presence of smaller micelles with lower aggregation numbers. The chemical shifts of respective phospholipids were changed when 2 phospholipid classes were simultaneously present. The PC-PE mixture showed a broad peak, suggesting the formation of larger micelles with higher aggregation numbers. Two sharp peaks appeared in the simultaneous presence of PC and phosphatidic acid. When PC and phosphatidic acid were present, TCNQ was gradually solubilized with the increase in phospholipid concentration (Fig. 2, bottom). Therefore, the micelles with different aggregation numbers are assumed to be formed by steps into a definite association, as in the case of surfactants (21,22). Thus the 2 peaks in the ^{31}P NMR spectrum may reflect 2 species of small aggregates with lower aggregation numbers. These results indicate that PC may interact with PE or phosphatidic acid.

The simultaneous presence of 2 phospholipid classes changed their respective cmc (Fig. 2), suggesting that mixed micelles of PC with PE or phosphatidic acid were formed due to their interaction. The phospholipid-phospholipid interaction suggested by the results of hydration (Table I) and cmc (Fig. 2) experiments is supported by the results from ^{31}P NMR.

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Volatile Sulfur Compounds and Other Headspace Constituents of North Sea Fish Oils

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ABSTRACT

Headspace fractions of industrial oils produced from North Sea fish have been studied with emphasis on their sulfur-containing constituents, suspected inhibitors in the subsequent, catalytic oil hardening process. Fourteen individual, volatile sulfur compounds have been identified, accounting for virtually the total amount of such compounds in the volatile fraction. Sulfides, linear and cyclic di- and polysulfides, and a homologous series of methyl thiolesters, together make up the chemical patterns. Gas chromatography combined with mass spectrometry revealed a complex, but surprisingly constant pattern of the total, volatile fractions of several North Sea fish oils. About 100 individual compounds, including those containing sulfur, have been fully or partly identified. The structural characteristics and possible origin of the various types of compounds are discussed briefly.

INTRODUCTION

Fish oils are evaluated industrially by, among other things, their readiness to undergo catalytic hydrogenation (hardening), a process subject to marked inhibition by various factors. Among these, the sulfur content obviously plays a preeminent role (1,2). Several studies have indicated that certain water-soluble sulfur compounds, notably cysteine and methionine, rapidly undergo degradation to simple sulfur-containing products through bacterial activity in spoiling fish, whereas autolysis seems to be of minor importance in this connection (3,4). Despite the proven production under these circumstances of a few sulfur volatiles such as hydrogen sulfide, methanethiol and dimethyl sulfide, little is known about sulfur compounds present in industrial fish oils. On the assumption that insight into the chemical nature of the fat-soluble sulfur compounds present in or produced during the processing of spoiling fish may prove helpful in their removal or inactivation as inhibitors in the industrial hardening process, a systematic study of such compounds was undertaken in our laboratory. This paper reports the identification of the individual sulfur compounds encountered in the *volatile* fractions of typical, unrefined fish oils of North Sea origin. The studies were conducted by subjecting the oil headspaces to gas chromatographic (GC) analysis with simultaneous recording of both the total pattern of volatiles and, selectively, the sulfur-containing-constituents, supplemented by gas chromatographic/mass spectrometric (GC/MS) analysis. Though of less importance in the present context, an array of sulfur-free fish oil volatiles were encountered during our studies. They are discussed briefly at the end of this paper.

MATERIALS AND METHODS

All oils studied in this work were of industrial origin,

comprising a typical North Sea production oil of mixed, i.e., ill-defined origin and history (T40), and, for the sake of comparison, 4 other oils of similar character, collected from different Danish factories. A mackerel oil (E1) from the Faroe Islands was included in our studies.

All oils were analyzed for their contents of free fatty acids (FFA) and sulfur; sulfur analyses were performed by oxidative combustion-microcoulometry in a Dohrmann C-300 system, calibrated with thiophene (5). Sulfur volatile contents were determined by comparing sulfur analyses of the oil samples before and after keeping the magnetically stirred oil specimens at 10^{-6} mm Hg at 40 C for 1 hr. Headspace controls, performed as described next, ascertained that the removal of sulfur volatiles was complete under these conditions.

The headspace for GC was collected by placing 1 g of oil in a small, capped vial equipped with one hypodermic needle connected to a 1-atm nitrogen gas supply and another fitted onto an adsorption tube (about 12 cm × 0.7 mm id, packed with about 10 mg of 60-80 mesh Tenax GC [poly 2,6-diphenyl-*p*-phenylene oxide], a registered trademark of Enka N.V., The Netherlands). Suction (80 mm Hg) was applied for 5 min to the exit of the Tenax tube allowing the collection of about 50 mL of headspace, while the magnetically stirred oil sample was kept in a bath at 120 C. GC was performed on a Perkin-Elmer Sigma 1 model, equipped with both flame ionization and flame photometric detectors, FID and FPD, the FPD operated in the sulfur mode with linear output. A 40-m SCOT glass column, 0.5 mm id, coated with SP 2100, a methylsilicon stationary phase (manufacturer: SGE Inc.), was used. Carrier gas was He, 20 cm/sec; temperature program was 30-250 C at 4 C/min. The headspace sample was thermally desorbed from the Tenax tube at 325 C for 120 sec, and was trapped within the first few centimeters of the column, cooled to -78 C, by a Unijector injection system, operated in the concentrator/headspace mode (manufacturer: SGE Inc.). The column effluent was split 1:1 between the 2 detectors. Helium, 20 mL/min, was added as a make-up gas; the detectors were kept at 350 C.

The GC/MS analyses were performed on a VG Micromass 7070 F mass spectrometer equipped with a VG 2035 Data System and a Pye Unicam Series 204 gas chromatograph. The column was attached to the ion source at 200 C by all-glass transfer lines, kept at 200 C. The column and injector system (350 C) were as already specified.

RESULTS AND DISCUSSION

Sulfur-Containing Volatiles

The quantity of sulfur present in the headspaces of 3 production oils, with total sulfur contents of 20, 35 and 102 ppm, amounted to 19, 21 and 27%, respectively, of the total sulfur contents.

The GC pattern of the volatile sulfur compounds in the headspace of oil sample T40 (FFA=5.9%, S=35 ppm), is reproduced in Figure 1. Fourteen components comprising all the major ones have been structurally identified through their GC and MS characteristics, verified upon comparison with the data of authentic specimens, synthesized according to standard procedures. Peak numbers (Fig. 1) and structures are interrelated in Table I, which also contains the MS characteristics as determined in this laboratory. It appears that the observed constituents fall into 2 major structural groups: mono-, di-, or polysulfides and methyl thiolesters of lower, unbranched or terminally branched alkanoic acids. It is notable that dimethyl sulfide, reported in spoiling fish (3,4), was never observed in headspaces of oils studied in this laboratory, whereas methanethiol and hydrogen sulfide were occasionally observed by our technique, but only in small amounts.

In all oil specimens studied, dimethyl disulfide (peak 19) constitutes the quantitatively dominant sulfur compound,

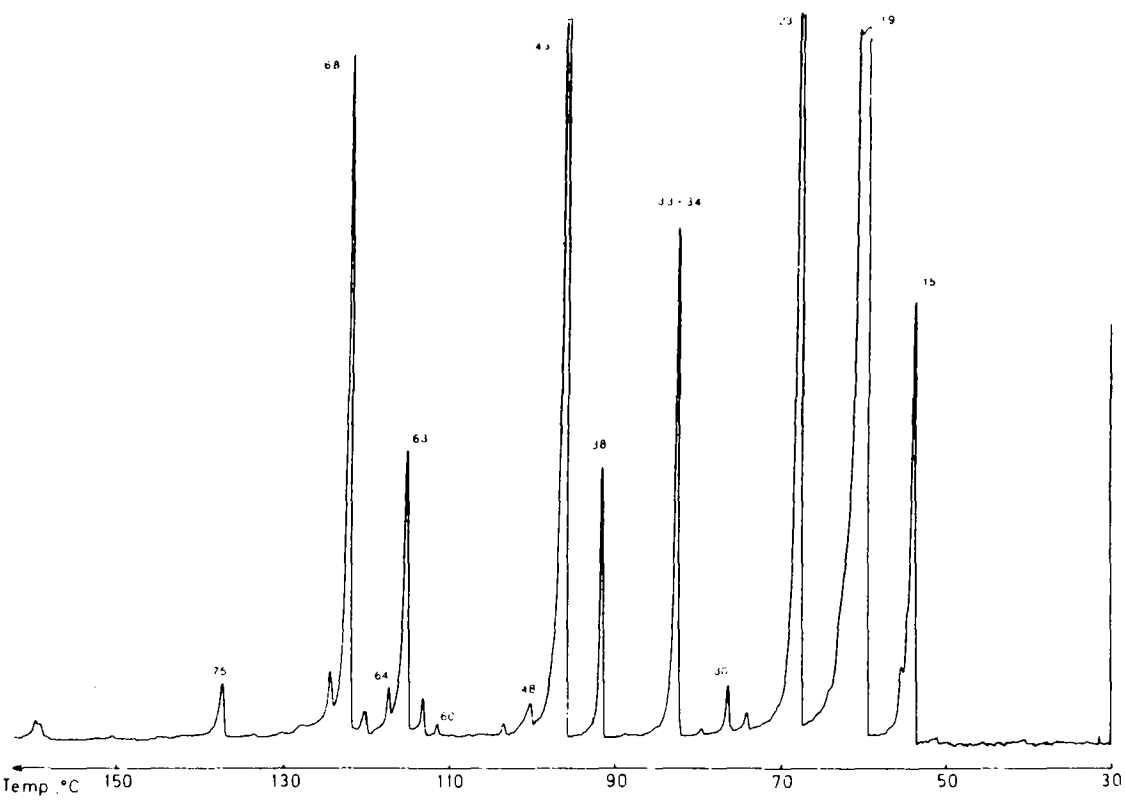
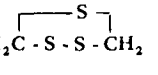


FIG. 1. Gas chromatogram of volatile sulfur-containing constituents in a representative industrial oil (T40) produced from North Sea fish; the chromatogram was recorded using a specific flame photometric sulfur detector. Peak numbers relate to the structures specified in Table I.

TABLE I

Volatile Sulfur Compounds in a Typical, Industrial North Sea Fish Oil

Peak no. (Fig. 1)	Structural formula	Mass spectrum: 8 highest peaks above *m/z* 20: *m/z* (% rel. int.)
15	CH_3COSCH_3	43(100), 90(24), 47(9), 45(7), 48(5), 44(4), 46(3), 75(2)
19	CH_3SSCH_3	94(100), 79(54), 45(37), 46(28), 47(17), 48(10), 61(10), 64(9)
23	$CH_3CH_2COSCH_3$	29(100), 57(76), 104(17), 27(16), 47(10), 75(8), 48(8), 45(7), [M - 15 (∿0)]
30	$(CH_3)_2CHCOSCH_3$	43(100), 71(50), 41(29), 29(26), 27(19), 48(16), 118(9), 75 (8), [M - 15 (∿0)]
33	$CH_3(CH_2)_2COSCH_3$	43(100), 71(63), 41(25), 27(19), 47(8), 75(7), 39(6), 118(5), [M - 15 (4)]
34	$CH_3SCH_2SCH_3$	61(100), 108(67), 45(28), 35(18), 27(13), 46(10), 47(8), 110(6)
38	$(CH_3)_2CHCH_2COSCH_3$	57(100), 85(78), 41(46), 29(36), 43(25), 75(17), 39(11), 47(9), [$M^{+\cdot}$ (3); M - 15(2)]
43	CH_3SSSCH_3	126(100), 79(44), 45(33), 47(23), 111(15), 64(15), 128(13), 46(13)
48	$CH_3(CH_2)_3COSCH_3$	57(100), 85(70), 29(55), 41(45), 117(15), 27(14), 75(9), 47(9), [$M^{+\cdot}$ (<1)]
60	$(CH_3)_2CH(CH_2)_2COSCH_3$	43(100), 99(58), 81(45), 71(24), 41(18), 75(16), 131(14), 29(13), [$M^{+\cdot}$ (<1)]
63		78(100), 124(96), 45(46), 60(28), 46(20), 59(15), 126(13), 80(10)
64	$CH_3(CH_2)_4COSCH_3$	43(100), 99(48), 71(33), 41(16), 29(15), 27(10), 55(9), 75(8), [$M^{+\cdot}$ (∿0); M - 15 (7)]
68[a]	$CH_3SSCH_2SCH_3$	61(100), 45(29), 140(13), 35(13), 46(12), 47(8), 63(6), 79(6)
75	$CH_3SSSSCH_3$	79(100), 158(76), 45(62), 64(43), 47(41), 126(40), 46(24), 94(22)

[a]MS data quoted from the literature (19).

most likely resulting from oxidation of initially formed methanethiol, an established bacterial degradation product of methionine. Dimethyl trisulfide (peak 43), previously recorded from contaminated fish (6), constitutes another major component, probably of related origin (cf. ref. 6). Both the di- and trisulfide are commonly encountered in foodstuffs, the trisulfide possessing a remarkably low odor threshold (0.01 ppb of water) (7). The thermally labile dimethyl tetrasulfide (peak 75) seems unprecedented as a product of natural derivation whereas the dimethylmercaptal of formaldehyde (peak 34) has been formerly reported as an odorous constituent of white truffle (8). 2,3,5-Trithiahexane (peak 68) may well be rather widely distributed within naturally derived products with reported occurrences in, e.g., cooked cabbage (7), linear sulfides, a cyclic counterpart, 1,2,3-trithiolane (peak 63), occurred in oil samples, representing another structure with precedent in natural product chemistry, viz., as a minor constituent of the marine red alga *Chondria californica* (10).

Several methyl thiolesters have been discovered in the last few years, resulting from poorly understood biological processes (11). The remarkable series of such esters, consistently present in North Sea fish oils, comprises derivatives of every C_2-C_6 straight-chain and C_4-C_6-isocarboxylic acid (Table I). There is reason to believe that higher homologs are absent, or present in marginal amounts at the most, inasmuch as synthetic, long-chain thiolesters (including C_{16}), when added to the oil samples in quantities comparable to those of the naturally occurring esters, presented no difficulties with regard to detection and identification. Five of the esters, peaks 30, 38, 48, 60 and 64, have previously been encountered, viz., as constituents of hop oil in which they occur accompanied by methyl 2-methylbutanethiolate and methyl heptanethiolate (12), 2 esters not observed in fish oils. Three unidentified sulfur compounds, with apparent molecular weights of 104, 132 and 132, were previously reported in the volatile fraction of sterile fish muscle inoculated with *Pseudomonas perolens* (6). Retention times and molecular weights now suggest that these unknowns may, in fact, be the methylthiol esters of propionic, isovaleric and valeric acids (peaks 23, 38 and 48, Fig. 1). Upon comparison of the original MS with those from the present study, Professor L.M. Libbey, Department of Food Science and Technology, Oregon State University, Corvallis, has confirmed that 2 of the published, volatile sulfur unknowns (6) are, in fact, the methylthiol esters of propionic and valeric acid (personal communication).

The general pattern of the sulfur volatiles in the oil T40 remains surprisingly similar in 5 additional oil samples collected from different factories and varying in total S contents from 20 to 110 ppm. Again, homogeneous oils produced from sand-eel (47 ppm S), pilchard (53 ppm S), sprat (31 ppm S) and Norway pout (153 ppm S) all exhibited patterns very similar to that presented in Figure 1.

Headspaces produced after addition of tri-*n*-hexylamine to the oil samples did not contain volatile sulfur-substituted amines. Apart from the disappearance of the thermolabile dimethyl tetrasulfide and a minor sulfur constituent of unknown structure, the sulfur volatiles profile remained virtually constant over the temperature range 120-220 C. Heating of a sealed, nitrogen-covered oil sample at 140 C for 4 hr produced no other change in the pattern of sulfur volatiles.

An oil pressed from uncooked herring, frozen immediately after the catch and stored at 0 C for 13 days, contained traces of dimethyl disulfide (peak 19) as the only detectable sulfur compound in its headspace. When a freshly caught herring was kept at 12 C for 11 days before analysis, the headspace from the oil of the uncooked fish was similar to that of Figure 1, yet devoid of the cyclic trisulfide (peak 63) and supplemented with hydrogen sulfide and/or methanethiol. In the oil headspace produced after cooking, the complete pattern of Figure 1 had been restored. Hence, it can be concluded that the characteristic sulfur headspace pattern in fish oil is not, in general, a result of thermal processes taking place during the industrial cooking of fish.

The structural pattern of the volatile sulfur compounds in industrial fish oils (Table I) suggests that hydrogen sulfide and/or methanethiol play a decisive role in their formation. Convincing evidence exists for the formation in

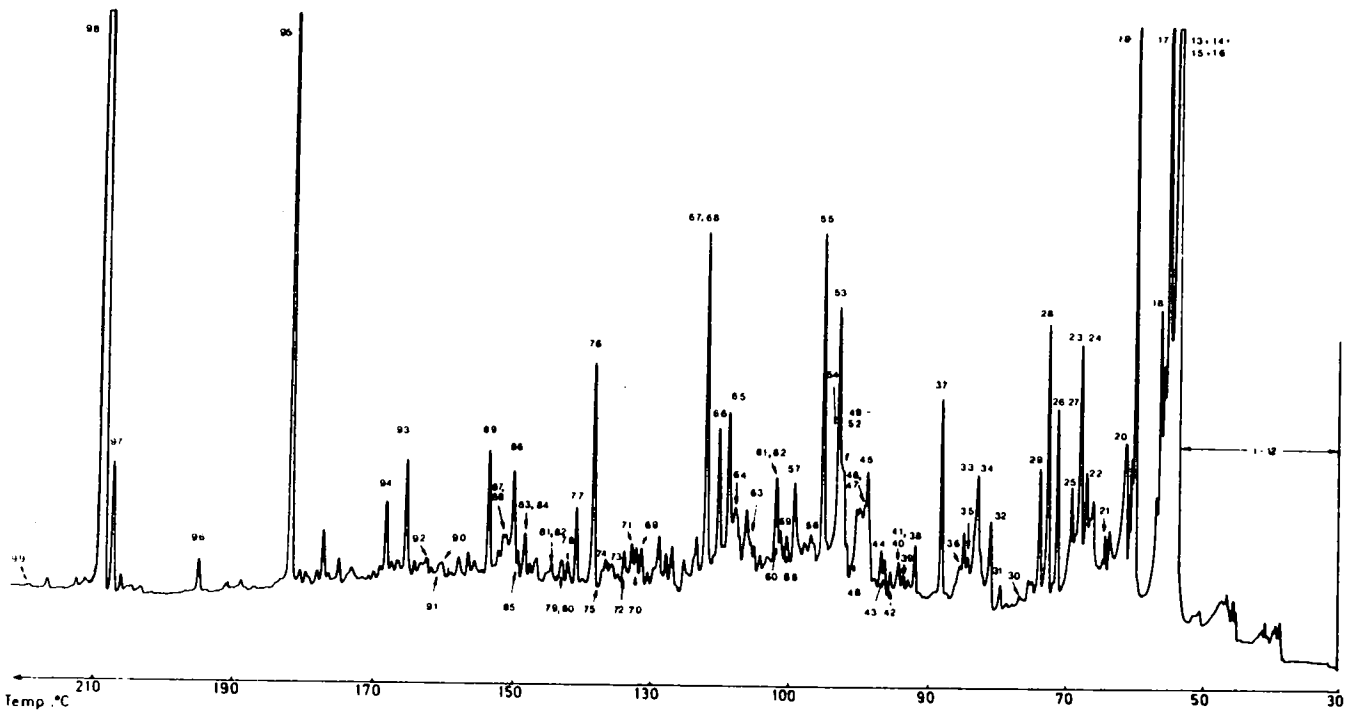


FIG. 2. Gas chromatogram of the total volatile fraction of a representative industrial oil (T40) produced from North Sea fish; the gas chromatogram was recorded using a flame ionization detector. Peak numbers relate to the structures and compositions specified in Table II. The quantities of compounds 1-12 are not representative of those present in the oil sample, due to poor capacity of the adsorption tube for small, polar molecules. The poor resolution in this region of the gas chromatogram results from difficulties in the application of such compounds to the column by our transfer technique.

spoiling fish of hydrogen sulfide and methanethiol by bacterial decomposition of cysteine and methionine, respectively (3,4). Reaction, conceivably nonenzymic, between methanethiol and activated carboxylic acids, resulting from bacterial activity, seems a likely origin of the various methyl thiolesters. With regard to the sulfides, structural inspection of components 34, 63 and 68 (Table I) suggests that they all derive from methanethiol or hydrogen sulfide, and a formaldehyde equivalent. It may be significant in this connection that formaldehyde is, in fact, a reported constituent of marine organisms, including fish (13,14), formed by enzymic degradation of trimethylamine oxide (13,15), present in substantial quantities in saltwater fish and rapidly degraded during fish spoilage prior to the steep increase in the sulfur contents of the oil (1). In this connection, it appears relevant that a labile component of the nonvolatile sulfur fraction of fish oil was identified in this laboratory as $CH_3SCH_2OCH_2OCH_2OH$ by MS of its trimethylsilyl derivative (E. Kelstrup, unpublished data).

The way is now open to study the effect of the individual and combined volatile sulfur compounds on the catalytic hydrogenation, a process of paramount interest in the industrial hardening of fish oil. Studies in this laboratory have revealed that the fraction of nonvolatile sulfur compounds in oils produced from spoiling North Sea fish is of extreme chemical complexity, consisting of a vast number of compounds varying widely in molecular size, polarity and stability.

Non-Sulfur Volatiles

During the above studies of the volatile sulfur constituents in headspaces of oils produced from spoiling North Sea fish, we decided to subject the GC patterns of other volatiles, as well, to closer scrutiny. As in the case of the sulfur-containing species, the total GC patterns of several fish oils, widely varying in their origin and contents of FFA and sulfur, exhibited surprisingly small variations. Hence, the headspace pattern of oil T40, reproduced in Figure 2, can be regarded as characteristic of other fish oils produced from North Sea fish. About 100 individual components, including the sulfur volatiles, have been fully or partly identified by MS. Structure assignment rests on comparison with published spectra or, in several cases, with spectra recorded in this laboratory. The various components are given in Table II according to the order in which they emerge from the GC column. The GC pattern of the headspace of the same oil, collected at 160 C rather than 120 C, is unchanged, except for a number of novel peaks attributable to components of high polarity. Among these, butyric acid, isovaleric acid, phenol and 2 isomeric cresols have been unequivocally identified.

The observed abundance of alkanes, ranging in carbon numbers from 5 to 18, as well as of alkenes and/or alicyclic hydrocarbons, is remarkable. Within this class, pristane, $C_{19}H_{40}$ (peak 98), greatly dominates (Fig. 2), whereas farnesane, $C_{15}H_{32}$ (peak 93), another branched alkane isoprenoid, is present in more moderate concentrations. In addition to the aliphatic hydrocarbons, a series of their aromatic counterparts, ranging from benzene to variously substituted methyl-, ethyl- and propylbenzenes, occur as fish oil volatiles, albeit in lower concentrations. The occurrence of indole (peak 87), a nitrogenous maverick, is surprising. This picture is not at variance with previous reports on the occurrence of alkanes in fish (16). These hydrocarbons are metabolized much more slowly than those of the aromatic series (17), a fact which may account for the distribution pattern observed in our studies and elsewhere (18). It is striking that, apart from a few low-molecular weight alcohols and carbonyl compounds (peak range 1-12, Table II), the non-sulfur oil volatiles are devoid of oxygen. While these compounds are mostly well known products of microbial activity, products expected from autoxidation of lipids (e.g., C-6 alcohols or aldehydes) are curiously missing. The reason might be that fish storage in

TABLE II

Volatile Constituents of a Representative Industrial Oil Produced from North Sea Fish

Peak no. (Fig. 2)	Structure or name
1-12[a]	CH_3CHO; C_5H_{12}; $(CH_3)_2CO$; CH_3CH_2OH; C_5H_{10}; $CH_3(CH_2)_2OH$; $CH_3COC_2H_5$; $CH_3CH(OH)C_2H_5$; $CHCl_3$; $(CH_3)_2CHCH_2OH$; $(CH_3)_2CHCH_2CHO$; C_6H_6
13	C_6H_{14}[b]
14	C_6H_{12}[b]
15	CH_3COSCH_3
16	$C_2H_5COC_2H_5$
17	C_7H_{12}[b]
18	n-C_7H_{16}
19	CH_3SSCH_3
20	$(CH_3)_2CHCH_2CH_2OH$
21	$C_6H_5CH_3$
22	C_7H_{12}[b]
23	$CH_3CH_2COSCH_3$
24	C_8H_{14}[b]
25	C_8H_{14}[b]
26	n-C_8H_{18}
27	$Cl_2C=CCl_2$
28	C_8H_{14}[b]
29	C_8H_{14}[b]
30	$(CH_3)_2CHCOSCH_3$
31	$C_6H_5C_2H_5$
32	m- and/or p-$C_6H_4(CH_3)_2$
33	$CH_3(CH_2)_2COSCH_3$
34	$CH_3SCH_2SCH_3$
35	$C_6H_5CH=CH_2$
36	o-$C_6H_4(CH_3)_2$
37	n-C_9H_{20}
38	$(CH_3)_2CHCH_2COSCH_3$
39	C_9H_{16}[b]
40	$C_{10}H_{20}$[b]
41	C_9H_{16}[b]
42	$C_6H_5CH_2CH_2CH_3$
43	CH_3SSSCH_3
44	m- and/or p-$C_6H_4(CH_3)C_2H_5$
45	C_9H_{18}[b]
46	o-$C_6H_4(CH_3)C_2H_5$
47	$C_{10}H_{16}$[b]
48	$CH_3(CH_2)_3COSCH_3$
49	$C_{10}H_{18}$[b]
50	$C_6H_3(CH_3)_3$[b]
51	$C_{10}H_{20}$[b]
52	$C_{10}H_{16}$[b]
53	$C_{10}H_{16}$[b]
54	$C_{10}H_{20}$[b]
55	n-$C_{10}H_{22}$
56	$C_6H_3(CH_3)_3$[b]
57	$C_{11}H_{24}$[b]
58	$C_{10}H_{20}$[b]
59	$C_{11}H_{22}$[b]
60	$(CH_3)_2CH(CH_2)_2COSCH_3$
61	$C_{11}H_{20}$[b]
62	$C_{11}H_{22}$[b]
63	1,2,4-Trithiolane
64	$CH_3(CH_2)_4COSCH_3$
65	$C_{11}H_{18}$[b]
66	$C_{11}H_{18}$[b]
67	n-$C_{11}H_{24}$
68	$CH_3SSCH_2SCH_3$
69	$C_{12}H_{24}$[b]
70	$C_{12}H_{24}$[b]
71	$C_{12}H_{26}$[b]
72	$C_{12}H_{22}$[b]
73	$C_{12}H_{22}$[b]
74	$C_{12}H_{24}$[b]
75	$CH_3SSSSCH_3$
76	n-$C_{12}H_{26}$
77	$C_{13}H_{28}$[b]
78	$C_{13}H_{24}$[b]
79	$C_{13}H_{24}$[b]
80	$C_{13}H_{26}$[b]
81	$C_{13}H_{24}$[b]
82	$C_{13}H_{26}$[b]
83	$C_{13}H_{24}$[b]
84	$C_{13}H_{26}$[b]
85	$C_{13}H_{24}$[b]
86	$C_{13}H_{26}$[b]
87	Indole
88	$C_{13}H_{24}$[b]
89	n-$C_{13}H_{28}$
90	$C_{13}H_{26}$[b]
91	$C_{14}H_{26}$[b]
92	$C_{14}H_{26}$[b]
93	2,6,10-$(CH_3)_3C_{12}H_{23}$ (farnesane)
94	n-$C_{14}H_{30}$
95	n-$C_{15}H_{32}$
96	n-$C_{16}H_{34}$[c]
97	n-$C_{17}H_{36}$[c]
98	2,6,10,14-$(CH_3)_4C_{15}H_{28}$ (pristane)
99	n-$C_{18}H_{38}$[c]

[a]Identified by repetitive mass spectrometric scanning without assignment of the components to individual GC peaks.

[b]Of undecided structure; compositions inferred from overall mass spectrometric characteristics.

[c]Mass spectra not recorded; identification based on retention time.

deep shipholds from catch to processing occurs under virtually anaerobic conditions. The data presented here do not permit conclusions to be drawn as to the origin of the individual, sulfur-free volatiles of Table II (petroleum, plankton, or other materials in the food chain).

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