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Freshness Assessments of Moroccan Sardine (*Sardina pilchardus*): Comparison of Overall Sensory Changes to Instrumentally Determined Volatiles

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Freshness of ice-stored sardine was assessed by two sensory methods, the quality index method (QIM) and the European Union freshness grading system, and by instrumental means using the method of aroma extract dilution analysis. Screening of sardine potent volatiles was carried out at three freshness stages. In the very fresh state, the plant-like fresh volatiles dominated the odor pattern, with the exception of methional. Overall odor changes in sardine throughout storage correlated with changes in the concentration of some potent volatiles: after 2 days of ice storage, (*Z*)-4-heptenal, (*Z*)-1,5-octadien-3-one, and methional imparted an overall "fishy" odor character to sardine, whereas at a lower sensory grade (B), the compounds (*E*)-2-nonenal and (*E*,*Z*)-2,6-nonadienal could be, in part, associated with the slightly rancid aroma top notes. Trimethylamine was detected as a highly volatile odorant using solid-phase microextraction (SPME) headspace analysis of refrigerator-stored sardine. Intensity and sensory characteristics of some SPME determined volatiles, for example, 3-methylnonane-2,4-dione, were closely related to overall odor changes. SPME headspace analysis may be useful in the characterization of off-flavors in fish.

KEYWORDS: Sardine; *Sardina pilchardus*; seafood; freshness; volatile compounds; sensory; AEDA; SPME; headspace

INTRODUCTION

Freshness is a major quality attribute of seafood. Although numerous sensory, chemical, microbiological, and instrumental tools exist for the determination of spoilage, methods for the reliable determination of what freshness is are few and not generally applicable (*1*). Fish at different freshness stages are often sold at average prices. In Belgium and The Netherlands, a fresh fish market strategy for some fish species has been initiated, the fish being packaged under a quality label "Silver Sealed" after sale in the auction (*2*). Furthermore, management of the raw material on storage is a fundamental part of production planning in the food fish industry.

Sensory analysis has been the most commonly used method to evaluate fish freshness, but the need to develop rapid and objective methods to reinforce the conclusions reached by sensory means has been emphasized. Ongoing research in many laboratories of the European Union (EU) aims at developing indices for freshness assessment that are simple to apply and easy to use, both in trade and at the industrial level. A joint project (EU FAIR CT.96.3253) has been trying to develop flow injection/gas diffusion methods (FIGD) for trimethylamine (TMA-N) and total volatile basic nitrogen (TVB-N) determination in fish (*3*).

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Volatile compounds contributing to the characteristic odor of fish can be measured to evaluate the freshness of fish (*4*). Recommendations from a European concerted action project, "Evaluation of fish freshness" (AIR3 CT94-2283), stressed the need to further characterize "fresh" fish odors for various species to guide the development of selective sensors for specific detection of fresh odor compounds. Qualitative and quantitative analysis of fish volatiles by gas chromatography (GC) is necessary, with particular emphasis on correlating individual components to sensorial perception (GC−olfactometry).

The flavor of fresh seafoods, including both fish and shellfish, is primarily affected by lipoxygenase-derived lipid-based volatiles (*5*). Various volatile compounds have been identified, providing a good understanding of the chemical basis of the odor of saltwater and freshwater fish (*6*). Josephson et al. (*7*) monitored the overall volatile pattern of whitefish during storage and suggested different classes of compounds for indexing freshness/spoilage. The classes were fresh long-chain alcohols and carbonyls (C_6−C_9) to index freshness and short-chain alcohols, sulfur compounds, amines, sweet esters, aromatics, and dienals as indicators of spoilage. In fresh capelin (*Mallotus villosus*), Ólafsdóttir et al. (*8*) identified 2,6-nonadienal and 1,5-octadien-3-one as the most influential fresh odor compounds, using GC−olfactory sniffing and retention times for identification. The same authors (*9*) monitored sulfur compounds in capelin using direct headspace analysis. The capelin samples

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Table 1. Freshness Grading of Fatty Fish Species According to the EU Freshness Grading Scheme

	freshness category			not admitted
criteria	extra	A	B	C
skin	bright pigmentation, bright iridescent colors; clear distinction between dorsal and central surfaces	loss of luster and shine; duller colors; less difference between dorsal and ventral surfaces	dull, lusterless, insipid colors; skin creased when fish curved	very dull pigmentation; skin coming away from flesh
skin mucus	aqueous, transparent	slightly cloudy	milky	yellowish grey, opaque mucus
consistency of flesh	very firm, rigid	fairly rigid, firm	slightly soft	soft (flaccid)
gill covers	silvery	silvery, slightly red or brown	brownish and extensive seepage of blood from vessels	yellowish
eye	convex, bulging; blue-black bright pupil, transparent "eyelid"	convex and slightly sunken; dark pupil; slightly opalescent cornea	flat; blurred pupil; blood seepage around the eye	concave in the center; gray pupil; milky cornea
gills	uniformly dark red to purple; no mucus	less bright color; paler at edges; transparent mucus	becoming thick, discolored opaque mucus	yellowish; milky mucus
smell of gill	fresh seaweed; pungent; iodine	no smell or seaweed; neutral smell	slightly sulfurous fatty smell, rancid bacon cuttings or rotten fruit	rotten sour

were put in plastic bags equipped with a septum and sealed. The plastic bags were kept at room temperature for 30 min before samples were withdrawn from the bags by piercing the septum with an airtight syringe. Samples of 1 mL were injected onto an HP-1 capillary column and detected by an FPD sulfur-sensitive detector. Semiquantitative information on the change of components in capelin samples was based on comparison with an external standard.

Few studies have focused on identifying volatiles that actually contribute to seafood flavor. Among a series of investigations on potent odorants from boiled fish, Milo and Grosch (*10*) showed by the use of two GC−sniffing methods, aroma extract dilution analysis (AEDA) and gas chromatography−olfactometry of static headspace samples (GCO-H), quantification of potent volatiles by means of an isotope dilution assay, and calculation of odor activity values that methanethiol and dimethyl disulfide followed by (*E*,*Z*)-2,6-nonadienal and 3-methylbutanal were the most potent odorants for the nasal perception of fresh boiled cod (*Gadus morrhua*), whereas (*Z*)-1,5-octadien-3-one and methional showed the highest retronasal odor activity values.

It is worth mentioning that most of the previous investigations on fresh fish volatiles used headspace sampling methods. Lower boiling volatiles may exhibit poor if no recoveries using such methods. No application of distillation methods in studies of fresh fish volatiles could be found in the literature. Hence, there is a need to assess the importance of less volatile flavor compounds for the flavor of fresh fish.

In Morocco, small pelagic fisheries are mainly represented by the species *Sardina pilchardus* (539,839 tons total catches in 2000). This darkflesh fish species is known to be highly perishable. Sardine consumption, as fresh, is high in Morocco, but a significant part is intended for the Moroccan canning industry. Morocco is known to be the first exporter of canned sardines.

The present investigation was aimed at studying the relationship between sensory analysis and instrumental profiles for assessing freshness in sardine (*S. pilchardus*). The main goal was to find key indicator volatiles for differentiating early freshness stages. Grading of sardine during ice storage was carried out by sensory means using both the EU freshness grading scheme (*11*) and the quality index method (QIM) (*12*). For the same storage trial, high-vacuum distillation/aroma extract dilution analysis (AEDA) of volatiles (*13*) from samples of sardine corresponding to three different freshness stages was performed. Overall changes in fish during storage were compared to individual potent volatiles determined by AEDA. Finally, sensory and SPME headspace analyses were applied to refrigerator-stored sardine in an attempt to monitor freshness-related volatiles in the headspace from whole sardine.

MATERIALS AND METHODS

Chemicals. Trimethylamine−HCl (TMA) was from Sigma. Pure samples of methional, 2-acetyl-1-pyrroline, (*E*,*E*)-2,4-nonadienal, (*E*,*Z*)-2,6-nonadienal, 3-methylnonane-2,4-dione, and (*E*,*E*)-2,4-decadienal were gifts from Prof. Dr. Peter Schieberle (Deutsche Forschungsanstalt für Lebensmittelchemie, Garching, Germany). The remaining compounds were all previously identified.

Storage Trial for AEDA. Fresh sardines of the species *S. pilchardus* (10 kg) were obtained (∼5 h after catching) in the Rabat wholesale fish market. Sardines were caught off the coasts of Mehdia (north central Moroccan Atlantic coast) in April 2002 (average temperature = 19 °C). The mean length and weight of fish were 16 cm ± 2 cm and 33 ± 2 g, respectively. The fish were then transported to our laboratory in crushed ice. In the laboratory, the fish were immediately placed in a self-draining polystyrene box with crushed ice (fish-to-ice ratio 1:2), replenishing melted ice daily. The box was stored in a refrigerator at 4 °C. Samples (10 sardines) were taken on a daily basis, up to 5 days of storage (at which fish were deteriorated) for sensory evaluation. AEDA was carried out on three samples taken (1) just upon arrival of fish to the laboratory (S1), (2) after 48 h of storage (S2), and (3) on the fourth day (96 h) (S3).

Storage Trial for SPME Analysis. SPME trials were carried out on two samples (2 kg each) of sardine caught off the north central Moroccan Atlantic coast in February 2002 (average temperature = 23 °C). The samples were obtained from a point of sale in Rabat (∼5−6 h after catching). In the laboratory, fish were kept under refrigeration at a temperature of 4 °C (no ice added). Sampling was carried out daily, up to fitness for consumption. Before SPME analyses were begun, specimens of samples were taken for sensory evaluation.

Sensory Evaluation. For both storage trials (AEDA and SPME), fish had been inspected at each time of sampling and subsequent freshness grades were assigned by three panelists including two veterinarians (the authors) familiar with quality control and quality assurance of fish and fish products. The freshness state of sardine was assessed both by the EU grading scheme for bluefish and by QIM. The EU freshness grading scheme (*11*) distinguishes four categories of fish, that is, E (extra), A, B, and not admitted (C), using the following criteria: skin, skin mucus, consistency of flesh, gill covers, eye, gills, and smell of gills (**Table 1**). The QIM is based on significant sensory parameters (*12*) for whole fish using many weighted parameters and a score system from 0 to 4 demerit points. Scores are added to give an overall sensory score, the so-called quality index. QIM gives scores of zero to very fresh fish and an increasingly larger total result as fish deteriorates. We used the scheme developed for European sardine (*S.*

Table 2. Quality Index Method (QIM) Scheme for Sardine (*S. pilchardus*)

	parameter	characteristic	demerit points
general appearance	surface appearance	very bright, iridescent	0
		bright	1
		less bright	2
		slightly opaque, dull	3
	stiffness	flexible (prerigor)	0
		tense (rigor)	1
		less tense	2
		soft	3
	flesh firmness	firm, elastic	0
		firm, hard	1
		less elastic	2
		soft	3
eyes	clarity (cornea)	clear, transparent	0
		slightly opalescent	1
		opalescent or bloodstained	2
	pupil	black and circular	0
		black and distorted	1
		grey and distorted, bloodstained	2
	shape	slightly convex, normal	0
		plane, flat	1
		concave, sunken	2
cover	bloodiness	no visible (0%)	0
		slight (<10%)	1
		some(<50%)	2
		wide (>50%)	3
gills	color	red or dark red	0
		brownish red	1
		discolored (brownish)	2
	odor	seaweedy	0
		slightly seaweedy	1
		slightly acre or rancid or sweet	2
		rancid oil or ammonia or sour	3
abdomen	postgill (belly-burst)	intact, firm	0
		intact, soft	1
		stretch marks, soft	2
		torn or ruptured	3
flesh	appearance	smooth, translucent	0
		slightly opaque, opaque	1
		flattened, bood-stained	2
		total	0–28

pilchardus) at the "Institute of Seafood Quality Investigations" (IPIMAR), Portugal (*14*) (**Table 2**). In this scheme, odor description is limited to the gills of sardine. For the purpose of this study, separate odor descriptors for skin were developed but not included in calculating total demerit points.

Isolation of the Volatiles for AEDA. Fish (six sardines, 200 g) were manually beheaded, gutted, and filleted. The fillets were cut into small pieces, soaked in methylene chloride (150 mL), and then homogenized using an Ultra-turrax for 3 min. To the suspension was added 50 mL of methylene chloride, and the solvent was filtered off. The remaining material was further washed with 50 mL of methylene chloride and filtered. The extract was dried over anhydrous sodium sulfate and concentrated to ~100 mL by distilling off the solvent on a Vigreux column (50 × 1 cm) at 40 °C.

The vacuum distillation method described by Sen et al. (*15*) with the modifications previously described (*16*) was used to recover sardine volatiles. The concentrated extract was poured into a 250 mL distillation flask and frozen for 30 min in liquid nitrogen. After sublimation of the volatiles and of the solvent in vacuo (9×10^{-3} mbar), the temperature of the water bath was increased to 35 °C and the sublimation was continued for a further 1 h. The condensate of the first cooling trap was dried over anhydrous sodium sulfate, filtered, and then concentrated by distilling off the solvent on a Vigreux column (50 × 1 cm) and then by microdistillation.

Capillary Gas Chromatography (AEDA). High-resolution gas chromatography (HRGC) was performed by means of a Hewlett-Packard 5890 gas chromatograph series II equipped with a flame ionization detector (FID). Separation was achieved on DB-5 and DB-FFAP fused silica capillaries (each 30 m × 0.30 mm, 0.25 μm film thickness) supplied from J&W Scientific, Folsom, CA.

The samples were applied by the on-column injection technique at 35 °C. After 1 min, the temperature of the capillary was raised at 40 °C/min to 60 °C, held 5 min isothermal, and then raised at 4 °C/min to 240 °C (220 °C for the DB-FFAP). Hydrogen was used as a carrier gas at a column flow rate of 2 mL/min and 15 psi of head pressure. The FID was set at a temperature of 250 °C. Nitrogen was used as a makeup gas for FID at 20 mL/min. The data from the HP 5890 were recorded on an HP 3396 A integrator. Retention data of the compounds are presented as retention indices (RI).

HRGC—Olfactometry. The final portion of the GC column was passed through an unused heated block (250 °C copper insert) as previously described (*17*). Analysis of the aroma concentrates was done using the following dilution series: the original extract (800 μL) was stepwise diluted (1 + 1, v/v) by addition of methylene chloride. Aliquots of each dilution (1 μL) were analyzed by GC using the DB-5 capillary column. The potent odorants were located in the capillary gas chromatography by AEDA. Compound detection and odor description were done by the primary author. Chromatographic conditions were the same as those described above.

SPME Headspace Analysis. SPME fibers with 50/30 m thicknesses of divinylbenzene/Carboxen/poly(dimethylsiloxane) (DVB/Carboxen/PDMS) coating and the manual holder were obtained from Supelco Co. (Bellefonte, PA). Before initial use, the fiber was conditioned for 4 h at 270 °C in the split/splitless injection port of the GC. Before each extraction, the fiber was held at 230 °C for 2 min.

Isolation of Headspace from Whole Sardine. A glass vessel (21 cm length, 6 cm diameter) was specially designed by a glass blower (Manching, Germany). The top-capped vial of the vessel had a septum that could be pierced by the SPME fiber attachment needle, and the side lid allowed the introduction of a whole fish for sampling headspace.

The mean length and weight of fish used in the trials were 19 ± 1 cm and 45 ± 3 g, respectively. Before each sampling, specimens of fish were withdrawn from the refrigerator and allowed to stand at room temperature for 15 min while sensory evaluation was performed. When fish reached an average internal temperature of 15 °C, it was then introduced in the sampling vessel, the gills being maintained open by means of an adjusted small metal clip. After the vessel had been closed, the SPME fiber was exposed to the headspace above the fish. Exposure time was set to 15 min.

GC Parameters. After sampling, the fiber was placed in the injection port of the GC for 5 min at 230 °C. The purge was off for the first 2 min of desorption. Separation was achieved on the same previously described capillaries. The temperature program was started at 35 °C, held for 2.5 min, then increased by 8 °C/min to 240 °C (200 °C for DB-FFAP), and held for 2 min. The GC conditions for FID were the same as above. Odorants were located in the capillaries by eluate sniffing through an unused heated detector block as previously described.

RESULTS AND DISCUSSION

Storage Trial: AEDA. The results of freshness grading and odor evolution of iced stored sardine are shown in **Table 3**. Sardine at the time of purchase had a QIM score of 1, corresponding to E grade using the general EU grading method. Concomitant pH determinations (nonpublished data) yielded a value of 6.1, indicating that fish was in the rigor-mortis phase at time of purchase. The very fresh state (grade E) is limited to 1 day of storage, but fish were still of excellent freshness up to the second day. This corresponded to an overall QIM score of 9, the sardine being assessed as high A using the EU grading scheme. A total QIM score of 22 can be regarded as the

Table 3. Freshness Assessment of Iced-Stored Sardine by EU-Grading and QIM, with Emphasis on Odor Development

	freshness stage			
days of storage	EU grade	QIM score	odor description	
			gills	skin
0	E	1	seaweedy	neutral to green
1	E	3	seaweedy (less pronounced)	pleasant sweet odor, slightly green
2	high A	9	slightly seaweedy	green, more fishy in character
3	low A	17	slightly acrid and rancid	green, slightly fishy
4	B	22	acrid and rancid	fishy, slightly rancid
5	C	27	rancid, ammoniacal, sour	sulfurous odor of deterioration

borderline of saleability of sardine. Drawbacks of the EU scheme appear when sensory attributes of fish at days 2 and 3 of storage are compared: After 3 days of storage, sardine could not be regarded as very fresh, as compared to the second day, but still could not be graded B, whereas using the QIM a difference of 8 total demerit points was noticed. QIM revealed changes in sensory attributes more than the EU grading method.

In this study, the parameters and descriptors used in the QIM scheme developed for European sardine (*14*) (**Table 2**) were found to be appropriate, but for the purpose of comparing overall odor changes to individual potent volatiles, the odor parameter was described separately for gills and skin (**Table 3**). The use of separate descriptors was important in subsequent SPME trials, where local practices of fish commercial handling and distribution (icing often not done properly) were simulated.

The early freshness stages of iced-stored sardine are characterized by a pleasant seaweedy odor of the gills and a faint green odor in skin. The "fishy" odor character in skin and the gradual loss of seaweedy odor of gills are good indications that fish are of lower sensory grades (low A or QIM score >17). The odor of sardine on day 4 started to be somewhat objectionable, although fish were still fit for consumption. Putrid spoilage odors were typical of the rejection grade C.

Sardine used in this trial had a shelf life of 4 days as determined by sensory evaluation. Longer storage periods (6−10 days) have been reported in other studies (*18*, *19*). Factors such as season and inadequate handling practices of fish onboard and after landing may accelerate the rate of spoilage.

Samples used in AEDA were chosen to represent three freshness stages (**Table 3**): S1 (grade E; QIM score 1), S2 (grade high A; QIM score 9), and S3 (grade B; QIM score 22). The results of AEDA from the storage trial are shown in **Table 4**. It appears that most of the compounds identified by AEDA have been previously found in salt-ripened anchovy (*20*).

From sample S1, taken just before laboratory ice storage, the undiluted flavor isolate exhibited a distinct green-like odor, which lacked the seaweed character. This could be attributed to removal of gills during sampling. AEDA applied to this sample revealed 10 odorants in the flavor dilution range of 16−256, of which (*Z*)-1,5-octadien-3-one and methional showed the highest FD factors. Other important odorants of fresh sardine were 1-octen-3-one and (*E*)-2-nonenal (**Table 4**). Lipoxygenase-derived volatiles dominated the overall volatile pattern at this freshness stage, which is in accordance with early published work on fresh saltwater fish flavor (*21*). C_9 carbonyl compounds, such as 2,6-nonadienal, which were previously found to be characteristic only for freshwater and euryhaline fish (*22*), were, on the basis of the current and previous investigations (*23*), important odorants of fresh small pelagic fish (sardine and anchovy). Additionally, even at this early freshness stage, methional was a potent volatile of sardine. Milo and Grosch (*10*) found the combination of methional and (*Z*)-1,5-octadien-3-one in an aqueous solution to impart a fishy odor.

After 2 days of ice storage (**Table 4**), the flavor isolate from sample S2 had a more intense green odor with slight fishy top notes. At this freshness stage, the odorants showing a substantial increase of the FD factor were (*Z*)-4-heptenal and (*Z*)-1,5-octadien-3-one. Higher FD factors were determined for some oxidatively derived volatiles, for example, (*E*,*Z*)-3,5-octadien-2-one and (*E*,*E*)-2,4-decadienal. In addition, 2−4-fold higher concentrations were found for (*Z*)-3-hexenal, methional, diacetyl, and 2,3-pentanedione as compared to sample S1. Such changes may in part explain the odor characteristics of sardine, as assessed using the QIM. The increase in concentration of (*Z*)-1,5-octadien-3-one, methional, and (Z)-4-heptenal is likely to be responsible for the development of the "fishy" character in sardine. Although the concentration of some peroxidation products of omega unsaturated fatty acids increased as well, it did not affect the overall green odor of sardine after 2 days of ice storage.

The flavor isolate from sample S3 had a distinct fish-like odor with slight oxidized and rancid aroma notes. Compared to sample S2, AEDA showed that the concentration of (*E*)-2-nonenal was 16-fold higher. Higher FD factors were found for (*E*,*Z*)-2,6-nonadienal, 3-octen-2-one, and (*E*,*E*)-2,4-heptadienal and, to a lesser extent, for 1-octen-3-one, (*E*,*E*)-2,4-nonadienal, 3-methylnonane-2,4-dione, (*E*)-2-decenal, (*E*,*Z*)-2,4-decadienal, methional, and (*E*,*Z*)-3,5-octadien-2-one. 2-Acetyl-1-pyrroline, which was previously identified in ripened anchovy (*20*) and in fresh boiled cod (*10*), was also detected in this sample. The differences in FD factors of the highly volatile compounds diacetyl and 2,3-pentanedione are difficult to interpret due to probable losses in the concentration steps of the flavor isolates. Because of their low thresholds and increase in concentration, (*E*)-2-nonenal and (*E*,*Z*)-2,6-nonadienal are likely to be responsible for the rancid notes perceived at this stage, whereas (*Z*)-4-heptenal rather participates in the expression of the overall fishy odor.

Storage Trial for SPME Headspace Measurements. We used the extra-long (2 cm) Carboxen/DVB/PDMS fibers to account for low concentration of target volatiles in sardine headspace and to have the highest overall sensitivity. Sampling time (15 min) was a compromise between short-time sampling, which better estimates "true" headspace (*24*), and exhaustive SPME.

Except for TMA, all of the compounds detected from the SPME extraction (**Table 6**) were identified in AEDA. The fewer number of detected volatiles could be explained by the sampling procedure: one fish was used in SPME as compared to six in AEDA. The relatively large volume of the SPME sampling vessel (designed for both small and other medium-size species) was an additional factor of decreased sensitivity.

SPME headspace analysis of whole sardine volatiles is likely to better represent the equilibrium headspace concentration, that is, samples of both skin and gill volatiles. Overall odor assessments of refrigerator-stored sardine are shown in **Table 5** and results of SPME headspace in **Table 6**. Odors perceived by GC eluate sniffing were assessed for intensity using a five-point scale to have a rough estimation of potential changes in concentration of influential volatiles in relation to overall odor changes. Deibler et al. (*25*) described a method to use SPME sampling to conduct a dilution analysis (Charm analysis or AEDA) of a static headspace by varying fiber thickness and

Table 4. Volatile Compounds Identified in Sardine (*S. pilchardus*) at Different Freshness Stages

compound	RI[a] on capillary DB-5	RI[a] on capillary DB-FFAP	odor quality[b]	FD factor[c] S1	FD factor[c] S2	FD factor[c] S3
diacetyl		968	butter-like	<16	128	32
2,3-pentanedione	697	998	butter-like	16	128	32
(*Z*)-3-hexenal	800	1145	green	16	128	32
(*E*)-2-hexenal	858		green	nd	nd	<16
(*Z*)-4-heptenal	900	1234	fatty-fishy	<16	512	512
acetic acid		1434	pungent	nd	nd	<16
methional	906	1446	boiled potato-like	64	256	512
2-acetyl-1-pyrroline	912	1322	roasty; popcorn-like	nd	nd	16
1-octen-3-one	964	1296	mushroom-like	32	64	256
(*Z*)-1,5-octadien-3-one	976	1362	geranium-like	256	2048	2048
(*E*,*E*)-2,4-heptadienal	1003		fatty	nd	nd	32
3-octen-2-one[d]	1036		fatty-spicy	16	16	128
(*E*,*Z*)-3,5-octadien-2-one	1096	1480	fatty-fruity	16	128	256
(*Z*)-2-nonenal	1148	1491	fatty-green	nd	nd	<16
(*E*,*Z*)-2,6-nonadienal	1150	1567	cucumber-like	16	16	128
(*E*)-2-nonenal	1156	1517	cucumber-like; green	32	32	512
(*E*,*E*)-2,4-nonadienal	1203	1680	fried fat-like	nd	<16	32
3-methylnonane-2,4-dione	1246	1703	fruity-sweet	<16	<16	32
(*E*)-2-decenal	1261	1641	fatty	nd	64	256
(*E*,*Z*)-2,4-decadienal	1306	1731	fatty-green	nd	16	64
(*E*,*E*)-2,4-decadienal	1309	1792	fried fat-like	16	256	128
trans-4,5-epoxy-(*E*)-2-decenal	1371	1988	metallic	nd	32	16

[a] Calculated retention index on capillaries DB-5 and DB-FFAP. [b] Odor quality perceived at the sniffing port. [c] FD factors determined on DB-5 capillary column; an FD factor of <16 means that the volatile was detected only by eluate sniffing of the original extract; nd = not detected. [d] Tentatively identified on the basis of odor quality and RI on capillary DB-5.

Table 5. Changes in Odor Characteristics during Refrigerator Storage (4 °C) of Sardine

total QIM score	odor description: gills	odor description: skin
1	seaweedy	neutral
2–3	less pronounced seaweedy odor	sweet and fatty
10–12	slightly seaweedy-neutral	sweet and fatty (more pronounced)
14–16	slightly acrid	fried fat
22	acrid and rancid	rancid-fishy
27	rancid, ammoniacal, sour	sulfurous odor of deterioration

length to achieve various adsorbent volumes. However, the commercial availability of fibers with nominal thicknesses that match closely their actual dimensions is a prerequisite to the adoption of the technique for routine use. In using HS-SPME, Ulrich et al. (*26*) simulated the "dilution" for the AEDA by shortening the adsorption time, but the adsorption selectivity of the fiber and the substance discrimination at short adsorption times have to be taken in account for quantitative analyses such as AEDA.

Only TMA was detected as a highly volatile odorant in the headspace of sardine. Its amine-like fishy odor was perceived with the same intensity up to a QIM score of 10 (grade A), at which it was no longer detected. TMA originates from the breakdown of TMA-oxide by bacterial enzymes and is therefore related to bacterial spoilage of refrigerated marine fish (*3*). The fact that TMA was detected in the headspace of sardine only up to grade A seems contradictory to its actual accumulation during the ultimate stages of storage. This apparent discrepancy could in part be explained by the pH changes during storage that affect the odor activity of TMA. It should be emphasized that SPME headspace measurements were not carried out on sardine at the rejection stage, as TMA was detected in spoiled hake (*Merluccius merluccius*) under similar experimental conditions (unpublished data). Owing to the rather high odor threshold of TMA in water (30 ppm) (*6*), it is unlikely to be an influential odorant of sardine. In stored boiled cod (*10*) TMA was not found to belong to the most important odorants, although it was perceived by GCO-H.

Sardine of grade E (QIM ≤ 3) had a seaweedy odor in the gills, whereas sweet and weak fatty notes could be perceived on skin (**Table 5**). SPME headspace profile was characterized by distinct odor impressions from TMA, an unknown compound, 3-methylnonane-2,4-dione, and, to a lesser extent, (*E*,*Z*)-3,5-octadien-2-one and (*E*,*E*)-2,4-decadienal. The plant-like fresh volatiles dominated the odor pattern at this grade. (*Z*)-1,5-Octadien-3-one was detected when the QIM reached a total score of 3, but due to its low recognition threshold (0.001 ppb) (*27*), it is a significant contributor to the overall odor of sardine. The odor characteristics of 3-methylnonane-2,4-dione and (*E*,*Z*)-3,5-octadien-2-one in the GC effluent were very reminiscent of the aroma notes perceived on the skin of sardine (**Table 5**), which is consistent with early oxidative changes in the lipid fraction of sardine when fish are stored without ice, as opposed to the odor changes of ice-stored sardine (AEDA trial, **Table 4**).

Subsequent loss in freshness (sardine of grade A; QIM ≤ 14) was characterized in GC eluate sniffing by more intense odors from (*E*,*Z*)-3,5-octadien-2-one, 3-methylnonane-2,4-dione, and (*Z*)-1,5-octadien-2-one, along with fried-fat-like odors imparted by 2,4-nonadienal and 2,4-decadienal. (*E*,*Z*)-2,6-Nonadienal was detected at this freshness stage. Such changes are likely to explain the acrid and fried-fat notes that developed in sardine.

When sardine reached the borderline of saleability, rancid and fishy odors dominated the odor pattern. The corresponding results from sniffing experiments indicated a probable increase in concentration for methional, 1-octen-3-one, and other oxidatively derived volatiles. This is further supported by the results of AEDA (increase in FD factors) (**Table 4**). Such changes are likely to be responsible for the objectionable odors of sardine at this stage.

Some of the potent volatiles determined by AEDA, for example, (*Z*)-4-heptenal and (*E*)-2-nonenal, were not detected in the SPME headspace experiments. Furthermore, 3-methyl-

Table 6. SPME Headspace Analysis of Sardine Volatiles at Different Freshness Stages during Refrigerator Storage (4 °C)

				QIM score					
		RI on capillary		grade E[b]			grade A		grade
compound	odor quality[a]	DB-5	DB-FFAP	1	2	3	10	14	22
trimethylamine	amine-like; fishy			+++	+++	+++	+++	–	–
(*E*)-2-hexenal	green	850		+	+	+	+	+	+
methional	boiled potato-like	900		–	–	–	–	–	++++
1-octen-3-one	mushroom-like	972	1295	–	–	–	–	–	+
(*Z*)-1,5-octadien-3-one	geranium-like	980	1372	–	–	++	++	+++	++++
(*E*,*E*)-2,4-heptadienal	fatty	994		–	–	+	+	+	+
(*E*,*Z*)-3,5-octadien-2-one	fatty-fruity	1095	1443	++	++	++	++	+++	++++
unknown	fatty	1133		+++	+++	+++	–	–	–
(*E*,*Z*)-2,6-nonadienal	cucumber-like	1148	1578	–	–	–	+	++	+++
(*E*,*E*)-2,4-nonadienal	fried fat-like	1193		–	–	–	++	++	++++
3-methylnonane-2,4-dione	fruity-sweet	1240	1716	++	++	++	++++	++++	+++++
(*E*,*Z*)-2,4-decadienal	fatty-green	1293		–	–	+	++	++	+++
(*E*,*E*)-2,4-decadienal	fried fat-like	1309	1824	++	++	++	++	+++	++++
trans-4,5-epoxy (*E*)-2-decenal	metallic	1307	2006	–	–	–	–	–	+

[a] Odor quality of the compound perceived at the sniffing port; intensity of odor was evaluated using the following scale: +++++, very intense odor; +, slightly perceived odor; –, odorless at the sniffing port. Results are expressed as the mean of two sniffing runs. [b] Grade assigned to fish using the EU grading scheme for fatty fish.

nonane-2,4-dione was not a potent volatile in ice-stored sardine (FD factor ≤ 32), whereas it was detected with an intense odor in the SPME headspace of sardine stored without ice. Its sweet-fruity odor dominated the overall odor perceived in the skin of refrigerator-stored sardine (**Table 5**). We have previously discussed the occurrence of this volatile in anchovy (*23*). It is formed by singlet oxygen ($^1O_2$) mediated oxidation of furanoid fatty acids (*28*) occurring in the lipid fraction of several fish species. An ongoing investigation (unpublished data) revealed that 3-methylnonane-2,4-dione is also found in hake (*M. merluccius*).

Conclusion. The odor of ice-stored sardine in the very fresh state (up to day 2) is dominated by pleasant seaweedy and faint green top notes. AEDA showed that lipoxygenase-derived plant-like volatiles along with methional were the most potent odorants at this stage. Increase in concentration of (*Z*)-4-heptenal followed by (*Z*)-1,5-octadien-3-one and methional correlated with the appearance of an overall "fishy" odor in sardine of lower sensory grade (high A, QIM = 9). At a storage stage corresponding to grade B, distinct fish-like and oxidized aroma notes were likely imparted by (*E*)-2-nonenal and (*E*,*Z*)-2,6-nonadienal. The QIM proved to be more reliable in assessing sensory changes of sardine as compared to the EU grading scheme.

SPME headspace measurements of the volatiles from sardine stored in a refrigerator without ice revealed a good correlation between the early onset of overall fatty-oxidized aroma notes and the intensity/odor attributes of individual volatiles, for example, 3-methylnonane-2,4-dione and (*E*,*Z*)-3,5-octadien-2-one. TMA was detected as a highly volatile odorant of sardine by this method.

The present investigation has provided information on the volatile pattern of sardine at different freshness stages and compounds that may be regarded as indicators of a given freshness stage. The results of SPME headspace measurements suggest that this method may be useful in the characterization of off-flavors in fish.

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