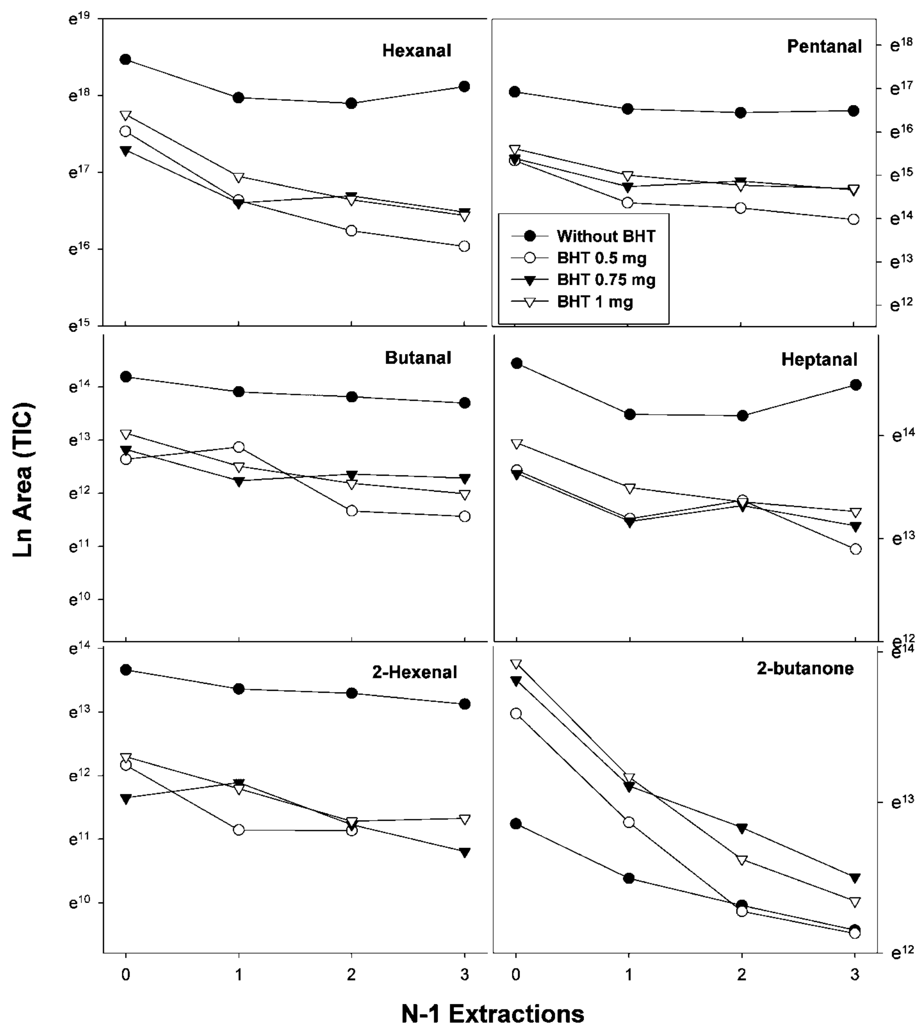

Figure 1. Effect of BHT addition on the extraction of volatile compounds in fermented sausages by multiple HS-SPME. Results are shown as the abundance (total ion current, TIC) of the compound versus the number of extractions minus one.

in the highest proportion, hexanal and heptanal, showed an increment in the area at the fourth extraction. In addition, the area of the aldehydes obtained without BHT was higher than that in the presence of BHT from the first extraction to the latest. This could be due to an oxidation of the sausage during the homogenization process. On the other hand, butan-2-one that is generally formed in fermented sausages from the bacterial metabolism by carbohydrate fermentation showed a different behavior (*14*). Its area was lower in the absence of BHT, probably due to a competition phenomenon produced by the high concentration of hexanal and heptanal formed through the oxidation process. In this sense, when BHT was added, higher recoveries of butan-2-one were obtained. In general, no significant differences in the total area of the compounds extracted were observed with the different quantities of BHT assayed. In summary, the addition of BHT inhibits the oxidation process produced during the sample preparation and during the analysis by multiple HS-SPME. In the conditions assayed in this experience, sample quantity, vial, etc., the addition of 0.75 mg BHT during sample preparation was selected for the multiple HS-SPME analysis of volatile compounds in fermented sausages.

The next step in the optimization process was to study better conditions to analyze the volatile compounds by multiple HS-SPME. As indicated previously, the amount of compound in the sample is crucial and should be small enough to be able to observe an exponential decay in peak area with the number of extractions. But in many cases, the compounds present in very low quantities are not extracted and it is necessary to increase their concentration in the headspace by adding NaCl that produces a salting out effect. This fact is shown in **Figure 2**. The effect of NaCl addition was studied by also adding BHT to prevent the lipid oxidation. Smaller differences were detected among the NaCl concentrations assayed (**Figure 2**). The total area extracted for each volatile compound was significantly higher in the presence of 0.35 and 0.5 g NaCl. In general, the highest recoveries were obtained between 0.35 and 0.5 g NaCl. This fact can be explained by the saturating concentration of NaCl that is 0.3 g/mL. Also, in the case of hexen-2-al that it is present in very low amounts, it was observed that, in the presence of 0.1 and 0.25 g NaCl, it was not detected after the second extraction by multiple HS-SPME. In conclusion, the addition of a saturating concentration of NaCl is necessary to analyze the volatile compounds that are in lower concentration by multiple HS-SPME.

Multiple HS-SPME is generally carried out under an equilibrium situation where the variables affecting the extraction rate, extraction time, and agitation do not affect it. The extraction time necessary to reach equilibrium was determined. It was obtained that, in both vials assayed, the 10 mL and 100 mL HS vials, the extraction time to reach equilibrium was 90 min (data not shown). In many cases, the compounds reached equilibrium

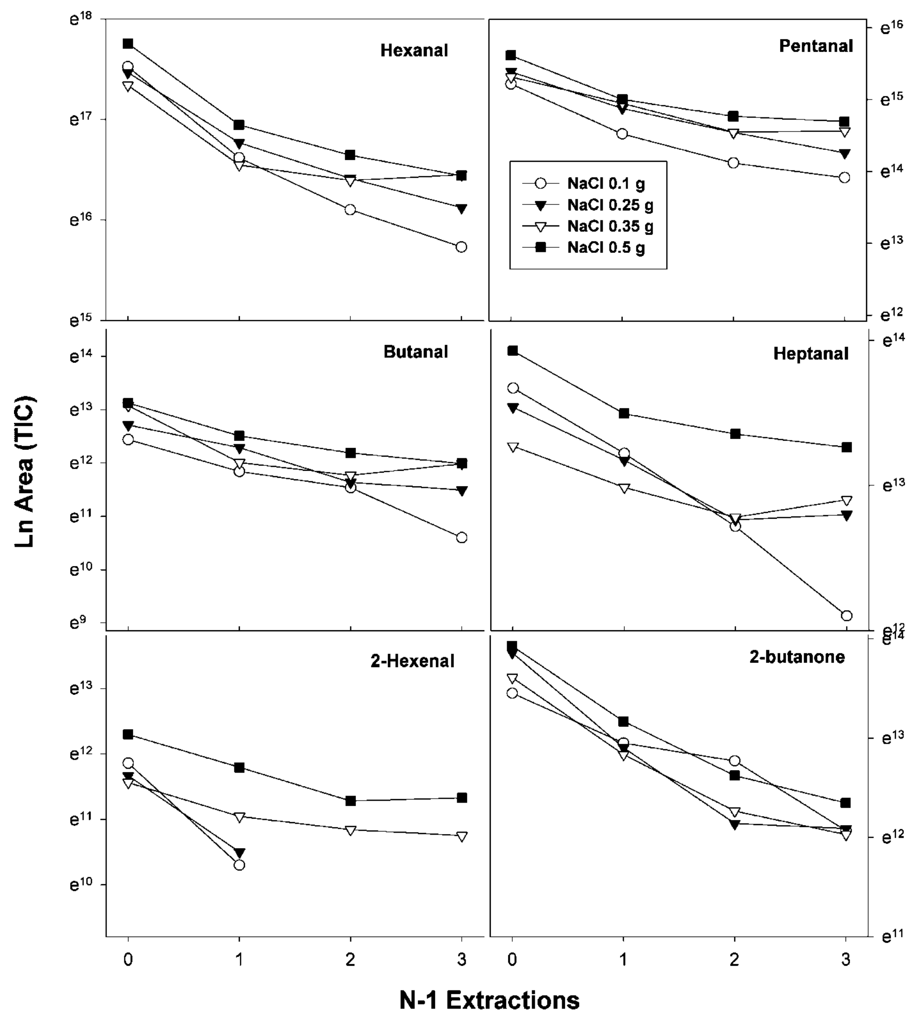

Figure 2. Effect of NaCl addition on the extraction of volatile compounds in fermented sausages by multiple HS-SPME. Results are shown as the abundance (total ion current, TIC) of the compound versus the number of extractions minus one.

at 60 min but the compounds in highest concentration, such as hexanal, needed more time to reach equilibrium. In these experiences, we decided to select 90 min as the optimum extraction time. Although, it has been reported that a reduction in the analysis time can be performed under a nonequilibrium situation while the variables affecting the extraction rate, extraction time, and agitation are kept constant (*9*).

Other factors evaluated were the quantity of sample analyzed and the ratio of sample volume/total volume (V_s/V_t). The results are shown in **Table 2** where those compounds showing a linear decay and a correlation coefficient higher than 0.75 are presented. The quantity added to the vials assayed (10 mL and 100 mL HS) affected the number of compounds who showed a linear decay. The highest sample quantity analyzed showed a lower number of compounds with a linear decay, probably due to a saturation of the headspace in the successive extractions. In addition, the lowest quantity analyzed showed also a lower number of linear descendent compounds probably due to their proximity to the LOD.

In the 10 mL HS vial, the best results were obtained with a sample quantity of 0.07 g where 25 compounds showed a linear decay. However it was remarkable that none of the acid compounds showed a linear decay and they can not be analyzed by multiple HS-SPME. Also, the two sulfur compounds methanethiol and methional showed an exponential decay but the results were not reproducible as also happened with ethanol and acetone (**Table 2**).

In the 100 mL HS vial, the best results were obtained with a sample quantity of 0.07 g and 0.1 g where 18 and 19 compounds showed a linear decay, respectively. In the 100 mL HS vial, the same compounds as in the 10 mL HS vial did not show a linear decay or the results were not reproducible, but in addition, several aldehydes, heptanal, octanal, nonanal, one of the isomers of hepta-2,4-dienal, and octen-2-al, did not show the linear decay or the results were not reproducible.

Another experience without adding water in the sausage preparation was performed in the 10 mL HS vial under the same conditions that resulted in the optimum for the quantitative determination of volatile compounds. In this case, the quantity of sample added varied from 0.07 to 0.15 g, and in all cases, the results obtained were not adequate to quantify the volatile compounds by multiple HS-SPME (data not shown). At the lowest quantity of sample used 0.07 g, none of the volatiles showed an exponential decay. When the sausage quantities were increased, several volatile compounds showed an exponential decay but the results were not reproducible with correlation coefficients lower than 0.75. The addition of water produced a dilution of the sample and allows the partition of volatiles between lipids and aqueous phases and, then, between aqueous and gas phases. In these

Table 2. Slopes and Correlation Coefficients in the Different HS Vials Using Different Amounts of Dry Fermented Sausage

		10 mL HS vial						100 mL HS vial					
		0.03 g		0.07 g		0.13 g		0.07 g		0.1g		0.15 g	
number	compound	*m*	r^2	*m*	r^2	*m*	r^2	*m*	r^2	*m*	r^2	*m*	r^2
1	metanethiol	*a*											
2	ethanol												
3	propanal			−0.15	0.957					−0.10	0.774		
4	acetone	−0.04	0.777										
5	butanal	−0.32	0.805	−0.34	0.912			−0.10	0.899	−0.24	0.887		
6	butan-2-one	−0.77	0.980	−0.57	0.970	−2.20	0.774	−0.19	0.988	−0.20	0.934	−0.14	0.973
7	ethyl acetate	−0.83	0.804	−1.43	0.961	−1.08	0.986	−0.30	0.950	−0.31	0.994	−0.32	0.937
8	3-methylbutanal	−0.80	0.949	−0.66	0.939	−1.78	0.760	−0.32	0.994	−0.38	0.990	−0.24	0.997
9	acetic acid												
10	pentan-2-one	−1.46	0.929	−1.45	0.931	−0.88	0.954	−0.44	0.987	−0.60	0.949	−0.28	0.985
11	pentanal			−0.33	0.832			−0.30	0.976	−0.33	0.952	−0.17	0.940
12	pentane-2,3-dione	−0.41	0.849	−0.45	0.939			−0.20	0.865	−0.26	0.944		
13	ethyl butanoate	−0.62	0.845	−0.69	0.911	−1.65	0.791	−0.90	0.914	−0.85	0.942	−0.41	0.776
14	hexanal	−0.43	0.919	−0.40	0.887			−0.44	0.973	−0.48	0.966	−0.28	0.971
15	2-methylpropanoic acid												
16	butanoic acid												
17	(*E*)-hexen-2-al	−0.32	0.884	−0.27	0.912			−0.17	0.804	−0.17	0.770	−0.11	0.779
18	heptan-2-one	−0.39	0.900	−0.35	0.832			−0.49	0.793	−0.33	0.886	−0.31	0.962
19	heptanal	−0.25	0.800	−0.21	0.845			−0.13	0.868				
20	methional												
21	(*E*)-hepten-2-al	−0.22	0.832	−0.22	0.817			−0.17	0.792	−0.13	0.941		
22	oct-1-en-3-ol	−0.55	0.857	−0.57	0.767			−0.33	0.873	−0.19	0.879		
23	octanal			−0.26	0.785								
24	(*E*,*E*)-hepta-2,4-dienal			−0.33	0.799					−0.16	0.828		
25	hepta-2,4-dienal	−0.21	0.924	−0.15	0.849								
	sum of hepta-2,4-dienal isomers	−0.19	0.859	−0.25	0.827								
26	hexanoic acid												
27	phenyl acetaldehyde	−0.23	0.978	−0.18	0.929	−6.45	0.857			−0.12	0.893		
28	(*E*)-octen-2-al	−0.27	0.891	−0.24	0.834			−0.14	0.875				
29	octan-1-ol			−0.68	0.809			−0.48	0.897	−0.26	0.843	−0.18	0.787
30	nonan-2-one	−0.32	0.835	−0.31	0.784			−0.24	0.875	−0.16	0.941	−0.15	0.855
31	nonanal			−0.22	0.855								
32	(*E*)-nonen-2-al			−0.17	0.835	−7.02	0.700						
33	ethyl octanoate	−0.28	0.882	−0.46	0.809	−2.64	0.546	−0.25	0.838	−0.10	0.864		
34	octanoic acid												

[a] Slope not shown as it showed no linear decay or an r^2 value <0.7.

conditions, the volatilization of volatiles is improved because volatiles are generally hydrophobic.

In summary, the highest number of volatile compounds in dry fermented sausages that were analyzed by multiple HS-SPME were obtained in the 10 mL HS vial with 0.07 g of sample in the presence of 1 mL water, 0.75 mg BHT, and 0.5 g NaCl.

The linearity of the total peak area of the volatile compounds versus the mass of these volatiles was studied using standard solutions in methanol at seven concentration levels. These dilutions were analyzed in triplicate using the same procedure as for the sample. **Table 3** shows the linear ranges of the volatile compounds studied, correlation coefficients, slopes and intercepts of the equations, limits of detection (LODs), and limits of quantification (LOQs). The correlation coefficients obtained ranged from 0.835 to 0.999, and they were acceptable in all cases.

Quantification and detection limits were calculated from the area of the first extraction of a blank as indicated previously. The detection limits obtained for the volatile compounds were lower than the olfactory thresholds of these compounds (*3*).

Application of Multiple HS-SPME for the Quantitative Determination of Volatile Compounds in Fermented Sausages. The total area for each volatile compound in the dry fermented sausage analyzed by multiple HS-SPME was calculated using eq 1. The concentration of the volatile compounds was calculated using the external standard calibrations. The results obtained are shown in **Table 4** where the most abundant compounds were the aldehydes, hexanal, propanal, and pentanal. A higher proportion of almost all the volatile compounds were detected in the fermented sausage manufactured with nitrate. Only four compounds, phenyl acetaldehyde, octen-2-al, octan-1-ol, and nonen-2-al, did not show significant differences between batches. These results are in accordance with the volatile estimated data obtained for the same sausages with direct SPME analysis (*14*), where a higher proportion of aldehydes derived from the lipid oxidation process was obtained for the sausage containing nitrite and submitted to a rapid fermentation process. On the other hand, the results obtained in the concentration of the volatile compounds derived from amino acid catabolism and staphylococci esterase activity are not in accordance with the estimated proportion by headspace SPME. The reason for these differences could be that by using multiple HS-SPME the matrix effect is avoided (*10*) and the concentration reported is present in the HS and in the solid matrix sample. In contrast, in HS-SPME, the volatile compounds are affected by the matrix composition who affect the partition coefficients between the matrix and the headspace. Finally, the contribution of the quantified volatile compounds to the flavor of dry fermented sausages will depend not only on their concentration in the matrix but also on the matrix composition.

Table 3. Linearity, Correlation Coefficients, LODs, and LOQs of the Standard Compounds Studied by Multiple HS-SPME

number	compound	linear range (ng)	correlation coefficient (r^2)	slope (A_t/ng × 10^{-3})	intercept[a] (A_t × 10^{-3})	LOD (ng)	LOQ (ng)
3	propanal	0.52 - 33.34	0.954	40.06	19.34	0.88	1.89
5	butanal	0.49 - 15.63	0.982	34.61	59.66	0.50	1.22
6	butan2-one	1.09 - 35.00	0.898	13.27	424.11	0.84	1.33
7	ethyl acetate	0.60 - 38.33	0.919	77.85	798.26	0.91	2.06
8	3-methyl butanal	0.52 - 33.33	0.995	15.92	−0.59	0.25	0.43
10	pentan-2-one	0.52 - 2.11	0.928	1000.00	171.09	0.01	0.03
11	pentanal	0.50 - 32.50	0.958	48.64	178.85	0.24	0.37
12	pentane-2,3-dione	0.67 - 42.92	0.980	49.07	243.46	0.03	0.05
13	ethyl butanoate	0.55 - 8.85	0.988	117.82	47.60	0.03	0.06
14	hexanal	0.57 - 36.67	0.982	83.57	139.86	1.36	2.96
17	(*E*)-hexen-2-al	0.54 - 34.58	0.975	71.85	−87.52	0.06	0.11
18	heptan-2-one	0.53 - 34.17	0.983	264.67	−221.54	0.14	0.37
19	heptanal	0.52 - 33.75	0.972	95.17	−99.13	0.72	1.62
21	(*E*)-hepten-2-al	0.54 - 35.00	0.974	39.10	−54.07	0.20	0.36
22	oct-1-en-3-ol	0.54 - 8.65	0.999	44.89	11.18	0.11	0.18
23	octanal	0.60 - 38.75	0.975	34.96	−26.23	1.76	3.02
27	phenyl acetaldehyde	0.19 - 12.50	0.835	247.09	1000.00	0.03	0.05
28	(*E*)-octen-2-al	0.55 - 35.42	0.957	34.17	−68.81	0.21	0.48
29	octan-1-ol	0.52 - 33.33	0.968	8.79	−3.68	0.41	0.82
30	nonan-2-one	0.54 - 8.65	0.996	115.15	−45.48	0.09	0.20
31	nonanal	0.53 - 34.17	0.937	22.39	−18.52	3.66	3.74
32	(*E*)-nonen-2-al	0.56 - 36.25	0.954	18.74	−34.81	0.40	0.67
33	ethyl octanoate	0.57 - 36.67	0.998	78.52	−2.42	0.01	0.02
24+25	sum of hepta-2,4-dienal isomers	0.61 - 39.17	0.982	33.38	−48.22	0.05	0.12

[a] A_t is the total area of compound calculated using eq 1 obtained in the multiple HS-SPME analysis.

Table 4. Results of the Analysis by Multiple HS-SPME of Two Dry Fermented Sausages Cured with Nitrate or Nitrite

number	compound	nitrate (ng/g dry sausage)[a]	nitrite (ng/g dry sausage)	*P* value
3	propanal	2228.1 ± 574.5	23880.7 ± 825.2	0.001
5	butanal	61.3 ± 20.1	720.7 ± 79.7	0.001
6	butan-2-one	155.6 ± 56.4	1642.5 ± 464.0	0.005
7	ethyl acetate	170.8 ± 87.2	1277.2 ± 241.2	0.002
8	3-methyl butanal	501.6 ± 87.3	833.5 ± 146.8	0.046
10	pentan-2-one	0.7 ± 0.2	8.3 ± 3.1	0.013
11	pentanal	962.3 ± 258.6	4791.5 ± 1681.3	0.018
12	pentane-2,3-dione	163.7 ± 70.0	652.9 ± 92.9	0.002
13	ethyl butanoate	11.5 ± 0.6	42.7 ± 8.5	0.003
14	hexanal	3895.4 ± 996.8	18578.4 ± 6755.0	0.027
17	(*E*)-hexen-2-al	37.8 ± 7.4	247.7 ± 63.9	0.009
18	heptan-2-one	21.9 ± 2.2	41.3 ± 3.3	0.001
19	heptanal	105.9 ± 26.1	420.2 ± 40.3	0.002
21	(*E*)-hepten-2-al	138.9 ± 23.0	671.8 ± 160.2	0.009
22	oct-1-en-3-ol	15.3 ± 12.4	1554.3 ± 488.6	0.009
23	octanal	183.9 ± 28.1	357.5 ± 91.0	0.034
27	phenyl acetaldehyde	37.7 ± 5.4	107.6 ± 36.6	0.116
28	(*E*)-octen-2-al	99.9 ± 14.5	220.4 ± 89.2	0.082
29	octan-1-ol	51.0 ± 6.5	284.4 ± 183.3	0.092
30	nonan-2-one	10.3 ± 0.6	21.8 ± 4.7	0.014
31	nonanal	76.2 ± 4.8	231.5 ± 41.9	0.003
32	(*E*)-nonen-2-al	52.3 ± 9.9	115.0 ± 40.9	0.071
33	ethyl octanoate	4.4 ± 0.7	31.0 ± 10.9	0.014
24+25	sum of hepta-2,4-dienal isomers	82.2 ± 13.5	505.3 ± 177.7	0.021

[a] Data are expressed as mean values and standard deviations, $n = 3$ samples.

In summary, the optimization of multiple HS-SPME for the quantitative analysis of volatile compounds in dry fermented sausages is necessary in order to obtain sensitive and reliable results. The high fat content of the solid food should be taken into account to prevent oxidation phenomenon during the sampling process. The optimized multiple HS-SPME is a technique successfully applied to quantitatively analyze the volatile compounds present in a solid food such as dry fermented sausage. Although, the matrix composition will be mainly responsible for the final flavor perception produced by these volatile compounds due to their effect on the partition coefficients between the matrix and the headspace. The results obtained demonstrate the suitability of multiple HS-SPME for volatile quantification in solid foods and its potential application to dry fermented sausages with acceptable sensitivity.

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