JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of Semivolatile Compounds in Baltic Herring (*Clupea harengus membras*) by Supercritical Fluid Extraction−Supercritical Fluid Chromatography−Gas Chromatography−Mass Spectrometry

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The on-line supercritical fluid extraction−supercritical fluid chromatography−gas chromatography method was applied to the determination of volatile compounds of raw and baked Baltic herring (*Clupea harengus membras*). The analytes were extracted with supercritical carbon dioxide at 45 °C and 10 MPa pressure. After extraction, the volatiles and coeluted lipids were separated on-line using supercritical fluid chromatography and the volatile fraction was introduced directly into a gas chromatograph. In all, 30 compounds were identified from fish samples with mass spectrometry. The most abundant compounds in the fresh Baltic herrings were heptadecane and 1-heptadecene. When the fish were stored for 3−6 days at 6 °C, the total peak area of the volatiles began to increase and the proportions of short chain acids (acetic, propanoic, 2-methylpropanoic, and 3-methylbutanoic) also increased. After 8−9 days of storage, 3-methylbutanoic acid comprised about 36 and 40% of all volatiles in raw and baked herring, respectively.

KEYWORDS: Baltic herring; off-flavor; supercritical fluid chromatography; supercritical fluid extraction; volatile compounds

INTRODUCTION

Baltic herring (*Clupea harengus membras*) is a subspecies of the Atlantic herring (*C. harengus*) and lives in the Northern parts of the Baltic Sea. It is a fatty fish, the oil content of which varies seasonally from 2 to 11%, being highest in autumn (*1*−*3*). The fatty acid composition is nutritionally interesting due to the high amount of long chain polyunsaturated fatty acids (*1*, *2*). The content of important mineral elements (Ca, Mn, and Fe) is also high (*4*). Despite these nutritional benefits, the main part of the annual catch of Baltic herring is used for animal feed in Finland. One reason for the low consumption as food is the characteristic aroma that influences the acceptance of this fish by consumers. Just after it is caught, Baltic herring has a very mild odor that is not unattractive. The characteristic odor starts to arise a little later and is noticeable when the fish arrive at the stores. The final strong odor is sometimes recognized to be a problem for the fish processing industry.

During recent years, supercritical fluid extraction (SFE) combined either off-line or on-line with gas chromatography (GC) has become popular for aroma analyses. SFE−GC has been especially used to analyze the volatile compounds of plant material (e.g., fruits, herbs, and spices), but the technique has also been shown to be applicable to samples of animal origin. The volatile compounds of meat samples have successfully been extracted using supercritical carbon dioxide (*5*−*9*). The volatile fraction, often containing coextracted nonvolatile lipids, has been first extracted with SFE, and the volatiles have typically been analyzed using various modifications of GC−mass spectrometry (MS). Snyder et al. (*10*) used on-line SFE−GC−MS to identify and quantify the volatile and semivolatile contaminants in meat samples. The on-line SFE−GC method has also been developed for the identification of free fatty acids and some other flavor compounds of Swiss cheese (*11*).

Fish flavor has conventionally been analyzed by different headspace techniques. The applications based on supercritical fluids have mainly been developed for oil analyses (*12*−*16*).

The purpose of this research was to analyze the volatile and semivolatile compounds of Baltic herring using an on-line SFE−supercritical fluid chromatography (SFC)−GC system. The volatiles were analyzed from raw and baked fish samples. Special attention was paid to the aging of fish and thus to compounds, the proportions of which increased during storage at +6 °C of herrings. The herrings were stored, to strengthen the off-flavor, for 9 days maximum, which was longer than normally used for raw fish.

MATERIALS AND METHODS

Sample Preparation. Three batches of Baltic herring (*C. harengus membras*) were used for analyses. The first batch, in May 1998, was

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10.1021/jf010829c CCC: $22.00
Published on Web 03/03/2002

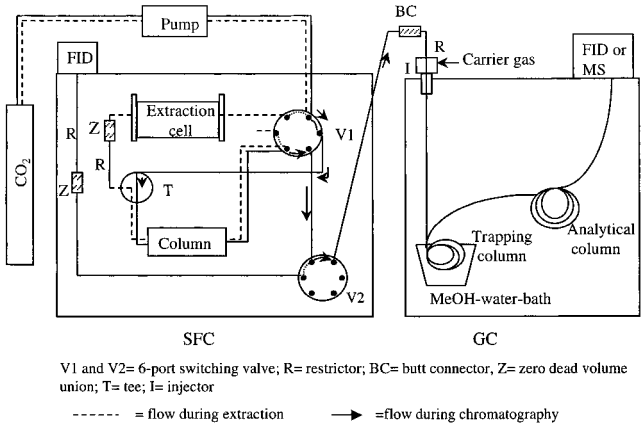

Figure 1. Schematic diagram of the on-line SFE–SFC–GC apparatus.

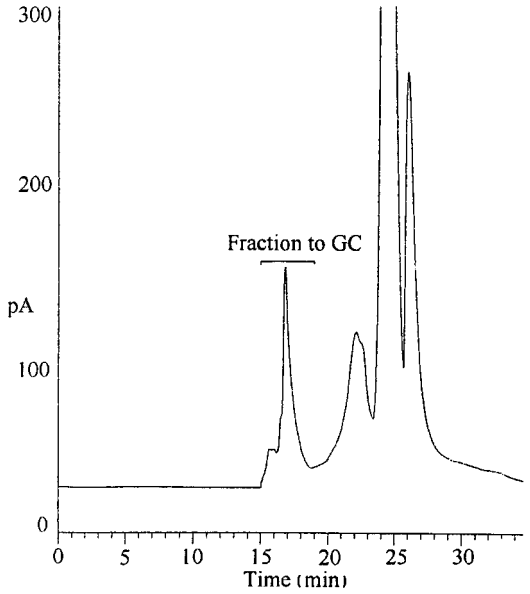

Figure 2. SFE–SFC–FID chromatogram of Baltic herring.

Table 1. Number of Samples Taken During Three Storage Periods of Baltic Herring

	days after catching								
fish batch	1	2	3	4	5	6	7	8	9
May 1998	R[a]	B[b]			R	B		R	B
Sep 1998	R	B	R	B			R	B	
Nov 1998	R	B			R,B	R	B	R	B

[a] Sample analyzed as raw. [b] Sample analyzed as baked.

filleted mechanically at a fish processing plant immediately after the landing of the local trawler. The next two batches of whole fish were purchased in September and November 1998 from a fish processing plant and headed and gutted manually in a laboratory.

Cleaned fish were placed in sealed plastic vessels and stored in a refrigerator at +6 °C for 8−9 days depending on the batch. The first analyses were made 1 day after they were caught, and the sampling continued as shown in **Table 1**.

Both the raw and the baked fish were analyzed. Approximately 100 g of Baltic herrings was oven-baked at 200 °C for 20 min and homogenized, and a 25 mg portion was put in an extraction cell (0.3 mL) together with Leco Dry matrix (Leco Corporation, St. Joseph, USA). Baking and homogenizing were carried out immediately before each analysis. When analyzing the raw herrings, a 40 mg portion was used. Analyses were repeated from 2 to 3 times.

Analysis of Volatiles. The volatile compounds were analyzed using an on-line SFE−SFC−GC system. The schematic diagram of the

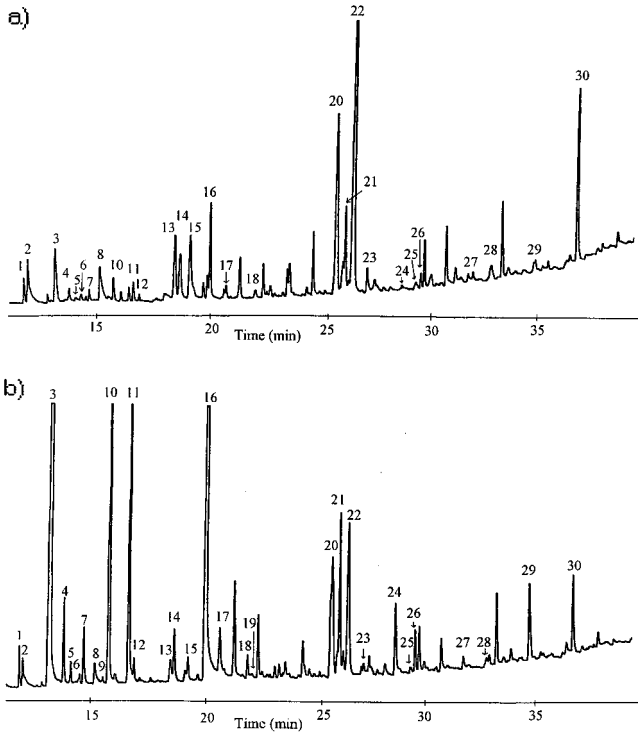

Figure 3. GC chromatograms of the volatiles from raw Baltic herring after 1 day (a) and 6 days (b) of storage.

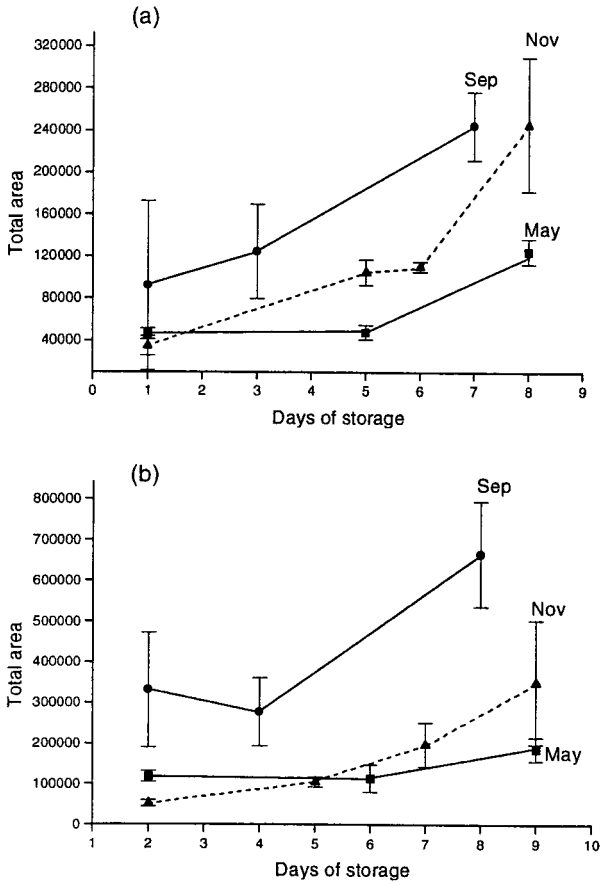

Figure 4. Effect of storage on the total volatile area of Baltic herring chromatogram analyzed as raw (a) and baked (b).

system is shown in **Figure 1**. A supercritical fluid chromatograph (Lee Scientific Series 600, Dionex, Salt Lake City, UT) was coupled to a gas chromatograph (HP 5890, Hewlett-Packard, Arondale, PA). The same SFC instrument was used for both extraction and chromatography

Table 2. Proportions of the Volatile Compounds Identified in Fresh and Stored Baltic Herring[a]

peak	compd	days after catching: 1 day (area %)[b]	7–8 days (area %)	identification[d]
1	2-butoxyethanol	0.55 ± 0.49	0.29 ± 0.16	MS
2	nonanal	3.30 ± 2.68	0.99 ± 1.26[c]	MS + RT
3	acetic acid	2.60 ± 1.18	15.64 ± 5.50[c]	MS
4	2,4-heptadienal	0.49 ± 0.42	0.55 ± 0.28	MS
5	1,5-octadien-3-ol	0.19 ± 0.24	0.18 ± 0.09	MS
6	2-ethylhexanol	0.24 ± 0.23	0.15 ± 0.18	MS
7	2,4-heptadienal	0.19 ± 0.30	0.30 ± 0.13	MS
8	decanal	3.58 ± 3.39	0.72 ± 0.82[c]	MS
9	3,5-octadien-2-one	0.20 ± 0.24	0.10 ± 0.09	MS
10	propanoic acid	0.78 ± 0.54	13.23 ± 7.33[c]	MS + RT
11	2-methylpropanoic acid	0.50 ± 0.37	6.37 ± 3.18[c]	MS + RT
12	2,2-dimethylpropanoic acid	0.24 ± 0.17	0.11 ± 0.08	MS
13	butanoic acid	2.82 ± 1.46	0.52 ± 0.49[c]	MS + RT
14	pentadecane	3.25 ± 2.85	1.37 ± 0.74[c]	MS + RT
15	1-pentadecene	4.94 ± 3.73	0.75 ± 0.64[c]	MS
16	3-methylbutanoic acid	2.25 ± 1.72	36.33 ± 9.31[c]	MS + RT
17	2,5-octadien-1-ol	0.46 ± 0.32	0.42 ± 0.18	MS
18	pentanoic acid	0.61 ± 0.67	0.38 ± 0.25	MS
19	hexadecane	0.19 ± 0.24	0.03 ± 0.06[c]	MS + RT
20	heptadecane	13.81 ± 3.09	2.27 ± 1.32[c]	MS + RT
21	heptadecene	3.17 ± 1.30	1.92 ± 0.91	MS
22	1-heptadecene	22.48 ± 20.39	4.51 ± 5.15[c]	MS
23	benzenemethanol	1.88 ± 1.27	0.33 ± 0.31[c]	MS
24	benzeneethanol	0.23 ± 0.24	1.72 ± 1.88	MS
25	2-ethylhexanoic acid	2.03 ± 2.77	0.24 ± 0.30[c]	MS
26	heptanoic acid	0.75 ± 0.18	0.40 ± 0.14[c]	MS
27	phenol	0.84 ± 0.92	1.57 ± 1.61	MS
28	octanoic acid	0.33 ± 0.37	0.15 ± 0.09	MS
29	isopropyl myristate	0.77 ± 0.98	0.15 ± 0.13	MS
30	nonanoic acid	5.00 ± 2.80	1.30 ± 0.55[c]	MS

[a] The fish were analyzed as raw. [b] Mean ± standard deviation for three fish batches. [c] Significant difference occurred during storage ($p < 0.05$). [d] MS + RT are mass spectra and retention time, which were consistent with those of reference compounds. MS is tentatively identified on the basis of mass spectra.

Table 3. Proportions of Volatile Compounds Identified in Fresh and Stored Baltic Herring[a]

peak	compd	days after catching: 2 days (area %)[b]	8–9 days (area %)	identification[d]
1	2-butoxyethanol	0.42 ± 0.39	0.21 ± 0.19	MS
2	nonanal	1.83 ± 1.42	0.57 ± 0.69[c]	MS + RT
3	acetic acid	2.69 ± 1.89	8.95 ± 3.58[c]	MS
4	2,4-heptadienal	0.31 ± 0.20	0.08 ± 0.06[c]	MS
5	1,5-octadien-3-ol	0.12 ± 0.16	0.06 ± 0.04	MS
6	2-ethylhexanol	0.17 ± 0.16	0.06 ± 0.06	MS
7	2,4-heptadienal	0.09 ± 0.13	0.05 ± 0.02	MS
8	decanal	1.52 ± 1.16	0.51 ± 0.68[c]	MS
9	3,5-octadien-2-one	0.16 ± 0.14	0.02 ± 0.02[c]	MS
10	propanoic acid	0.56 ± 0.36	12.64 ± 4.00[c]	MS + RT
11	2-methylpropanoic acid	0.81 ± 0.65	6.47 ± 2.69[c]	MS + RT
12	2,2-dimethylpropanoic acid	0.26 ± 0.20	0.10 ± 0.15	MS
13	butanoic acid	4.12 ± 1.24	1.00 ± 0.74[c]	MS + RT
14	pentadecane	1.32 ± 0.85	0.93 ± 0.67	MS + RT
15	1-pentadecene	3.77 ± 4.14	1.43 ± 1.13	MS
16	3-methylbutanoic acid	4.10 ± 4.00	40.59 ± 12.64[c]	MS + RT
17	2,5-octadien-1-ol	0.24 ± 0.13	0.14 ± 0.15	MS
18	pentanoic acid	0.59 ± 0.45	0.26 ± 0.21[c]	MS
19	hexadecane	0.32 ± 0.46	0.11 ± 0.11	MS + RT
20	heptadecane	21.43 ± 7.32	4.97 ± 2.79[c]	MS + RT
21	heptadecene	2.83 ± 2.35	0.75 ± 0.77[c]	MS
22	1-heptadecene	29.51 ± 15.54	8.35 ± 8.58[c]	MS
23	benzenemethanol	0.84 ± 0.39	0.35 ± 0.22[c]	MS
24	benzeneethanol	0.16 ± 0.15	0.76 ± 0.74	MS
25	2-ethylhexanoic acid	2.14 ± 3.12	0.44 ± 0.50[c]	MS
26	heptanoic acid	0.54 ± 0.12	0.29 ± 0.16[c]	MS
27	phenol	0.34 ± 0.24	3.49 ± 3.52[c]	MS
28	octanoic acid	1.22 ± 0.77	0.41 ± 0.34[c]	MS
29	isopropyl myristate	0.42 ± 0.31	0.15 ± 0.16[c]	MS
30	nonanoic acid	2.23 ± 2.00	0.58 ± 0.20[c]	MS

[a] The fish were baked before analysis. [b] Mean ± standard deviation for three fish batches. [c] Significant difference occurred during storage ($p < 0.05$). [d] MS + RT are mass spectra and retention time, which were consistent with those of reference compounds. MS is tentatively identified on the basis of mass spectra.

purposes. This was done by adding two valves and a tee in the oven of the chromatograph. The first valve (V1) (Rheodyne 7030, Cotati, CA) introduced the carbon dioxide flow either through an extraction cell into the ODS column or directly into the column. The second valve (V2) (Valco C6CWTY, Valco Europe, Schenkon, Switzerland) led the flow to the SFC−flame ionization detector (FID) or to a transfer line (50 cm × 100 μm i.d., 170 μm o.d.) leading to a GC injector. The GC interfacing system was similar to that previously described (*17*).

The volatiles were extracted at 45 °C and 10 MPa for 15 min. The extraction parameters (temperature, pressure, and time) were selected so that volatile or semivolatile compounds were not found in the repeated extraction. Pressure restriction for the extraction was achieved by using a 20 cm length of fused silica capillary tubing of 25 μm i.d. (Component Metal Service, Worcester, UK) after the extraction cell. Extracted compounds were collected at the front of an ODS C_{18} column, 100 mm × 1 mm i.d (Keystone, State College, PA) packed with 5 μm particles. To prevent the elution of the compounds, a pressure drop was created after the column by leading the CO_2 flow into the open air through the valve (V1). In the next step, the SFC was used to separate the lipid fraction from the volatile fraction at 45 °C and with the following pressure conditions: initial pressure 100 MPa, 2 MPa/min to 12 MPa, held at 12 MPa for 5 min, then 5 MPa/min to 30 MPa, and held at 30 MPa for 5 min. The first analytes (volatiles) were introduced into GC whereas lipids and the other, later eluting compounds were led to the FID (270 °C) of the SFC. After each analysis, the oven temperature was increased to 100 °C (20 MPa) in order to remove any lipid material possibly retained in the SFE−SFC system. The transfer line was occasionally cleaned by a CO_2 flow (20 MPa) and the GC oven was held at 250 °C.

The volatile fraction was collected into the trap column (SE-54, 2 m × 0.32 mm i.d. × 0.25 μm d_f, HNU Nordion, Helsinki, Finland) and prefocused before GC analysis. The trap column was kept in a methanol/water/ice bath during collection. An analytical column (EC-1000, 30 m × 0.25 mm i.d. × 0.25 μm d_f, Alltech Associates, Inc., Deerfield, IL) was connected to the trap column with a zero dead volume connector. The GC oven was programmed as follows: from 50 to 100 °C at a rate of 5 °C/min and from 100 to 250 °C at a rate of 3 °C/min. The GC injector and detector temperatures were 250 and 270 °C, respectively. The split flow and purge flow rates were 29 and 0.9 mL/min, respectively.

To identify the compounds, the SFC instrument was coupled with a gas chromatograph equipped with a mass selective detector (MSD, Shimadzu GCMS-QP500, Shimadzu Corporation, Kyoto, Japan). The configuration of the SFC−GC−MS system and the programs were the same as described for SFC−GC−FID. The MSD conditions were as follows: transfer line temperature, 250 °C; ionization voltage, 70 eV; mass range, *m*/*z* 35−310; and electron multiplier voltage, 1.3 kV. The herring volatiles were identified by comparing their mass spectra with those of reference compounds or with those in the Wiley mass spectral database (Shimadzu Corporation, Kyoto, Japan) and in previously published literature.

Statistical Analysis. Statistical analysis was performed using SPSS software, version 9.0. The differences between fresh and stored fish samples were tested using paired, two-tailed *t*-tests.

RESULTS AND DISCUSSION

The on-line SFC separation of a Baltic herring extract following the supercritical carbon dioxide extraction is shown in **Figure 2**. The first 15 min was the time of isolation where the volatiles and other eluted compounds were separated from the fish material and collected at the front of the ODS column. The extraction parameters, 45 °C and 10 MPa, were set to dissolve the whole volatile fraction in supercritical CO_2. Under these conditions, lipids also start to dissolve, which may cause problems in the direct gas chromatographic separation. Thus, the volatile fraction was separated from the coextracted lipids

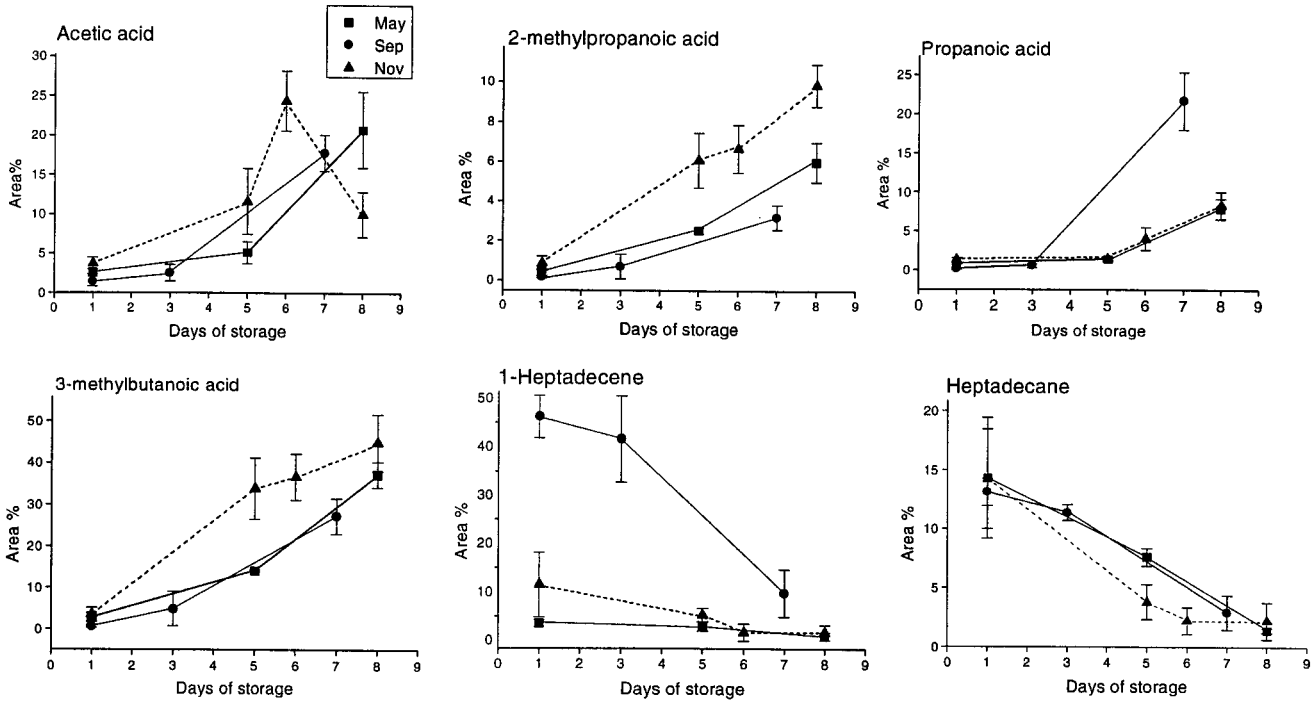

Figure 5. Effect of storage on the major volatile compounds of Baltic herring analyzed as raw. The fish batches were caught in May (■), September (●), and November (▲).

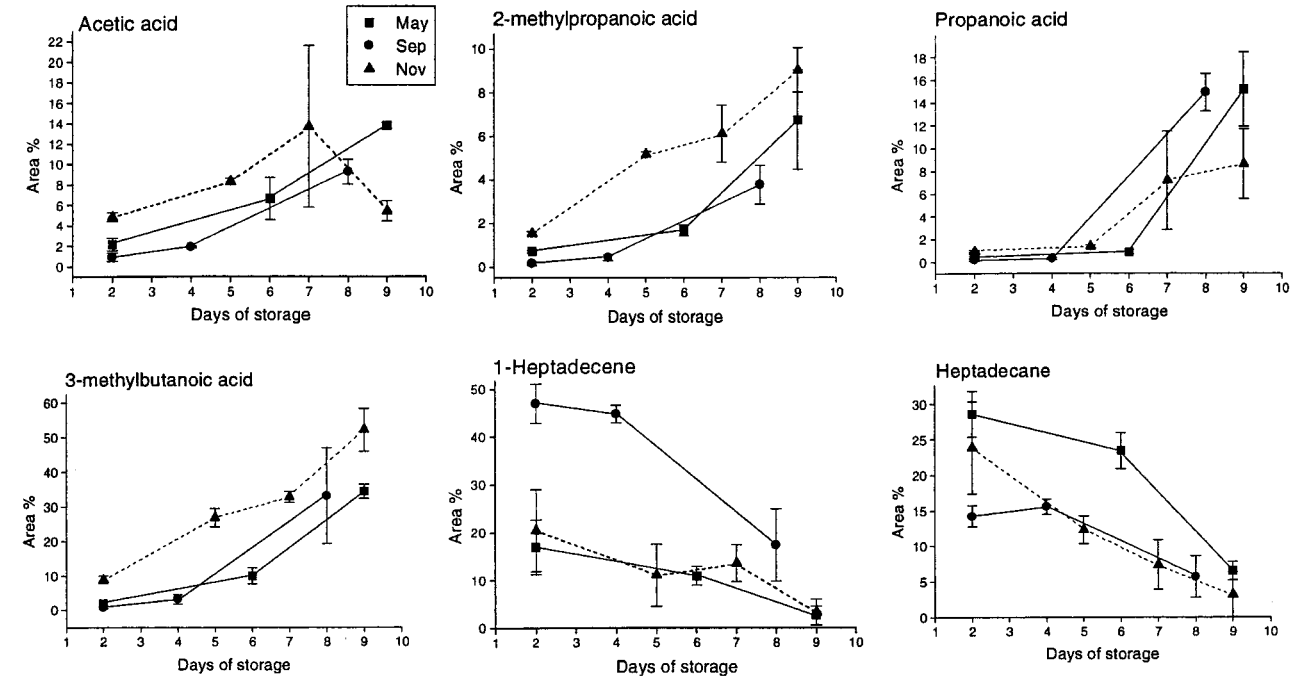

Figure 6. Effect of storage on the major volatile compounds of Baltic herring analyzed as baked. The fish batches were caught in May (■), September (●), and November (▲).

in the ODS column using supercritical CO_2 as the carrier fluid. The first fraction eluted from 15 to 19 min was directed to the GC for separation.

GC−FID chromatograms of fresh and stored raw Baltic herring are shown in **Figure 3a**,**b**, respectively. Baked fish gave analogous chromatograms, but the total area of peaks was higher despite the smaller sample amount used for the analysis. During storage, the total area of the peaks increased and the profile of the chromatogram changed significantly. The changes in the total peak areas of each herring batch are shown in **Figure 4**. The lowest peak areas after the storage period were obtained from fish caught in May, which were stored and analyzed as fillets. This may have inhibited the increase of volatile compounds formed by bacterial processes. The other fish samples were only headed and gutted. However, despite different area levels between fish batches, the total peak area of volatile compounds in each batch started to increase when the herring was stored from 3 to 6 days.

Identifications of the peaks and relative peak areas of raw and baked Baltic herrings are presented in **Tables 2** and **3**. The

relative peak areas are the mean values of three fish batches. In all, 49 volatile or semivolatile compounds were separated, 30 of which were identified. The identified compounds comprised a major percentage of total volatiles including alkanes, alkenes, acids, alcohols, aldehydes, ketones, and miscellaneous compounds. The peak numbers in the tables correspond to those in the chromatograms. The retention time of the first compound was approximately 12 min showing that some compounds with small molecular weights were lost during isolation. In all probability, those compounds were not retained in the ODS column after extraction but were vented out from the system through the column. The compounds such as hexanal, 1-octen-3-one, 1-octen-3-ol, and 1,5-octadien-3-one often identified from fish by headspace techniques (*18*−*20*) were not found in this investigation.

In the raw and baked herrings, 1−2 days old, the same compounds were identified. Heptadecane and 1-heptadecene were the most abundant compounds comprising over 30% of total peak area. N-alkanes (C_{10}−C_{17}) have been detected from fresh and oxidized whitefish (*21*, *22*) and menhaden oil (*23*, *24*), but their effect on flavor, even in high concentrations, has been noticed to be insignificant (*23*). The other abundant compounds were 1-pentadecene, nonanal, decanal, and short chain acids (acetic, butanoic, and 3-methylbutanoic). Trace amounts of 1-pentadecene have earlier been identified from tuna oil having a high docosahexaenoic acid content (*25*) and from oxidized whitefish, where it was seen to be one of the main components (*22*). Oxidized whitefish is also known to contain trace amounts of 1-heptadecene. The eight-carbon alcohols, 1,5-octadien-3-ol and 2,5-octadien-1-ol, found in low concentrations in fresh Baltic herring, are enzymatically derived from eicosapentaenoic acid via lipoxygenase action (*26*, *27*). They give a green, plantlike aroma to the fresh fish (*21*). Unsaturated carbonyls 2,4-heptadienals and 3,5-octadien-2-one were also detected, but their proportions were low. These compounds are nonenzymatically formed autoxidation products of unsaturated *n*-3 fatty acids (*28*, *29*).

During storage, the proportions of short chain volatile acids increased while the proportions of *n*-heptadecane and 1-heptadecene decreased significantly (**Tables 2** and **3**). After 7−9 days of storage, the most abundant compound was 3-methylbutanoic acid comprising 36% of all volatiles in the raw herring and 40% in the baked herring. The other main volatiles were acetic, propanoic, and 2-methylpropanoic acids. Acetic acid has been found in many fish species (*19*, *21*, *30*), but the other acids found in Baltic herring are not common in seafood. Acetic, propanoic, 2-methylpropanoic, butanoic, and 3-methylbutanoic acids have been identified from Alaska pollack (*31*) and smoked sardines (*32*). Volatile acids are important components of sauce made from fish and salt by fermentation (*33*, *34*). They are supposed to be formed from amino acids through bacterial fermentation, but lipid oxidation may also be responsible for some of the acids (*35*−*37*). Secondary oxidation of aldehydes can also form short chain acids (C_4−C_8) in fish (*29*). The volatile acids detected in the present study were probably produced via both mechanisms, oxidation and fermentation. In all probability, 2-methylpropanoic and 3-methylbutanoic acids were formed through fermentation because the content of branched fatty acids in Baltic herring is low (*2*).

On the first 1−2 storage days, the fish had the fresh, metallic odor characteristic to Baltic herring as stated by Luoma and Latva-Kala (*38*). During storage, the intensity of the odor increased, being very unattractive after about a week after catching. Hsieh et al. (*23*) reported that short chain fatty acids (C_2−C_6) gave a very intense and objectionable sweaty odor for menhaden fish oil. In addition, these fatty acids together with sulfur compounds were noticed to cause an offensive odor in solid fish waste (*39*). Obviously, acetic, propanoic, 2-methylpropanoic, and 3-methylbutanoic acids partially caused the unpleasant odor that increased during the storage of herrings.

The more detailed changes in the main compounds during the storage period in raw and baked fish are shown in **Figures 5** and **6**, respectively. The profiles of the curves representing three fish batches were rather similar, but there were differences between the levels. The fish caught in November had a higher concentration of acetic, 2-methylpropanoic, and 3-methylbutanoic acids than the other batches. In the same batch, the proportion of acetic acid seemed to reach the maximum within 6−7 days after which the level started to decrease. Despite decreasing trends of heptadecane and 1-heptadecene, these were still found in rather high amounts after the storage period. In fact, the areas of these compounds remained quite stable, and only their relative abundances decreased when the total area increased strongly. Between the herrings analyzed as raw and the herrings analyzed as baked, no major differences could be noticed in the main compounds.

CONCLUSION

A new on-line SFE−SFC−GC method for aroma analyses has been presented and was used for analyzing the volatile and semivolatile compounds of Baltic herring. Compounds seldom detected with other methods were found. The investigation showed that heptadecane and 1-heptadecene are abundant compounds in fresh Baltic herring. During storage, their proportions decrease while the proportions of volatile short chain acids increase strongly. Evidently, these acids partially cause the unpleasant odor that occurs when the herrings are stored but further investigation is required to study the influence of other compounds, sulfur-containing compounds, for example, on the odor of Baltic herring.

ABBREVIATIONS USED

FID, flame ionization detector; GC, gas chromatography; MS, mass spectrometry; MSD, mass selective detector; SFC, supercritical fluid chromatography; SFE, supercritical fluid extraction.

ACKNOWLEDGMENT

Ms. Mari Hakala is acknowledged for her assistance in the GC−MS analysis.

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Received for review June 26, 2001. Revised manuscript received November 23, 2001. Accepted December 18, 2001. This study was funded by the Ministry of Agriculture and Forestry and The Emil Aaltonen Foundation.

JF010829C