JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Quantitation of Selected Odor-Active Constituents in Dry Fermented Sausages Prepared with Different Curing Salts

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The odor-active compounds of dry-fermented sausages with added nitrite or nitrate as curing agents were identified by gas chromatography−olfactometry (GC-O) applying the detection frequency (DF) method. The quantification of these compounds in the sausage was determined by multiple headspace solid-phase microextraction (multiple HS-SPME). There were no specific odor-active compounds related to the use of nitrite or nitrate although there were differences in the DF value of several compounds. The nitrite-added sausages presented higher DF values for ethanol, 1-hexanol, propanoic acid, 2-heptenal, and nonanal while the nitrate-added sausages had higher DF values for phenylacetaldehyde and 3-methyl-butanal. Eighteen compounds were quantified by multiple HS-SPME. Most of them were above their air detection thresholds, but only hexanal, heptanal, and 1-octen-3-ol were in a concentration higher than their oil threshold values. These compounds would probably be the main contributors to the aroma of fermented sausages.

KEYWORDS: Cured flavor; detection frequency; olfactometry; multiple HS-SPME; nitrate; nitrite; dry fermented sausage

INTRODUCTION

Aroma is a very important characteristic for the overall quality of dry-fermented sausages. This aroma is completely different from that of thermally processed meats. Generally, the nature and amount of spices (pepper, garlic, and others) used in fermented sausages characterize the aroma (*1*). However, there are reactions and metabolic pathways that occur in the sausage meat matrix, which play an important role in the overall sausage aroma, as in the case of lipid oxidation and esterification reactions produced by microbial metabolism (*2*, *3*) and even amino acid catabolism and carbohydrate fermentation (*4*, *5*).

The effect of nitrite on the development of the characteristic cured flavor has been widely studied (*4*, *6*) although compounds responsible for this cured flavor have not yet been identified. Furthermore, while in Mediterranean countries nitrite and nitrate are used indistinctly as curing agents, in Northern European countries, only nitrite is used (*7*). In a previous work (*8*), a significant effect was reported of either nitrite or nitrate on the hydrolysis and oxidative processes of lipids, volatile compounds generation, and sensory characteristics of fermented sausages.

In the past few years, the use of solid-phase microextraction (SPME) (*9*) has permitted the isolation and identification of a high number of volatile compounds in fermented sausages (*10*) and allows one to monitor them throughout the process to study the effect of several factors on their generation (*8*). However, SPME is not an exhaustive extraction technique and the amount of extracted compound depends on the equilibrium partition coefficients between the sample and the headspace (HS) volume and also the HS and the fiber coating (*11*). As with many others HS techniques such as purge and trap, in SPME, the proportion of extracted compounds depends on the experimental conditions such as time, volume, fiber coating, temperature, etc. (*12*). Therefore, the quantitative profile obtained through these HS techniques cannot be compared with other works obtained under different conditions, as Stahnke (*2*) previously indicated.

Multiple headspace extraction (MHE) is a procedure originally developed for the quantification of volatile compounds in solid samples (*13*). 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 has the same aim as MHE, with the benefit of being a solvent-free technique, which provides more sensitivity and selectivity than direct HS sampling (*14*). 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 to the quantitative determination of volatile compounds in multilayer packaging (*14*, *15*) and red wine (*16*).

Little information is available on the main odorants responsible for the typical dry-cured aroma in fermented sausages. Gas chromatography (GC) analysis in combination with an olfactometry technique (GC-O) has been used to detect potent aroma-active compounds in fermented sausages (*1*−*3*). Only a

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10.1021/jf0631880 CCC: $37.00
Published on Web 03/24/2007

few studies have applied flavor dilution techniques to quantify the aroma intensity (*17*−*19*). However, there are no reports on other olfactometry techniques used on fermented sausages, despite the fact that it has been demonstrated that the detection frequency (DF) method (*20*, *21*) gives results that are directly related to the aroma intensity (*22*).

The odor-active compounds present in the HS of fermented sausages have never been quantified as their recovery depends on the extraction technique applied. In this sense, the aim of the present work was to determine the odor-active compounds that contribute to the overall aroma of fermented sausages manufactured with either nitrite or nitrate by the DF method and then to quantitatively determine these compounds by multiple HS-SPME.

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 (98%), 2,4-heptadienal (*E*,*E*) (90%), phenylacetaldehyde (90%), 2-octenal (*E*) (94%), nonanal (≈97%), 2-nonenal (*E*) (97%), diacetyl (97%), and 2-ethyl-furan (97%), which were obtained from Aldrich (St. Louis, MO).

Manufacture of Dry Fermented Sausages. Two batches of sausages, one containing sodium nitrite and another containing potassium nitrate, were manufactured and submitted to slow fermentation, as reported in Marco et al. (*8*). Dry-fermented sausages were made with lean pork (80%) and pork back fat (20%). The following additives were added in g/kg 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 meat was ground through a plate of 6 mm diameter holes, vacuum minced with the remaining ingredients, and inoculated with the starter culture SP-318 (Rhodia Iberia, group Rhône-Poulenc, Madrid, Spain) containing *Lactobacillus sakei*, *Pediococcus pentosaceus*, *Staphylococcus xylosus*, and *Staphylococcus carnosus*. The mixture was stuffed into collagen casings (Fibran, S.A., Girona, Spain, 75−80 mm diameter); the final weight of each sausage was 500 g. The sausages were kept in a refrigeration chamber at 3−5 °C for 27 h and then dried at 10 °C and 85−75% RH (relative humidity). The total drying time was 44 days. The sausages were then vacuum packed and stored at 4 °C for approximately 2 months, giving a total processing time of 111 days. At the end of the storage stage, three sausages chosen randomly from each batch were used to perform the analyses.

Extraction and Analysis of Volatile Compounds. Extraction of HS volatile compounds was performed using a SPME device (Supelco, Bellefonte, PA) with a 85 μm carboxen/polydimethylsiloxane StableFlex fiber (CAR/PDMS SF) using the extraction conditions previously optimized for use in fermented sausages by Marco et al. (*10*). For each experiment, 3 g of minced sausage was weighed into a 10 mL HS vial and sealed with a PTFE-faced silicone septum (Supelco). The vial was left for 1 h in a thermoblock (J. P. Selecta, Barcelona, Spain) at 30 °C for equilibration. The SPME fiber was then exposed to the HS for 3 h while maintaining the sample at 30 °C.

For the identification and confirmation of the volatile compounds, a GC HP 5890 series II (Hewlett-Packard, Palo Alto, CA) equipped with a HP 5972 mass selective detector (Hewlett-Packard) was used with the conditions described by Flores et al. (*23*). The compounds adsorbed by the fiber were desorbed in the injection port of the GC-mass spectrometry (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, 30 m, 0.25 mm i.d., film thickness 1.4 μm). The GC oven temperature program began at 38 °C and was 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 of 29−400 amu. The compounds were identified by comparison with mass spectra from the library database (Nist'98), Kovats retention index (*24*), and comparison with authentic standards.

GC-O. The compounds adsorbed by the fiber as described above were desorbed in the GC (GC 8000 Top, CE Instruments, Rodano, Milan, Italy) injection port for 6 min at 240 °C in splitless mode, and the split valve was opened after 1 min. The compounds were separated using a DB-624 capillary column (J&W Scientific, 60 m, 0.32 mm i.d., film thickness 1.8 μm). The capillary column was split (2:1) into deactivated and uncoated capillary tubing connected with the sniffing port and flame ionization detector (FID), respectively. Helium was used as the carrier gas with a linear velocity of 35.14 cm s^{-1}. The initial GC oven temperature was 38 °C and held for 13 min; the temperature was then increased to 100 °C at a rate of 3 °C min^{-1} and held for 10 min, then raised to 150 °C at a rate of 3 °C min^{-1}, raised to 210 °C at a rate of 5 °C min^{-1}, and held for 10 min. The FID detector temperature was set at 240 °C. The sniffing port was equipped with a humidified air make up.

The DF method was used to estimate the aromatic impact of each volatile compound (*20*, *21*) in the different batches. Six trained assessors evaluated the odors from the GC effluent by smelling and recording the retention time and odor descriptors of every perceived odor. A total of 18 assessments per batch were carried out, six individual sniffings for each of the three samples per batch. For each assessment, evaluation of the odor took place over two different time intervals (0−35 and 35−70 min) in order to avoid olfactory fatigue of the assessors. The detection of an odor in the sniffing port by less than three assessors was considered to be noise. The final DF value for each compound was obtained by summation of the 18 sniffings for each batch.

Compounds were identified using the following techniques: comparison with mass spectra and Kovats retention indices (*24*) obtained for the same samples in the GC-MS, comparison with the retention times of authentic standards injected in the GC-FID, and coincidence of the assessors descriptors with those in the Fenaroli's Handbook of Flavor Ingredients (*25*).

Determination of Odor-Active Volatile Compounds by Multiple HS-SPME. The principle of the MHE procedure was based on stepwise gas extractions at equal time intervals from a single sample (*13*). The result obtained by this method was an area value that corresponded to the total amount in the sample vial and independently from its distribution between both phases (*13*). The relation between the area and the corresponding amount of compound could be done either by external or by internal standard method. The external standard had to be prepared and analyzed under the same instrumental conditions; in this case, the vial had to contain the volatile compound only and not the sample matrix. Because no sample matrix had to be present, the vial contained the gas phase only, and consequently, the volatiles were analyzed as a homogeneous gas mixture in the vial (*13*). In this analysis, a few microliters of the volatile compounds were injected into the empty vial as a solution in a volatile solvent. In this system, to compensate in the calibration vial the missing sample volume, the addition of a similar volume of an inert material such as glass beads was recommended (*13*).

Multiple HS-SPME was based on the same principle except for the use of SPME for volatile compound extraction (*14*). In this way, after successive extractions, the concentration of analyte decayed exponentially and the total peak area (A_t) corresponding to an exhaustive extraction of the analyte was calculated from the following (*14*):

$$At = \frac{A_1}{1 - e^k} \quad (1)$$

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 vs the number of extractions minus one.

To calculate the initial mass of the analyte in the sample, a calibration by external standard method was performed. An external standard was prepared and analyzed under the same instrumental conditions.

Finally, the initial mass of the analyte in the sample (m_0) was calculated as follows:

$$m_0 = \frac{A_t \cdot m_s}{A_{ts}} \quad (2)$$

where A_t and A_{ts} are the total area of compound in the sample and in the external standard, respectively, calculated using eq 1. m_s is the initial mass of the external standard added. All of the details, as well as the conditions that must be fulfilled to perform multiple HS-SPME, have been reviewed by Ezquerro et al. (*14*).

For the extraction procedure, a sausage slice was diced and frozen in liquid nitrogen for mincing, so that the sausage could be finely pulverized. One gram of the minced sausage was weighed in a 30 mL screw top glass vial with 15 mL of double-distilled water added together with 15 μL of a 5% solution (w/v) of butylated hydroxytoluene (BHT) in methanol to avoid lipid oxidation. The sample was then homogenized for 1 min in 20 s pulses, using a Polytron PT 1200 (Kinematica AG, Switzerland). One milliliter of the homogenate was placed in a 10 mL HS vial, sealed with a PTFE-faced silicone septum (Supelco) with 0.5 g of NaCl, and equilibrated at 30 °C for 1 h. The sample was then extracted four consecutive times by exposing the SPME fiber CAR/PDMS SF to the HS for 1 h at 30 °C for each extraction. This procedure was done per triplicate on each batch.

After each extraction, the fiber was desorbed in the GC injection port of the GC-MS using the same conditions described above. Data were acquired in selected ion monitoring (SIM) mode using a specific ion for each of the quantified compounds (see ions in **Table 1**).

For the external standard method used for quantification, stock standard solutions of pure compounds were prepared in methanol. A mixture of these stock solutions was prepared in methanol, then the mixture was diluted several times, and the several dilutions were analyzed by multiple HS-SPME using the same procedure applied to the sample. The concentration of the external standard shown in **Table 1** was the one with the highest correlation coefficient in the exponential decay. The concentration of the external standard in **Table 1** and the slope obtained in the exponential decay were used for the quantification of the compound in the samples using eq 2. The multiple HS-SPME procedure was applied using similar conditions as for the sausage homogenates. In this case, 5 μL of the standard solution was 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 (*13*). The same extraction procedure used in the sample homogenate was also applied to the standard calibration vial.

Statistical Analysis. The effect of the different curing agents in the quantified odor-active compounds was tested by analysis of variance using the statistic software Statgraphics plus (version 5.1).

RESULTS

GC-O Analysis. Ninety-seven volatile compounds were detected by SPME-GC-MS. From all of these compounds, it is not clear which of them actually contribute to the aroma of dry-fermented sausages and are present in quantities above their detection thresholds.

Among the 97 compounds detected, 55 different aroma-active zones were described in the HS of the dry-fermented sausages (see **Table 2**). Of them, 26 compounds were identified by matching mass spectra, Kovats indices of an authentic standard, and odor descriptions from bibliographies; another 18 were identified by mass spectra and Kovats indices of an authentic standard; only one compound was tentatively identified by mass spectrum; and finally, 10 compounds were not identified. Of the identified odor-active compounds, 15 were aldehydes, eight were alcohols, seven were organic acids, seven were ketones, six were esters, and two were furans. All of these compounds had already been detected in the HS of dry-fermented sausages (*8*, *10*).

From these 55 odor-active compounds, only 13 presented DF values higher than 17 in at least one of the batches (see **Figure 1**). Five of these were most likely derived from lipid oxidation: pentanal, hexanal, 2-pentyl-furan, octanal, and 2-nonenal, which contributed with herbal type aromatic notes (**Table 2**); one compound was derived from lipid β-oxidation, 1-octen-3-ol (mushroom odor); two compounds were derived from carbohydrate fermentation, acetic acid (vinegar odor) and butanoic acid (cheese odor); two compounds were derived from amino acid catabolism, methanethiol (rotten eggs odor) and 3-methylbutanoic acid (cheese and feet odor); and three were esters, ethyl butanoate, ethyl 2-methylpropanoate, and ethyl pentanoate, which were always described as fruity, mainly strawberry notes (**Table 2**).

GC-O showed other aroma-active zones (17 compounds) that also contributed to the aroma of fermented sausages with DF values around 10 (**Figure 1**). This means that the assessors detected these compounds at least half of the times that they appeared. Five of these compounds came from lipid autoxidation: propanoic acid, 1-hexanol, heptanal, 2,4-heptadienal, and octanoic acid; two came from lipid β-oxidation, 2,3-pentanedione and 2-nonanone; one came from carbohydrate fermentation, ethanol; two came from amino acid catabolism, methional and phenylacetaldehyde; two were esters, ethyl 2-methylbutanoate and ethyl hexanoate; limonene was a contaminant from foodstuff; and finally, there were four unidentified compounds (two of which had a nutty odor).

It is worth pointing out that the assessors identified the presence of nine odor-active compounds as meat-related flavors (**Table 2**). In order of elution, the volatile compounds responsible for these aromas were 3-methylbutanal (rancid, dry-cured ham), 1-pentanol (roasted, roasted meat), 2-hexenal (salty meat, dry-cured ham), heptanal (citrus, soap, rancid cured ham), 2-heptanol (plastic, pork scratchings), methional (brothy, rancid),

Table 1. Ions Used for Quantification in the SIM Method and Concentration of the Standard Solutions Prepared for the External Standard Calibration

KI[b]	compound	quantification ion (*m*/*z*)	standard concn (μg/μL)
466	acetone	43	*a*
473	methanethiol	47	
603	butanal	72	
635	ethyl acetate	43	0.078
697	acetic acid	60	
727	2-pentanone	43	0.772
735	pentanal	58	0.075
730	2,3-pentanedione	57	1.000
824	1-pentanol	55	
829	hexanal	56	1.608
854	2-methyl-propanoic	43	
878	butanoic acid	60	
902	2-hexenal (*E*)	41	0.006
920	1-hexanol	56	1.600
936	2-heptanona	43	0.006
941	heptanal	70	0.018
970	methional	48	
1016	2-heptenal (*E*)	41	0.007
1025	1-octen-3-ol	57	1.640
1035	octanal	41	0.007
1057	2,4-heptadienal	81	0.007
1073	2,4-heptadienal (*E*,*E*)	81	0.007
1114	phenylacetaldehyde	91	0.008
1116	2-octenal (*E*)	41	0.033
1128	1-octanol	41	
1140	2-nonanone	58	0.006
1151	nonanal	98	0.013
1199	phenylethyl alcohol	91	
1226	2-nonenal (*E*)	41	0.006
1289	octanoic acid	60	

[a] Standard solution not prepared as this compound did not follow an exponential decay in the sausage sample. [b] Kovats index of the compounds injected in the GC-MS using a DB-624 capillary column (J&W Scientific; 30 m, 0.25 mm i.d. and film thickness 1.4 μm).

Table 2. Odor-Active Compounds Identified in the HS of Slow-Fermented Sausages with Different Curing Salts and Quantified by Multiple HS-SPME

						threshold range (mg/kg)[e]			quantity (mg/kg)[g]		
N[a]	GC-O descriptor	KI_{GC-O}[b]	compound	KI_{std}[c]	R[d]	air	oils	ref[f]	nitrite	nitrate	p[h]
1	acetone, alcohol	466	acetone	470	a	1–630	100–1768				
2	rotten eggs, cauliflower	473	methanethiol	473	a	0.0002–0.04					
3	bread dough, yeast	523	ethanol	520	b	0.64–640	50–500				
4	sweet, snacks	603	butanal	602	b	0.00084–8.8	0.15				
5	cheese, snacks	633	diacetyl (2,3-butanedione)	633	b	0.005–0.02	0.0045–0.01	*2*, *3*, *18*, *19*			
6	fruity, toffees	636	ethyl acetate	634	a	0.34–623	10–100	*18*, *19*	1.30 ± 0.10	0.17 ± 0.03	0.0001
7	rancid, dry cured ham	694	3-methylbutanal	691	b	0.002–0.004	0.013–13	*1–3*			
8	vinegar	707	acetic acid	707	a	0.025–25	0.124–0.75	*3*, *18*, *19*			
9	toasted, garlic	718	2-ethylfuran	719	b		8				
10	roasted, sweet	730	2-pentanone	730	b	6.7–30			1.00 ± 0.20	0.98 ± 0.04	ns
11	fresh cut grass, rancid	737	pentanal	737	a	0.12–17.5		*2*	4.4 ± 0.6	2.1 ± 0.3	ns
12	butter, cheese	743	2,3-pentanedione	740	a	0.01–0.06		*2*, *3*	17.0 ± 7.0	9.6 ± 0.3	ns
13	strawberry	789	ethyl 2-methyl-propanoate	785	a	0.0003–0.038	0.0012	*2*, *3*, *17*			
14	cheese	820	propanoic acid	818	b	0.003–3	3.84	*3*, *18*, *19*			
15	roasted, roasted meat	824	1-pentanol	820	b	0.02–5	0.85				
16	fresh cut grass, rancid	830	hexanal	830	a	0.02–0.33	0.032–0.2	*1–3*, *17–19*	9.5 ± 1.0	9.5 ± 0.3	ns
17	strawberry	841	ethyl butanoate	841	a	0.0027–0.2	0.028	*1–3*, *18*, *19*			
18	fatty, savory snacks	854	2-methylpropanoic acid	853	a	0.005–0.24	0.755	*3*, *18*			
19	strawberry	875	ethyl 2-methylbutanoate	876	a	0.00006–0.01	0.00026	*2*, *3*, *17*, *18*			
20	cheese	879	butanoic acid	881	a	0.0004–0.04	0.109–0.205	*2*, *3*, *18*, *19*			
21	salty meat, dry cured ham	902	2-hexenal (*E*)	903	b	0.05–1.8	0.85	*3*	0.08 ± 0.02	0.09 ± 0.01	ns
22	green grass, plastic	920	1-hexanol	918	a	0.04–4	0.4		0.14 ± 0.01	0.12 ± 0.02	ns
23	strawberry	927	ethyl pentanoate	924	a	0.0003–0.33		*2*, *3*			
24	cheese, feet, dirty socks	929	3-methylbutanoic acid	928	a	0.00022–0.014	0.022–0.066	*2*, *3*, *18*, *19*			
25	medicinal, fruity	935	2-heptanone	931	a	0.045–3.3	1.5–392	*3*	0.055 ± 0.009	0.046 ± 0.002	ns
26	citrus, soap, rancid cured ham	941	heptanal	940	b	0.06–0.26	0.25	*1–3*	0.6 ± 0.2	1.0 ± 0.3	ns
27	plastic, pork scratchings	944	2-heptanol	944	b	0.1					
28	roasted nuts, fried snacks	966	unknown 1								
29	meat broth, rancid, savory snack	970	methional (3-methyl-thio-propanal)	970	a	0.00006–0.06	0.0002–0.2	*17*			
30	rotten, strawberry, sweet	994	unknown 2								
31	onions, savory, rancid	1011	2-pentylfuran		c	0.27	2				
32	rancid, dirty	1016	2-heptenal (*E*)	1014	b	0.034–2.8	1.5		0.8 ± 0.1	1.3 ± 0.4	ns
33	mushroom	1025	1-octen-3-ol	1028	a	0.012–0.11	0.034–0.9	*1*	0.63 ± 0.04	0.75 ± 0.07	ns
34	sweet, fruity, cherry	1031	ethyl hexanoate	1029	a	0.003–0.09	0.04	*3*, *17*			
35	geranium, herbal, floral	1036	octanal	1037	a	0.005–0.02		*2*, *3*, *18*	0.6 ± 0.2	1.3 ± 0.1	0.0203
36	citrus, orange	1051	limonene	1048	a			*1*, *18*, *19*			
37	roasted, butter, soap	1057	2,4-heptadienal	1060	a						
38	cooked meat, nutty	1081	2,4-heptadienal (*E*,*E*)	1079	a	0.057	10				
			sum of 2,4-heptadienal isomers		a				0.11 ± 0.04	0.25 ± 0.06	0.0259
39	roses	1114	phenylacetaldehyde	1112	b	0.0006–0.002	22		0.16 ± 0.04	0.43 ± 0.06	0.0008
40	dry cured ham, dry cured sausage	1116	2-octenal (*E*)	1116	b	0.009–0.25	7	*3*, *17*	0.35 ± 0.06	0.40 ± 0.10	ns
41	rancid nuts, woody	1121	unknown 3								
42	mushroom	1128	1-octanol	1126	a	0.05–0.7		*1*			
43	roasted, burnt	1140	2-nonanone	1140	b	0.075–5.5		*2*, *18*, *19*	0.10 ± 0.01	0.02 ± 0.01	0.0000
44	plastic, soap	1151	nonanal	1151	b	0.005–0.23	1	*2*, *3*, *17*	0.8 ± 0.4	1.2 ± 0.3	ns
45	waxy, smoke	1152	unknown 4								
46	green, fresh	1156	unknown 5								
47	rotten cheese, rotten orange	1177	unknown 6								
48	rancid, dry cured ham	1178	heptanoic acid	1177	a	0.022–2.6					
49	roasted nuts, fried snacks	1180	unknown 7								
50	burnt plastic, stable	1197	unknown 8								
51	winery	1199	phenylethyl alcohol	1197	b	0.005–0.16		*18*			
52	plastic, salty, rancid	1207	unknown 9								
53	herbal, crushed leaves	1223	unknown 10								
54	cucumbers, herbal, woody	1226	2-nonenal (*E*)	1224	a	0.0001–0.025	0.9	*2*, *3*	0.07 ± 0.02	0.35 ± 0.08	0.0074
55	rancid, woody	1289	octanoic acid	1283	b	0.00006–0.5	161				

[a] Number of the aroma-active zones in order of chromatographic elution. [b] Kovats index of the aroma-active zones eluted from the GC-FID using a DB-624 capillary column (J&W Scientific; 60 m, 0.32 mm id., and 1.8 μm film thickness). [c] Kovats index of the authentic standards injected in the GC-FID with the same column as above. [d] R, reliability of identification; a, identification by mass spectrum, coincidence with the KI of an authentic standard and by coincidence with odor description (according to cited bibliography or reference (*25*); b, identification by mass spectrum and by coincidence with the KI of an authentic standard; and c, tentatively identified by mass spectrum. [e] Threshold values in air and vegetable oils, as according to ref *31*. [f] Cited references. [g] All of the results are given as the mean of three samples with the standard deviation. [h] *p* value of analysis of variance.

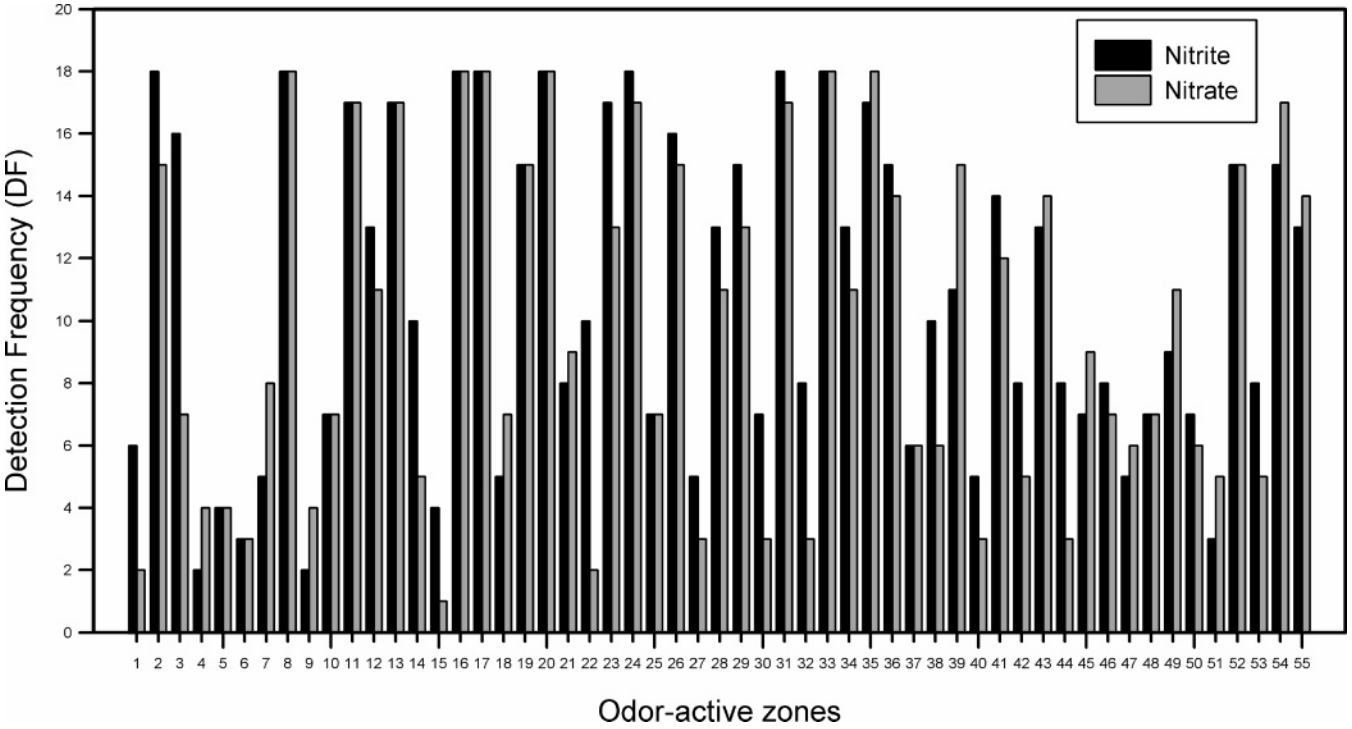

Figure 1. Aromagram from the HS of slow-fermented sausages processed using nitrate or nitrite. The DF values were obtained by six assessors and three sausages per batch. The numbers on the *x*-axis correspond to the aroma descriptors indicated in **Table 2**.

2,4-heptadienal (*E*,*E*) (cooked meat, nutty), 2-octenal (cured, dry ham, dry sausage), and heptanoic acid (rancid, dry-cured ham).

The use of the DF method did not show any difference in the odor-active compound profile between nitrite- or nitrate-added batches but only differences in the ratio of some of them. Thirteen compounds presented higher DF values in nitrite-added sausages. Among them, five presented differences higher than five DF units: ethanol, 1-hexanol, propanoic acid, 2-heptenal, and nonanal. Another eight showed differences of three or four DF units: acetone, methanethiol, ethyl pentanoate, 1-pentanol, 2,4-heptadienal (*E*,*E*), 1-octanol, and unknown 10. On the other hand, in the nitrate-added sausages, phenylacetaldehyde and 3-methylbutanal were four and three DF units higher, respectively.

Multiple HS-SPME. After the identification of the odor-active compounds in the HS of fermented sausages, multiple HS-SPME was applied to quantify them. To perform this technique, it is necessary to start with very small amounts of sample, so that the exponential decay of the peak area can be seen from the beginning of the experience (*14*). Dry-fermented sausages present a very heterogeneous matrix in contrast to wine or packaging. Therefore, weighing a very small amount of minced sausage would not give a representative sample. To avoid this, a minimum sample weight of 1 g was chosen and different dilutions in water were tried. A dilution of 1 g of minced sausage with 15 mL of water was chosen because it presented the maximum number of compounds with exponential area decay. To this dilution, NaCl in saturating amounts was added to promote the salting-out of the compounds to the HS. Also, BHT was added to avoid lipid oxidation phenomena, as from previous experiences during the optimization (data not shown), it was observed that the amount of aldehydes increased during successive extractions.

Area quantification was done using the SIM method to increase the sensitivity, as the yield of volatile compounds extracted was very low due to the low quantity of sausage used in the extraction and to the fact that the SPME fiber was exposed for only 1 h. Therefore, many of the volatile compounds found in the HS of sausages in the GC-O by extraction with the SPME for 3 h were not detected by multiple HS-SPME. Thus, from the 55 odor-active compounds detected by GC-O, only 30 of them were detected in the multiple HS-SPME as indicated in **Table 1**.

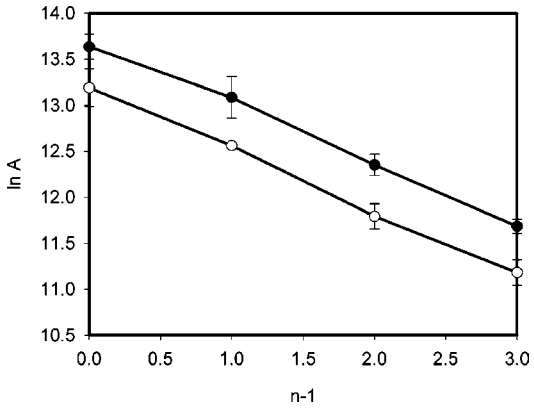

Figure 2. Exponential decay of ethyl acetate area (*A*) in the successive extractions (*n*) of slow-fermented sausages. Nitrite added sausage, ●; nitrate added sausage, ○.

Only 18 odor-active compounds presented exponential area decay when multiple HS-SPME was applied. Because an exponential decay of the peak area with the number of extractions is necessary to quantify the analytes (see **Figure 2**), the linearity of the plots for each odor compound was studied for sausage batches and standards. **Table 3** shows the slopes and correlation coefficients (R^2) for each compound with at least a 99% confidence level in the sausages and standards. The other compounds not shown in **Table 3** are those for which the area increased during extraction or the correlation coefficients were low; therefore, they cannot be quantified by multiple HS-SPME under the optimized conditions.

Table 3. Correlation Coefficient of the Exponential Decay of the Peak Areas Obtained by Multiple HS-SPME from Slow-Fermented Sausages with Different Curing Salts and Standard Solutions

		nitrite		nitrate		standard	
N[a]	compound	slope	R^2	slope	R^2	slope	R^2
6	ethyl acetate	–0.6586	0.9944	–0.6794	0.9935	–0.1226	0.9126
10	2-pentanone	–0.2197	0.9249	–0.2080	0.9170	–0.1011	0.9545
11	pentanal	–0.1463	0.9637	–0.2236	0.9784	–0.0535	0.8675
12	2,3-pentanedione	–0.0959	0.9104	–0.1429	0.9676	–0.2254	0.9264
16	hexanal	–0.3154	0.9105	–0.3553	0.9714	–0.6609	0.9418
21	2-hexenal	–0.3327	0.8851	–0.2850	0.9087	–0.5040	0.9706
22	1-hexanol	–0.3522	0.9848	–0.3229	0.9957	–1.1966	0.8424
25	2-heptanone	–0.3900	0.8870	–0.4589	0.9564	–0.2439	0.7978
26	heptanal	–0.2610	0.9230	–0.2012	0.8364	–0.1958	0.7366
32	2-heptenal	–0.2682	0.9025	–0.1683	0.7293	–0.6816	0.9067
33	1-octen-3-ol	–0.2607	0.9260	–0.2894	0.9802	–2.3988	0.8646
35	octanal	–0.2709	0.9386	–0.1910	0.8356	–0.2588	0.7674
37 + 38	sum of 2,4-heptadienal isomers	–0.1967	0.9541	–0.1080	0.9479	–1.9211	0.9862
39	phenylacetaldehyde	–0.1554	0.9855	–0.1359	0.9779	–0.2438	0.6890
40	2-octenal	–0.2495	0.9443	–0.1676	0.9178	–0.7251	0.9039
43	2-nonanone	–0.3584	0.9077	–0.3343	0.9124	–0.6573	0.9345
44	nonanal	–0.2267	0.9690	–0.1727	0.8573	–0.3639	0.7730
54	2-nonenal	–0.2590	0.9021	–0.0385	0.7919	–1.0813	0.9698

[a] Number of compounds in order of chromatographic elution that correspond to the numbers indicated in **Table 2**.

The proposed multiple HS-SPME method was applied to the nitrite- and nitrate-added sausages per triplicate, and the quantification of the 18 odor compounds is shown in **Table 2**. Only a few of them presented significant differences between batches. The content of ethyl acetate and 2-nonanone was higher in the nitrite-added batch, while octanal, 2,4-heptadienal isomers, phenylacetaldeyde, and 2-nonenal were higher in the nitrate-added sausage.

With regards to the detection threshold of the 18 odor-active compounds quantified, most of them were above their air detection threshold; therefore, they could contribute significantly to the aroma of fermented sausages. Other compounds like 2-pentanone, ethyl acetate, and 2-heptanone showed concentrations below or close to their air threshold values; therefore, their contribution to the overall aroma would be lower. Taken into account that dry-fermented sausages contain a high percentage of fat, the oil thresholds were also considered; however, only a few of them were available. In this case, only hexanal, heptanal, and 1-octen-3-ol showed a quantified concentration higher than the oil thresholds. Probably these compounds would be the main contributors to the aroma of fermented sausages.

DISCUSSION

Around 300 different volatile compounds have been isolated from the HS of dry-fermented sausages using different techniques (*26*−*28*). In this work, 97 volatile compounds were detected although only 55 were detected as odor-active compounds by the DF method. As previously stated, the aroma of fermented sausage has been evaluated by GC-O using aroma extract dilution analysis (*17*−*19*). The most potent odorants were hexanal, methional, and 2-acetyl-pyrroline (*17*). On the other hand, without taking into consideration the contribution of aroma compounds derived from spices, the most potent odorants were 3-methyl-butanoic acid, ethyl butanoate, propyl 3-methyl butanoate, and acetic acid (*18, 19*). However, because of the different regional processing conditions in Spanish-fermented sausages, the main potent odorants were 2,3-butanedione, 3-methyl-butanoic acid, ethyl propanoate, 2-phenylethanol, and acetic acid (*18*). In accordance with our results, several compounds such as hexanal, 3-methyl-butanoic acid, and acetic acid have generally been reported as potent odorants in fermented sausages, and probably, they are essential contributors to the overall dry cured aroma, although the contribution of other esters, aldehydes, and acid compounds should be considered.

Generally, an essential role in the dry fermented sausages flavor has been attributed to the branched chain aldehydes (*29*). In contrast, GC-O studies show that other compounds make a greater contribution to the cured character of dry-fermented sausages (*17*−*19*), as was also seen in the present study.

In a previous work, Marco et al. (*8*) observed that the addition of nitrate or nitrite to slow-fermented sausages produced a significant preference for the aroma of the nitrate-added sausages when compared with the nitrite-added sausages. The DF method was applied to both sausage batches in order to determine the possible differences in aroma-active compounds between them (**Figure 1**). However, all 55 odor-active compounds were detected simultaneously in both sausage batches. That is, no specific compound was responsible for the differences in aroma due to the curing agent, as was also indicated by Stahnke (*3*).

The results obtained from the multiple HS-SPME are not completely in accordance with those obtained by the DF method; only the higher phenylacetaldehyde concentration in the nitrate-added batch was detected by both DF and multiple HS-SPME methods. To relate the concentrations determined by multiple HS-SPME with the DF values, the threshold of the compounds should be taken into consideration. However, dry-fermented sausage is a complex matrix with a high content in proteins and fat; therefore, not only the air thresholds but also the oils thresholds that are more similar to the matrix should be taken into account. The presence of oil results in a significant increase of the threshold value as seen in **Table 2**. In general, those compounds with low thresholds and present at concentrations above their oil threshold should be frequently detected as in the case of 1-octen-3-ol, hexanal, and heptanal. By contrast, odor compounds present at high concentrations but with high detection threshold or compounds that have low DF but are present in low quantities would not be able to give an aromatic sensation, such as ethyl acetate and 2-pentanone (**Figure 1**).

It should be taken into account that using multiple HS-SPME, the matrix effect is avoided (*13, 14*); therefore, the concentration detected by this method will be present not only in the HS but

also in the solid matrix sample. In contrast, during the olfactometry analysis, the volatile compounds were extracted from the HS of the sausage and the profile and proportion of these compounds depend on many factors, among them, the partition coefficients between the matrix sample and the HS. Therefore, the comparison between these methods, SPME-GC-O, and multiple HS-SPME cannot be done directly. Furthermore, the results obtained by applying the multiple HS-SPME give new data about the concentration of the odor-active compounds present in a slow-fermented sausage avoiding the matrix effect.

In a previous work (*8*), the effect of nitrite and nitrate on the sample matrix was reported. In this work, a higher concentration of triglycerides was found in the samples with added nitrate. Taking into consideration that triglycerides constitute 92% of the total lipid concentration (*8*) and that the volatile compounds have a higher solubility in fat (*30*), the lower concentration of triglycerides in the sample with added nitrite would favor the release of volatile compounds to the HS. Therefore, a higher proportion of triglycerides in the nitrate-added sausages can dissolve a higher proportion of volatile compounds. This would be the reason for finding a higher volatile compound concentration by multiple HS-SPME in some volatile compounds in the nitrate-added sausages.

In conclusion, this study has shown that the qualitative aroma profile of nitrite- and nitrate-fermented sausages is very similar. The fermented sausage aroma is very complex, and 13 compounds were the main contributors as seen by their high DF values. However, only some of these compounds were quantified by multiple HS-SPME and their concentrations are not comparable with their DF values. This is due to the matrix (lipid−protein) effect, which affects the proportion of these compounds in the HS. Therefore, the concentration of volatile compounds obtained by multiple HS-SPME may not be sufficient to determine the sensory acceptance of dry-fermented sausages.

ABBREVIATIONS USED

BHT, butylated hydroxytoluene; CAR/PDMS SF, carboxen/polydimethylxylosane StableFlex; DF, detection frequency; GC, gas chromatograph; GC-O, gas chromatrography−olfactometry; HS, headspace; MS, mass spectrometry; MHE, multiple headspace extraction; SPME, solid-phase microextraction.

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Received for review November 6, 2006. Revised manuscript received February 12, 2007. Accepted February 17, 2007. Financial support from Grants AGL2005-00713 (MEC, Spain) and GV05/067 (Generalitat Valenciana, Spain) are fully acknowledged. The scholarship (I3P program) from CSIC/MEC to A.M. is also acknowledged.

JF0631880