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COMPARISON OF HEADSPACE SOLID-PHASE MICROEXTRACTION AND NITROGEN PURGE AND STEAM DISTILLATION FOR DETERMINATION OF TERPENES AND OTHER HAM VOLATILE ORGANIC COMPOUNDS

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Distinctive and unique ham flavor is developed through the long ripening period under specific temperature, relative humidity, and air-flow [1]. During ham maturation, various chemical processes occur, including water loss, increase in dry matter and salt concentration, complex enzymatic and non-enzymatic reactions such as lipolysis and proteolysis, Maillard reactions, and Strecker degradations [1, 2]. These changes form non-volatiles, mainly free amino acids and small peptides, as well as volatile organic compounds (VOCs) such as aldehydes, carboxylic acids, alcohols, ketones, esters, sulfur and nitrogen compounds, terpenes, alkanes and alkenes, and aromatic and cyclic hydrocarbons [1, 3]. Smoke-cured ham also contains low-molecular-weight phenol derivatives (lignin monomer derivatives) derived from the smoke or smoke flavourings produced during lignin pyrolysis [4]. However, only a limited number of volatiles contribute to dry-cured ham aroma, depending on the sensory threshold [5]. In spite of the negative effect of lipid oxidation, the typical aroma of dry-cured ham is related to the initiation of lipid oxidation and the subsequent generation of volatiles [6]. Although the higher molecular-weight fat-derived compounds have a high perception threshold, they play a small but direct role in total perceived flavor. Different extraction techniques can be used for VOC isolation, such as hydrodistillation (HD), steam distillation (SD), simultaneous distillation-extraction (SDE), solid-phase microextraction (SPME), purge and trap methods, static and dynamic headspace methods, and solvent, and supercritical CO_2 extraction. However, isolation of flavor-important VOCs from the lipid matrix is demanding [7].

Nitrogen purge and steam distillation (NPSD), and headspace solid-phase microextraction (HS-SPME) followed by the analysis by gas chromatography and mass spectrometry (GC; GC-MS) were compared in this research for qualitative determination of Istrian dry cured ham VOCs for the first time. HS-SPME and NPSD techniques showed satisfactory and complementary results in research on ham VOCs. Hexanal and 2- and 3-methylbutanal, commonly found in European dry-cured hams [8], were not found in this research by NPSD, but only by HS-SPME, probably due to their low boiling points [8, 9]. These compounds, purged by the nitrogen, could have evaporated and escaped from the traps during the 6 h long extraction period while the sample was heated at 102°C. Only one sulfur compound (methional) was found by NPSD, although sulfur compounds can be formed by Maillard reactions [2] and additionally, in the salting phase of Istrian dry-cured ham garlic is used, which can also serve as the source of sulfur compounds [10]. The nitrogen compounds found by NPSD could be associated not only with Maillard reactions during dry curing but also with heating of the ham during the extraction [2, 11]. The applied PDMS/DVB fibre coating showed higher susceptibility towards terpenes, found in wide ranges and percentages in comparison to NPSD. Nitrogen compounds, esters, and furans were not isolated by HS-SPME.

Volatiles Isolated by HS-SPME. A total of 54 volatile compounds were identified by HS-SPME. The identified chemical groups (Table 1) are in agreement with those reported in European dry-cured hams extracted by SPME [12, 13]. The PDMS/DVB fibre is recommended for nonpolar compounds, semivolatiles, and volatiles with lower molecular weights [12] and exhibited good results in extracting the headspace volatiles of the ham, especially terpenes.

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TABLE 1. Headspace Volatiles of Istrian Dry-Cured Ham Isolated by HS-SPME with PDMS/DVB Fiber Coating

Compound	RI	Min.	Max.	Av.	SD.	Compound	RI	Min.	Max.	Av.	SD.
Terpenes						**Carboxylic acids**					
α-Thujene	932	0.5	0.8	0.57	0.21	3-Methylbutanoic acid	<900	0.1	0.6	0.37	0.25
α-Pinene	939	1.1	1.2	1.10	0.10	2-Methylbutanoic acid	<900	0.0	0.1	0.03	0.15
Sabinene	978	2.6	5.4	4.00	1.40	Hexadecanoic acid	1963	0.8	3.3	2.03	1.25
β-Pinene	982	0.0	1.8	0.93	0.90	(*Z*)-Octadec-9-en-oic acid	2147	5.0	6.7	5.23	1.36
β-Myrcene	993	0.1	1.7	0.93	0.85	**Alcohols**					
α-Phellandrene	1008	1.0	1.3	1.10	0.17	Oct-1-en-3-ol	981	1.2	5.2	3.47	2.05
δ-3-Carene	1015	4.5	4.8	4.47	0.35	Benzyl alcohol	1037	0.0	0.8	0.43	0.40
p-Cymene	1029	1.0	1.1	1.03	0.06	Undecan-1-ol	1164	0.0	0.4	0.23	0.21
Limonene	1033	2.7	19.3	13.33	9.23	Heptadecan-1-ol	2023	0.4	1.0	0.53	0.42
γ-Terpinene	1063	0.0	0.6	0.30	0.30	Octadecan-1-ol	2087	1.0	1.5	1.17	0.29
trans-Sabinene hydrate	1072	0.9	1.3	1.07	0.21	**Aliphatic hydrocarbons**					
Linalool	1102	2.8	3.4	3.07	0.31	Heptane	<900	0.0	0.6	0.30	0.30
Terpinen-4-ol	1181	1.2	1.7	1.47	0.25	Dodecane	1200	0.8	1.0	0.93	0.12
β-Fenchyl alcohol	1193	0.0	0.7	0.33	0.35	Tridecane	1300	0.0	0.5	0.17	0.29
α-Cubebene	1378	0.0	1.5	0.77	0.75	Tetradecane	1400	0.2	0.6	0.40	0.20
trans-Caryophyllene	1421	3.9	10.5	7.13	3.30	**Aromatic hydrocarbons**					
α-Humulene	1456	0.0	0.8	0.50	0.44	Ethenylbenzene	<900	0.4	0.9	0.60	0.26
β-Selinene	1487	0.0	1.6	0.77	0.80	1,3-Dimethylbenzene**	<900	0.0	0.5	0.17	0.29
α-Selinene*	1495	0.0	1.0	0.57	0.51	1,2-Dimethoxybenzene**	1150	0.0	0.7	0.40	0.36
β-Bisabolene	1511	0.0	1.2	0.67	0.61	Naphthalene	1185	0.0	0.6	0.30	0.30
Elemol	1552	0.0	0.2	0.10	0.10	3,4-Dimethoxytoluene	1244	0.0	0.5	0.17	0.29
Farnesene*	1553	0.0	0.8	0.30	0.44	**Phenols**					
Crayophyllene oxide	1584	0.0	0.8	0.43	0.40	2-Methylphenol	1059	0.0	0.4	0.23	0.21
Carbonyl compounds						3-Methylphenol	1080	0.0	0.7	0.40	0.36
3-Methylbutanal	<900	0.6	0.8	0.70	0.10						
2-Methylbutanal	<900	0.3	0.6	0.47	0.15						
Hexanal	<900	2.9	9.5	6.13	3.30						
Heptanal	903	0.5	0.8	0.57	0.21						
Benzaldehyde	965	0.4	1.1	0.83	0.38						
2-Methyloctan-3-one	985	0.0	1.1	0.70	0.61						
Octanal	1004	0.8	1.8	1.20	0.53						
Phenylacetaldehyde	1048	0.9	1.1	1.00	0.10						
2,3,4-Trimethyl-cyclopent-2-en-1-one	1092	0.0	0.7	0.40	0.36						
Nonanal	1106	2.7	4.2	3.30	0.79						
Hexadecanal	1819	1.0	1.4	1.13	0.23						

Min. – minimal percentage; max. – maximal percentage, av. – average percentage; SD. – standard deviation; *tentatively identified; RI – retention indices on HP-5MS column; **correct isomer not identified.

Based on the relative percentages, the major terpenes were limonene (2.7–19.3%), followed by *trans*-caryophyllene (3.9–10.5%), δ-3-carene (4.5–4.8%), sabinene (2.6–5.4%), and linalool (2.8–3.4%). These compounds mostly originated from added spices like black pepper, garlic, rosemary, and laurel. Aldehydes play an important part in the aroma of dry-cured ham with low perception threshold [11]. Among aldehydes, hexanal (associated with off-flavor, usually found in European hams [8]) was found by this method with the highest percentages (2.9–9.5%), followed by nonanal (2.7–4.2%), octanal (0.8–1.8%), tetradecanal (1.0–1.4%), benzaldehyde (0.4–1.1%), and phenylacetaldehyde (0.9–1.1%). Among the branched lower aldehydes, 2-methylbutanal and 3-methylbutanal were found (0.6–0.8% and 0.3–0.6% respectively). They are known to be odor active compounds in Europeans hams [8, 14] and are predominant in Iberian ham and aged Italian hams [5] in increasing percentages during aging. Dominant carboxylic acids extracted by this method were (*Z*)-octadec-9-enoic acid (5.0–6.7%) and hexadecanoic acid (0.8–3.3%). The free fatty acids accumulate during dry curing as a result of triglyceride hydrolysis, but due to the low volatility, their contribution to ham flavor might be of less importance [7]. Identified long chain alcohols were oct-1-en-3-ol (1.2–5.2%) and octadecan-1-ol (1.0–1.5%).

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TABLE 2. Volatile Compounds Isolated by Nitrogen Purge and Steam Distillation (NPSD)

Compound	RI	1st Trap				2nd Trap (3rd Trap)			
		Min.	Max.	Av.	SD.	Min.	Max.	Av.	SD.
Terpenes									
α-Pinene	939	–	–	–	–	0.0	0.4	0.18	0.21
Sabinene	978	–	–	–	–	0.9	1.8	1.05	0.54
δ-3-carene	1015	–	–	–	–	0.0	0.9	0.35	0.44
p-Cymene	1029	–	–	–	–	0.0	0.9	0.25	0.43
Limonene	1033	0.0	0.1	0.05	0.06	0.0	4.2	1.55	2.00
γ-Terpinene	1063	–	–	–	–	0.0	0.8	0.33	0.39
trans-Sabinene hydrate	1072	–	–	–	–	0.0	2.4	0.70	1.15
trans-Linalool oxide	1076	–	–	–	–	0.0	0.6	0.38	0.26
Linalool	1102	0.9	3.2	2.03	0.95	0.9	5.1	2.50	1.82
Terpinen-4-ol	1181	0.0	2.1	1.00	0.97	1.5	3.2	1.80	1.17
p-Cymen-8-ol	1187	0.0	0.5	0.28	0.22	–	–	–	–
α-Terpineol	1192	0.2	1.5	0.65	0.58	0.0	1.0	0.40	0.43
Carbonyl compounds									
Heptan-2-one	<900	–	–	–	–	0.0	1.9	0.80	0.80
Heptanal	903	0.4	0.7	0.43	0.19	0.0	3.1	1.03	1.41
Methional	907	0.0	1.6	0.65	0.70	0.0	0.6	0.38	0.28
2-Methyl-cyclo-pent-2-en-1-one	909	–	–	–	–	0.0	0.2	0.08	0.09
(*E*)-Hept-2-en-al*	958	–	–	–	–	0.0	0.7	0.23	0.33
3-Ethylcyclopentanone	960	–	–	–	–	0.0	0.4	0.18	0.21
Benzaldehyde	965	0.4	1.4	0.83	0.43	0.3	9.6	5.20	3.97
Octan-3-one	987	–	–	–	–	0.0	1.8	0.83	0.77
Octanal	1004	–	–	–	–	0.0	1.5	0.50	0.71
2,3-Dimethyl-cyclopent-2-en-1-one	1043	0.0	0.3	0.15	0.17	–	–	–	–
Phenylacetaldehyde	1048	2.9	9.8	5.40	3.12	0.0	0.6	0.28	0.32
(*E*)-Undec-2-en-al*	1060	–	–	–	–	0.0	1.0	0.38	0.48
Nonan-2-one	1093	–	–	–	–	0.0	0.6	0.38	0.26
Nonanal	1106	0.6	2.5	1.78	0.83	0.0	1.9	1.18	0.88
3-Methoxybenzaldehyde	1261	0.0	0.6	0.30	0.26	–	–	–	–
Cyclohexa-2,5-diene-1,4-dione	1469	–	–	–	–	0.0	0.1	0.05	0.05
Pentadecan-2-one	1699	0.4	1.5	0.83	0.50	–	–	–	–
Hexadecanal	1819	14.1	34.2	23.31	8.51	–	–	–	–
Octadecanal	2021	0.0	0.9	0.38	0.41	–	–	–	–
Carboxylic acids									
Hexanoic acid	974	0.0	0.2	0.10	0.08	0.6	1.5	0.78	0.49
Octanoic acid	1173	0.5	3.2	1.15	1.37	0.4	1.9	0.80	0.73
Nonanoic acid	1273	0.5	6.3	2.10	2.85	0.3	2.4	1.08	1.04
Alcohols									
2-Furan methanol	<900	0.3	1.1	0.68	0.35	–	–	–	–
Hexan-1-ol	<900	–	–	–	–	0.0	0.9	0.30	0.42
2-Butoxyethanol	906	0.0	3.4	1.10	1.56	–	–	–	–
1-Butoxypropan-2-ol	942	0.0	1.5	0.70	0.63	0.0	0.3	0.13	0.15
Heptan-1-ol	969	0.0	0.8	0.50	0.36	0.0	0.9	0.53	0.39
Oct-1-en-3-ol	981	0.0	2.1	0.90	0.89	0.0	8.3	3.58	3.82
Benzyl alcohol	1037	0.0	3.5	1.38	1.70	0.2	1.0	0.65	0.34
Octan-1-ol	1073	0.3	0.9	0.53	0.26	–	–	–	–
2-Phenylethanol	1115	0.3	0.7	0.50	0.18	–	–	–	–
Nonan-1-ol	1193	–	–	–	–	(0.0)	(2.2)	(1.03)	(1.01)

Volatiles Isolated by NPSD. Flavor-important volatile organic compounds were isolated by NPSD in three different cold traps depending on their volatility and solubility (simultaneous isolation and fractionation of the volatiles). Nitrogen served as the carrier gas but also provided an inert medium which prevented oxidation of unsaturated fatty acids, sugars, and amino acids during the heating of the samples [7, 15].

TABLE 2. (continued)

Compound	RI	1st Trap				2nd Trap (3rd Trap)			
		Min.	Max.	Av.	SD.	Min.	Max.	Av.	SD.
Aromatic and aliphatic hydrocarbons									
Ethylbenzene	<900	0.0	0.2	0.07	0.09	(9.0)	18.2)	(12.40)	(5.05)
Ethenylbenzene	<900	–	–	–	–	0.3	0.5	0.38	0.09
1,4-Dimethylbenzene**	<900	–	–	–	–	0.0	0.4	0.18	0.21
						(5.1)	(11.5)	(8.20)	(3.73)
1,3-Dimethylbenzene**	<900	0.0	0.3	0.15	0.17	0.8	1.9	1.13	0.52
						(36.8)	(41.9)	(39.5)	(2.58)
3-Ethyl-2-methyl-hexa-1,3-diene	1035	–	–	–	–	0.0	1.9	0.93	0.78
1,2-Dimethoxybenzene**	1150	0.0	0.8	0.50	0.36	–	–	–	–
1,3-Dimethoxybenzene**	1170	–	–	–	–	0.0	0.6	0.38	0.26
3,4-Dimethoxy-toluene**	1243	0.0	0.6	0.30	0.26	–	–	–	–
1,2,3-Trimethoxy-benzene	1316	0.0	0.5	0.28	0.21	–	–	–	–
Phenols									
Phenol	980	0.0	1.1	0.65	0.51	0.0	0.2	0.08	0.09
2-Methylphenol	1057	0.0	1.9	0.60	0.89	–	–	–	–
2-Methoxyphenol	1091	0.0	2.5	0.98	1.08	0.0	0.5	0.30	0.25
2,4-Dimethylphenol	1152	0.0	0.4	0.20	0.23	–	–	–	–
3,5-Dimethylphenol	1171	0.0	0.3	0.15	0.16	–	–	–	–
2-Methoxy-4-methylphenol	1194	0.0	1.9	0.63	0.86	–	–	–	–
4-Ethyl-2-methoxyphenol	1281	0.0	1.2	0.63	0.57	–	–	–	–
2,6-*bis*-(1,1-Dimethylethyl)-4-methylphenol	1514	1.9	5.2	4.03	1.51	1.8	3.4	2.43	0.95
						(0.0)	(5.5)	(2.83)	(2.75)
Nitrogen containing compounds									
2,3-Dimethylpyrazine	913	1.3	6.7	3.55	2.43	0.0	5.5	1.88	2.60
Ethylpyrazine	918	0.3	1.0	0.73	0.31	0.0	0.7	0.38	0.29
2,6-Dimethylpyrazine	921	0.3	1.9	0.88	0.71	0.0	0.7	0.38	0.28
2-Ethyl-6-methylpyrazine	999	0.9	2.8	1.45	0.90	0.0	1.9	0.93	0.78
2,3,5-Trimethylpyrazine	1004	1.5	5.3	2.75	1.73	0.0	5.1	3.10	2.44
2-Methyl-6-vinylpyrazine	1018	0.0	0.1	0.05	0.06	–	–	–	–
3-Acetyl-1*H*-pyrrole	1063	0.0	0.4	0.20	0.23	–	–	–	–
2,3,5,6-Tetramethylpyrazine	1088	0.3	4.4	1.65	1.89	0.0	0.9	0.45	0.36
Esters									–
Diethyl phtalate	1595	0.0	0.3	0.15	0.16	–	–	–	
Diisobutyl phtalate	1869	1.7	2.6	1.83	0.66	1.6	4.6	2.30	1.59
						(0.0)	(0.2)	(0.13)	(0.12)
Dibutyl phtalate	1963	0.7	2.1	1.45	0.70	0.0	5.8	2.20	2.53
						(0.0)	(12.5)	(6.50)	(6.26)
Ethyl hexadecanoate	1996	0.0	0.5	0.28	0.22	(0.0)	(5.4)	(2.80)	(2.71)
Furans									
2-Pentylfuran	993	–	–	–	–	0.0	5.8	1.83	2.71

Min. – minimal percentage, max. – maximal percentage, Av. – average percentage; SD. – standard deviation; *tentatively identified; **correct isomer not identified; RI – retention indices on HP-5MS column.
Data 3rd Trap are given in brackets.

A total of 74 volatile compounds was found by NPSD in Istrian dry-cured ham (Table 2). Different organic functional groups were identified: terpenes, carbonyl compounds, nitrogen-containing compounds, alcohols, phenols, esters, carboxylic acids, furans, and aromatic and aliphatic hydrocarbons. Individually, the major volatile compounds in the traps were hexadecanal (14.1–34.2%) in the 1st trap, benzaldehyde (0.3–9.6%) in the 2nd trap and 1,3-dimethylbenzene (36.8–41.9%) in the 3rd trap.

Water-soluble and/or less volatile components mainly condensed in the 1st trap, such as low- and high-molecular-weight aldehydes, nitrogen compounds, phenols, low-molecular-weight alcohols, terpenes, low-molecular-weight carboxylic acids, ketones, high-molecular-weight esters, as well as aromatic hydrocarbons. Hexadecanal (14.1–34.2%) was the most abundant aldehyde, followed by phenylacetaldehyde (2.9–9.8%), nonanal (0.6–2.5%), and methional (0.0–1.6%). The high-

molecular-weight aldehydes, due to their lower volatility, are less important for the flavor. On the other hand, the phenylacetaldehyde, due to the low threshold has a great impact on the flavor [16]. An increase in aldehyde percentages during ripening (such as phenylacetaldehyde, nonanal, and hexadecanal) has been observed for Iberian and Dalmatian dry-cured hams [7, 9]. Nitrogen-containing compounds were the next dominant group in the volatile profile of the 1st trap, mainly pyrazines and pyrroles (the meaty group commonly found in dry-cured products). Among them, the major ones were 2,3-dimethylpyrazine (1.3–6.7%) and 2,3,5-trimethylpyrazine (1.5–5.3%). Pyrazines show increasing occurrence during processing, having a pleasant, desirable aroma of dry-cured ham with characteristic odors [10]. The unbranched alcohols like octan-1-ol (0.3–0.9%), hexan-1-ol (0.0–0.9%), and oct-1-en-3-ol (0.0–8.3%) found in the 2nd trap appeared also in European hams [11, 14]. Alcohols are considered to be unimportant for the flavor (due to their relatively higher threshold), but their impact rises with increase in the carbon chain [17]. The NPSD method extracted also phenols such as 2,6-*bis*-(1,1-dimethylethyl)-4-methylphenol (1.9–5.2%), 2-methoxyphenol (0.0–2.5%), 2-methylphenol (0.0–1.9%), 2-methoxy-4-methylphenol (0.0–1.9%), 4-ethyl-2-methoxyphenol (0.0–1.2%), phenol (0.1–1.1%), 3,5-dimethylphenol (0.0–0.3%), and 2,4-dimethylphenol (0.0–0.4%). Identified organic acids were nonanoic acid (0.5–6.3%), octanoic acid (0.5–3.2%), and hexanoic acid (0.0–0.2%) with minor effect on aroma due to their high olfaction threshold [10].

Volatile compounds that were not condensed in the 1st cold trap mainly dissolved in diethyl ether of the 2nd cold trap. Found organic functional groups included terpenes, aldehydes, amino acids, nitrogen compounds, alcohols, esters, carboxylic acids, phenols, ketones, aromatic as well as aliphatic hydrocarbons, and furans. Terpenes were abundant, among them linalool (0.9–5.1%), terpinen-4-ol (1.5–3.2%), limonene (0.0–4.2%), and sabinene (0.9–1.8%). Aldehydes, also found in European hams, consist mainly of benzaldehyde (0.3–9.6%), heptanal, (0.0–3.1%), octanal (0.0–1.5%), and (*E*)-hept-2-enal (0.0–0.7%) [5]. Phthalates like diisobuthyl phthalate (1.6–4.6%) and dibuthylphthalate (0.0–5.8%) are not usually found in dry-cured hams. Esters, usually found in small quantities in dry-cured ham, have fruity notes, especially those formed from short-chain acids [5]. Heptan-2-one (0.0–1.9%) and nonan-2-one (0.0–0.6%) are usually found in European hams among methylketones. 2-Pentylfuran was identified (0.0–5.8%), and furans are not among those compounds of intense flavor. The aliphatic hydrocarbon 3-ethyl-2-methylhexa-1,3-diene was only detected in this trap (0.0–1.9%). Aliphatic and branched hydrocarbons are minor contributors to the meat flavor, and are not among the most odor-active compounds in dry-cured ham [14].

The 3rd cold trap containing pentane mainly served for trapping the volatile components that escaped from the previous cold traps. This trap mainly contained non-polar aromatic hydrocarbons: 1,4-dimethylbenzene (36.8–41.9%), ethylbenzene (9.0–18.2%), and 1,3-dimethylbenzene (5.1–11.5%). Other abundant volatiles condensed in this trap included dibuthyl phthalate (0.0–12.5%), 2,6-*bis*-(1,1-dimethylethyl)-4-methylphenol (0.0–5.5%), ethyl hexadecanoate (0.0–5.4%), and nonan-1-ol (0.0–2.2%).

In conclusion, the NPSD method gave satisfactory results with minor or no interference from the sample lipid matrix. Nevertheless, due to long the NPSD extraction period, some odor-active compounds found in European hams, such as 2-methylbutanal, 3-methylbutanal, and hexanal, were lost due to their lower boiling points. On the other hand, these compounds were extracted by HS-SPME, and the method showed great susceptibility towards terpenes, but nitrogen compounds were not isolated, in contrast to NPSD. Identified volatiles were associated with added spices (terpenes such as limonene, *trans*-caryophyllene, δ-3-carene, sabinene, or linalool), lipid oxidation (carbonyl compounds such as hexadecanal, phenylacetaldehyde, nonanal, hexanal, 2-methylbutanal, or 3-methylbutanal), and amino acid catabolism (nitrogen-containing compounds such as 2,3-dimethylpyrazine or 2,3,5-trimethylpyrazine).

Istrian Dry-Cured Ham Samples and Chemicals Used. After removal of the dust and molds, five authentic Istrian dry-cured hams were longitudinally cut, perpendicular to the medial part of the femur, and from each ham 65 g were taken for NPSD and 5 g for HS-SPME.

Headspace Solid-Phase Microextraction (HS-SPME). The isolation of headspace volatiles was performed using manual SPME fibre with a layer of polydimethylsiloxane/divinylbenzene (PDMS/DVB). For HS-SPME extraction, 5 g of finely grated dry-cured ham was placed in a 15 mL glass vial and hermetically sealed with PTFE/silicone septa. The vial was maintained in a water bath at 60°C during equilibration (15 min) and extraction (45 min). After sampling, the SPME fibre was inserted into the injector of the GC and GC-MS.

Nitrogen Purge and Steam Distillation (NPSD). The apparatus for NPSD consists of a 100 mL two-neck flask. One of the necks served as the inlet for the purging gas, oxygen-free nitrogen. The second neck was the outlet, for purged volatiles in nitrogen, into the cold traps maintained at –20 ± 5°C with an ice-$CaCl_2$ mixture. The fine cut sample (65 g) was placed in the two-neck flask, where it was constantly maintained at 102 ± 5°C within the oil bath. A slow stream of oxygen-free nitrogen gas was passed through the sample to purge the volatiles. The effluent stream was connected to a cold trap (1st trap), next through the second cold trap (2nd trap), containing 30 mL of ether, and finally through the third cold trap (3rd trap), containing 30 mL

of pentane. The volatiles were collected over a 6 h purging and distilling period. At the end of the experiment, the condensate of the first cold trap was extracted twice with 30 mL ether. The extracts were combined, dried over anhydrous sodium sulfate, and concentrated to a final volume of about 0.2 mL. The second and third cold traps were dried over $MgSO_4$ and concentrated as described above.

Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS). An Agilent Technologies gas chromatograph model 7890A equipped with flame ionization detector and capillary column HP-5MS (5% phenylmethylpolysiloxane, 30 m × 0.25 mm i.d., coating thickness 0.25 μm) was used for the analyses. Helium was used as the carrier gas, with flow rate 1 mL min^{-1}. Injector temperature was 250°C and detector temperature was 300°C. HP-5MS column temperature was programmed isothermal at 70°C for 2 min, then increased to 200°C at a rate of 3°C min^{-1} and held isothermal for 15 min. The injected volume was 1 μL and the split ratio was 1:50. An Agilent Technologies gas chromatograph model 7890A with mass selective detector model 5975C was used. MS operating conditions were: ionization voltage 70 eV; ion source temperature 280°C; mass scan range 30–300 mass units. GC operating conditions and the column were as in the previous paragraph.

The individual peaks were identified by comparison of their retention indices (relative to C_9–C_{25} *n*-alkanes) with those of available authentic samples and the literature [18] as well as by comparing their mass spectra (obtained by GC-MS duplicate analyses for each batch of five samples) with Wiley 275 MS and NIST02 libraries. The percentage composition of the samples was computed from the GC-FID peak areas (duplicate analyses for each batch of five samples) using the normalization method without correction factors.

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