Detection of Radiation-Induced Hydrocarbons in Irradiated Fish and Prawns by Means of On-Line Coupled Liquid Chromatography−Gas Chromatography

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Radiation-induced hydrocarbons were analyzed in a fatty (halibut) and a lean fish (cod) as well as in a prawn species by on-line coupled liquid chromatography (LC)−gas chromatography (GC) combined with mass spectrometry. In irradiated halibut which is known to contain mainly saturated and monounsaturated fatty acids, all expected radiolytic alkanes, alkenes, and alkadienes could be detected. The yields of the C_{n-1} and $C_{n-2:1}$ hydrocarbons were comparable to those found in irradiated lipids of terrestrial animals and plants. However, in cod and prawns which contain high levels of polyunsaturated fatty acids (PUFA), the C_{n-1} hydrocarbons were found in concentrations which were up to 10 times higher whereas the $C_{n-2:1}$ products were again comparable to those of terrestrial animals and plants. The identification of radiation-induced hydrocarbons in fish lipids was achieved by transfer of the hydrocarbons from the LC column to the gas chromatographic column in fractions differing in their degree of unsaturation. For the first time, radiation-induced hydrocarbons with more than four double bonds generated from polyunsaturated fatty acids (20:4ω6 and 20:5ω3) could be identified.

Keywords: *Food irradiation; detection; hydrocarbons; fish; liquid chromatography; gas chromatography; mass spectrometry*

INTRODUCTION

Various methods for the detection of irradiated fat-containing foods based on the analysis of radiation-induced hydrocarbons have been published in recent years. If triglycerides are irradiated, preferential cleavage occurs in the α or β position to the carbonyl groups and, as a result, two main hydrocarbons are formed from each fatty acid (Nawar, 1972). One hydrocarbon has one carbon atom less than the parent fatty acid (C_{n-1}) and the other hydrocarbon has two carbon atoms less and an additional double bond in position 1 ($C_{n-2:1}$). After separation from the lipid fraction, these hydrocarbons are determined by gas chromatography (Nawar et al., 1990; Spiegelberg et al., 1994). It has been successfully applied to various food like frog legs (Morehouse et al. 1991), shrimps (Morehouse and Ku, 1992), liquid egg (Spiegelberg et al., 1994), soft cheese (Schulzki et al., 1995), and exotic fruit (Spiegelberg et al., 1993). Interlaboratory trials have established that this procedure can be applied as a routine method for the detection of irradiated chicken, pork, and beef (Meier and Stevenson, 1993; Morehouse et al., 1993; Schreiber et al., 1993, 1994) as well as of soft cheese and exotic fruits like avocado, papaya, and mango (Schreiber et al., 1995, 1996).

Up to now, little is known about the detection of radiation-induced hydrocarbons in fish lipids (Dubravcic and Nawar, 1969). In contrast to terrestrial animal or vegetable fats, fish lipids in general contain high levels of long-chained and polyunsaturated (PUFA) fatty acids. There is also a great variety in the fat content as well as in the fatty acid composition of the different fish species. Therefore, one species with high fat content (halibut) and a lean species (cod) as well as a species of prawn were chosen for the present investigation.

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Sample preparation and analysis of the hydrocarbons were performed by on-line coupled LC−GC (Biedermann et al., 1992). Recently, it has been established that, in comparison to the classical column chromatography on Florisil (Spiegelberg et al., 1994), the LC−GC technique is the more effective method: The hydrocarbons are separated from the lipid on a normal phase HPLC (LC) column and transferred directly to the gas chromatograph (GC). A further improvement is the fractional transfer of the hydrocarbons to classes with a varying degree of unsaturation which facilitates their identification (Schulzki et al., 1995, 1996). Detection and identification are performed mass spectrometrically.

EXPERIMENTAL METHODS

Materials. Cod, halibut, and prawns were purchased at a retail store and stored at −18 °C. Duplicate samples were irradiated at a temperature of 15 °C with ^{60}Co γ-rays at a dose rate of 25 Gy/min. The average absorbed dose per sample was 4 kGy.

The methyl esters of the following fatty acids (Sigma Chemical Company, St. Louis, MO) were irradiated separately under the same conditions with doses of 50 kGy: stearic acid (18:0), oleic acid (18:1ω9), linoleic acid (18:2ω6), linolenic acid (18:3ω3), and arachidonic acid (20:4ω6).

Extraction of Fat and Fatty Acid Analysis. Lipid was extracted according to Folch et al. (1957) using a mixture of chloroform/methanol (2:1). The fatty acid profile (Table 1) was determined according to a modification of the DGF Method C-VI-11a (1981): 30−50 mg of fat was saponified with 3 mL of 0.5 M methanolic NaOH after addition of antioxidant BHT and methylated with 3 mL of BF_3−methanol under reflux. The fatty acid methyl esters (FAMEs) were extracted with 10 mL of *n*-heptane. After being mixed with anhydrous Na_2SO_4, the extract was kept at approximately 4 °C until GC analysis.

FAMEs were analyzed gas chromatographically using a DB-FFAP column (30 m × 0.25 mm i.d. × 0.25 μm film thickness, J&W Scientific, Folsom, CA). The samples were injected in

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Table 1. Main Fatty Acid of the Examined Fish and Prawn Samples

	fatty acid concentration in mg/100 mg of lipid										
sample	16:0	16:1ω7	18:0	18:1ω9	18:2ω6	18:3ω3	20:1ω9	20:4ω6	20:5ω3	22:1ω11	22:6ω3
halibut	10.3	8.3	1.5	14.4	1.7	–	18.3		3.4	12.5	3.2
cod	8.1	0.8	2.5	5.3	0.5	–	1.1	1.9	6.2	–	18.2
prawns	5.5	1.0	3.4	5.6	2.6	0.9	–	2.9	2.6	–	1.3

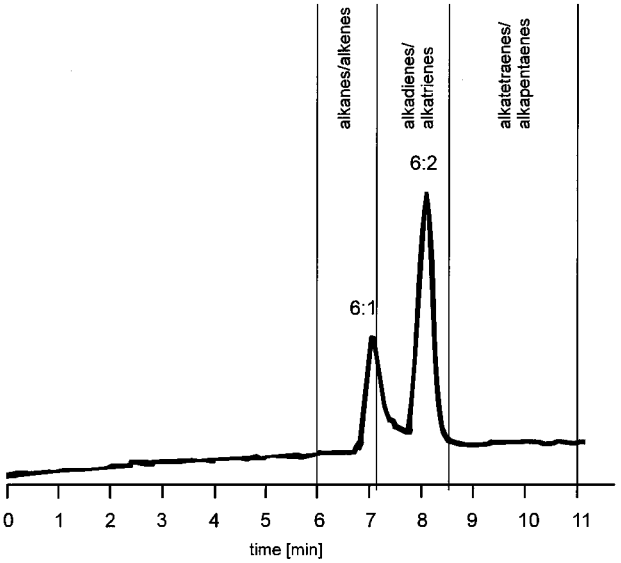

Figure 1. LC-UV chromatogram of a fish sample with standards 1-hexene (6:1) and 1,5-hexadiene (6:2) added. The time periods for the transfer of the selected hydrocarbon groups are indicated by the bars.

split mode at an injector temperature of 250 °C. The oven was programmed at a rate of 6 °C/min from 140 to 250 °C and kept isothermally for 7 min.

Separation and Fractionation of Hydrocarbons by On-Line Coupled LC−GC. The arrangement and principle of the coupled LC−GC system was described recently (Schulzki et al., 1995). The following modifications were made: A 125 mm × 4 mm i.d. cartridge packed with LiChrospher Si 60, 5 μm (E. Merck, Darmstadt, Germany) was used for LC separation. 20 μL of the 5% lipid solution in *n*-hexane was injected into the LC column to separate the hydrocarbons from the lipid. A selected hydrocarbon fraction was then transferred to the GC. The transfer times for the hydrocarbon classes of different unsaturation levels were determined by fractional transfer of a mixture of irradiated fatty acid methyl esters containing alkanes, alkenes, alkadienes, alkatrienes, alkatetraenes, and alkapentaenes. The GC retention times and mass spectra of these hydrocarbons served for identification of the radiolytic hydrocarbons in the samples. After transfer of the selected hydrocarbon fraction to the GC, the LC column was backflushed with *tert*-butyl methyl ether (17 min at a flow rate of 200 μL/min) and conditioned with *n*-hexane (15 min at 200 μL/min). A typical LC chromatogram with the added LC standards 6:1 and 6:2 is shown in Figure 1.

The on-column LC−GC interface and GC conditions were the same as described previously (Schulzki et al., 1995). Modifications were made for transfer times and closure times of the early vapor exit. After a transfer period of 150 s (transfer volume 500 μL) at an evaporation rate of 155 μL/min, the vapor exit was closed after 3.5 min (0.3 min is the dead time between transfer valve and vapor exit). For shorter transfer times, the vapor exit was closed earlier. Because long-chain hydrocarbons up to C22 had to be detected, the scanning range of the mass selective detector was set from 50 to 320 amu.

As standard substances, the saturated and the 1-unsaturated hydrocarbons were purchased from Sigma Chemical Company (St. Louis, MO). 8-Heptadecene and 1,7-hexadecadiene were synthesized by TeLa (Technische Lebensmittel- und Umweltanalytik GmbH, Berlin, Germany).

Table 2. Fatty Acids Contained in the Examined Samples and Expected Radiolytic C_{n-1} and $C_{n-2:1}$ Hydrocarbons

	radiolytic hydrocarbons	
fatty acid	C_{n-1}	$C_{n-2:1}$
16:0	15:0	1-14:1
16:1ω7	7-15:1	1,7-14:2
18:0	17:0	1-16:1
18:1ω9	8-17:1	1,7-16:2
18:2ω6	6,9-17:2	1,7,10-16:3
18:3ω3	3,6,9-17:3	1,7,10,13-16:4
20:1ω9	9-19:1	1,9-18:2
20:4ω6	4,7,10,13-19:4	1,3,6,9,12-18:5
20:5ω3	3,6,9,12,15-19:5	1,3,6,9,12,15-18:6
22:1ω11	10-21:1	1,9-20:2
22:6ω3	3,6,9,12,15,18-21:6	

RESULTS AND DISCUSSION

Fractionation of Hydrocarbons with Different Degrees of Unsaturation. The following fatty acids, stearic acid (18:0), oleic acid (18:1ω9), linoleic acid (18:2ω6), linolenic acid (18:3ω3), and arachidonic acid (20:4ω6), were analyzed separately after irradiation to determine the retention times of the radiolytic products. The main hydrocarbons C_{n-1} and $C_{n-2:1}$ as well as in smaller amounts, C_n (having the same number of carbon atoms as the parent fatty acid) and $C_{n-1:1}$ (having one carbon atom less than the parent fatty acid and an additional double bond in position 1) could be detected.

For analysis of the irradiated mixture containing all fatty acids mentioned above, the hydrocarbons were transferred for GC analysis in two fractions: a first fraction from 6:00 to 8:30 min (Figure 2A, top) and a second fraction from 8:30 to 11:00 min (Figure 2A, bottom). The hydrocarbons in both fractions were determined on the basis of the preceding individual analysis of irradiated fatty acid methyl esters and their mass spectra. The first hydrocarbon fraction contained the alkanes, alkenes, alkadienes and alkatrienes. But although the two isomers, 1,8-17:2 ($C_{n-1:1}$ from 18:1 fatty acid) and 6,9-17:2 (C_{n-1} from 18:2 fatty acid) had been expected, only one 17:2 peak was detected which could not be separated on the nonpolar capillary column used. Furthermore, 3,6,9-17:3 and 8-17:1 as well as 3,6,9-18:3 and 9-18:1 appeared as a single peak. The remaining alkatetraenes and alkapentaenes (see Table 2) were identified in the second fraction.

For separation of the coeluting peaks, the first hydrocarbon fraction was divided once more into two fractions. In the gas chromatogram of fraction 1.1, transferred from 6:00 to 7:10 min (Figure 2B, top), the alkanes and alkenes were detected completely. Fraction 1.2, transferred from 7:10 to 8:40 min (Figure 2B, bottom), contained all alkadienes and alkatrienes plus small amounts of 19:4. By this fractionation, a complete separation of the above mentioned hydrocarbon pairs

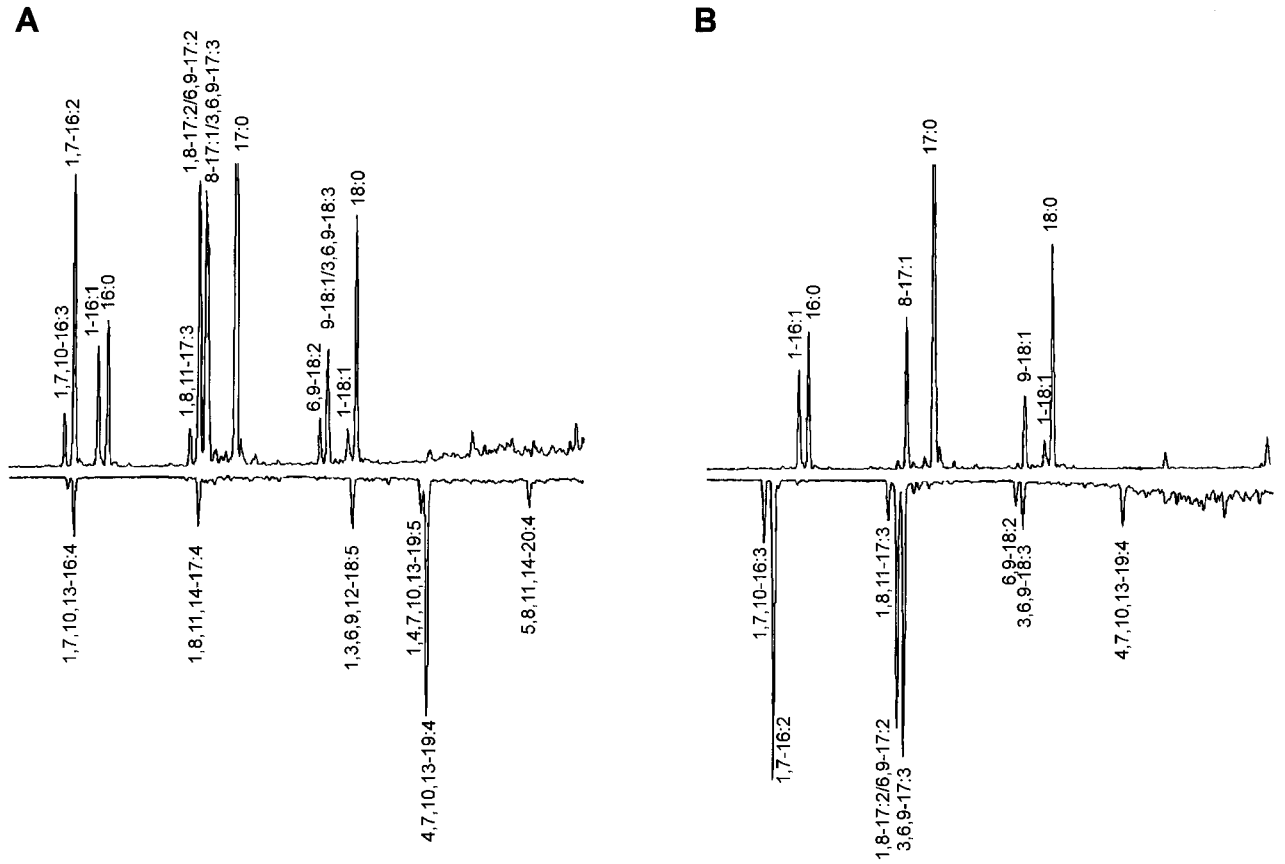

Figure 2. Fractionated transfer of the hydrocarbons from a mixture of the irradiated fatty acid methyl esters of stearic acid, oleic acid, linoleic acid, linolenic acid, and arachidonic acid. (A) First fraction transferred from 6:00 to 8:30 min (top), 2nd fraction from 8:30 to 11:00 min (bottom). (B) Fraction 1.1 from 6:00 to 7:10 min (top), fraction 1.2 from 7:10 to 8:40 min (bottom).

17:1/17:3 and 18:1/18:3 was achieved. A separation of the two 17:2 isomers was not obtained. To overcome this difficulty, a more polar capillary column should be tried.

By transferring smaller fractions, selective analysis of a single hydrocarbon type with respect to its unsaturation, for example of alkadienes, is possible. This offers also the possibility to separate the higher unsaturated hydrocarbons from alkanes and alkenes which are a typical result of radiation treatment and also frequently occur as contaminants from packaging material or food processing.

Fractionation of Radiation-Induced Hydrocarbons from Fish and Prawns. The halibut samples, being exemplary for a species with a high fat proportion, contained more than 10% oil. Most of the fatty acids were saturated and monounsaturated ones (Table 1). Therefore, the expected radiolytic C_{n-1} and $C_{n-2:1}$ hydrocarbons were mainly alkanes, alkenes, and alkadienes (Table 2) having been transferred with the first fraction (6:10 to 8:40 min).

The lean fish, cod, contained 0.4%−0.5% lipid and the prawns, 0.9%−1%. In contrast to the halibut extracts those of cod and prawns were of waxy consistency. Only 49% of the cod extract and 30% of the prawn extract could be attributed to triglycerides. In comparison to halibut, both species contained higher amounts of PUFA. According to their fatty acid composition, a broad spectrum of higher unsaturated hydrocarbons was expected (Table 2). Therefore, the hydrocarbons were transferred to GC in two fractions, the first one to separate alkanes to alkatetraenes and the subsequent one, to separate hydrocarbons with five and more double bonds.

Halibut. The non-irradiated controls of halibut contained the alkanes, 14:0, 15:0, 17:0, and pristane (Figure 3, top). Trace amounts of the alkenes 1-14:1 and 8-17:1 and higher concentrations of 1-15:1 were present, too. Furthermore, several other branch-chained or unsaturated hydrocarbons appeared in the controls which have not yet been identified. In the corresponding fraction of the irradiated samples (Figure 3, bottom), all of the expected C_{n-1} and $C_{n-2:1}$ hydrocarbons could be detected:

15:0 and 1-14:1 from 16:0 fatty acid

7-15:1 and 1,7-14:2 from 16:1ω7 fatty acid

8-17:1 and 1,7-16:2 from 18:1ω9 fatty acid

9-19:1 and 1,9-18:2 from 20:1ω9 fatty acid

10-21:1 and 1,9-20:2 from 22:1ω11 fatty acid

15:0 and 1-14:1 from 16:0 fatty acid and 8-17:1 from 18:1ω9 fatty acid which had been present already in the controls appeared in increased concentrations.

The calculation of the hydrocarbon yields per gram of precursor fatty acid and absorbed dose (Table 3) showed that both hydrocarbon types (C_{n-1} and $C_{n-2:1}$) were generated in the same order of magnitude, with slightly higher quantities for the C_{n-1} products. The average yields were 1.68 μg/g of fatty acid/kGy for the C_{n-1} and 1.36 μg/g of fatty acid/kGy for the $C_{n-2:1}$ hydrocarbons. These yields are comparable with those obtained for irradiated lipids of terrestrial animals and plants (Schulzki, 1996; Schulzki et al., 1997).

Cod. The first fraction of the non-irradiated control sample of cod (Figure 4, left) included, beside alkanes,

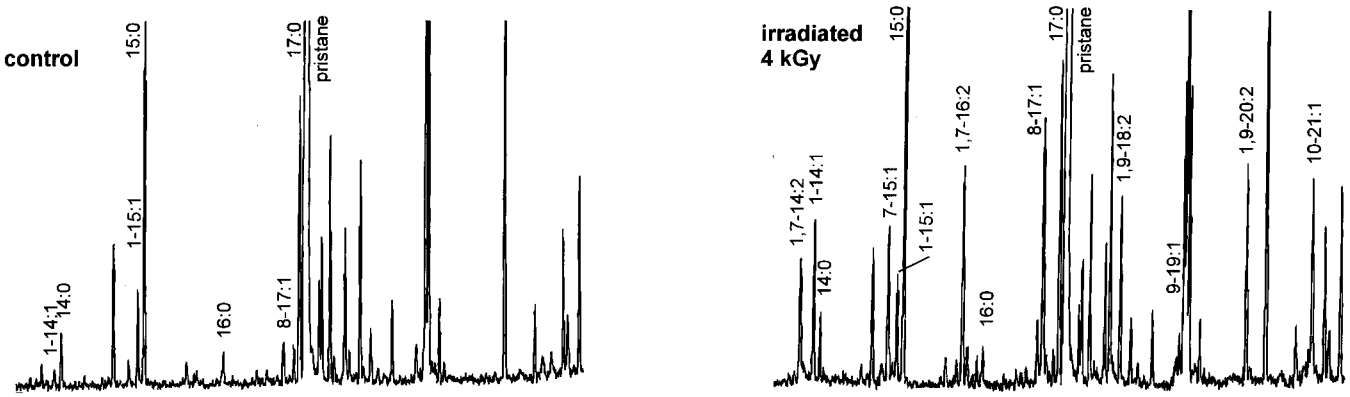

Figure 3. Gas chromatograms of the hydrocarbon fraction containing alkanes, alkenes, and alkadienes (6:10−8:40 min) from non-irradiated halibut (top) and halibut irradiated at 4 kGy (bottom).

Table 3. Calculation of the Concentrations of Radiolytic Hydrocarbons (Mean Values of Duplicates) per Gram of Precursor Fatty Acid and Absorbed Dose in the Lipid Fraction of Irradiated Halibut, Cod, and Prawns[a]

fatty acid	16:0	16:1 ω7	18:1 ω9	18:2 ω6	18:3 ω3	20:1 ω9	20:4 ω6	20:5 ω3	22:1 ω11
hydrocarbon	hydrocarbon yields in μg/g of fatty acid/kGy								
halibut									
C_{n-1}	nc	1.70	1.78	–	–	1.52	–	–	1.74
$C_{n-2:1}$	1.43	1.30	1.40	–	–	1.34	–	–	1.36
cod									
C_{n-1}	nc	19.19	23.35	*	–	17.77	18.72	18.79	–
$C_{n-1:1}$	1.72	nd	*	nd	–	nd	nd	nd	–
$C_{n-2:1}$	n.c.	nd	1.33	nd	–	nd	nd	nd	–
prawns									
C_{n-1}	nc	15.17	10.45	*	9.32	–	9.27	9.0	–
$C_{n-1:1}$	1.56	nd	*	nd	nd	–	nd	nd	–
$C_{n-2:1}$	nc	nd	2.27	nd	nd	–	nd	nd	–

[a] nd, not detected. nc, not calculated because relatively high levels of the hydrocarbon were already present in the non-irradiated sample. "–", precursor fatty acid present in too low amounts. "*", 1,8-17:2 and 6,9-17:2 not calculated because of coelution.

traces of the unsaturated hydrocarbons 1-14:1 and 8-17:1. In the corresponding fraction of the irradiated sample, the following C_{n-1} and C_{n-2}:1 products were detected:

15:0 and 1-14:1 from 16:0 fatty acid

7-15:1 from 16:1ω7 fatty acid

8-17:1 and 1,7-16:2 from 18:1ω9 fatty acid

6,9-17:2 from 18:2ω6 fatty acid

9-19:1 from 20:1ω9 fatty acid

4,7,10,13-19:4 from 20:4ω6 fatty acid

The concentrations of 15:0, 8-17:1, and 17:0 which had already been present in the controls increased considerably. Additionally, 1-15:1 was detected in the irradiated samples which is assumed to be the $C_{n-1:1}$ hydrocarbon from palmitic acid. The $C_{n-2:1}$ hydrocarbons were represented only by 1-14:1 and 1,7-16:2. Thus, $C_{n-2:1}$ products from the fatty acids, 16:1ω7, 18:0, 20:1ω9, and 20:4ω6, were missing.

In the second fraction of the irradiated samples (Figure 4, right), 3,6,9,12,15-19:5 was identified, being the C_{n-1} hydrocarbon from 20:5ω3 fatty acid. A small peak with the same retention time appeared in the second fraction of the control, too. Further higher unsaturated products, e.g., the $C_{n-2:1}$ hydrocarbon 1,3,6,9,12,15-18:6 from 20:5ω3 fatty acid or the C_{n-1} hydrocarbon 3,6,9,12,15,18-21:6 from 22:6ω3 fatty acid could not be identified.

The average yield of the C_{n-1} hydrocarbons generated in the lipid of cod was 19 μg/g of fatty acid/kGy which is more than 10 times higher than in halibut or terrestrial animals and plants (Table 3). This indicates that even radiolytic C_{n-1} hydrocarbons from fatty acids with concentrations of approximately 1% or greater of total lipids can be clearly identified at the given irradiation dose of 4 kGy, e.g., 7-15:1 from 1-16:1ω 7 fatty acid with a proportion of 0.8% of the total lipid, 9-19:1 from 20:1ω9 fatty acid with a proportion of 1.1% or 4,7,10,-13-19:4 from 20:4ω6 fatty acid with a proportion of 1.9%. The amount of $C_{n-2:1}$ hydrocarbon 1,7-16:2 was relatively small (1.33 μg/g of fatty acid/kGy) in comparison to the C_{n-1} products, however comparable to yields achieved in irradiated halibut or terrestrial animals. Additionally, the $C_{n-1:1}$ hydrocarbon 1-15:1 from 16:0 fatty acid was determined in a similar yield (1.72 μg/g of fatty acid/kGy) as the $C_{n-2:1}$ 1,7-16:2. For the remaining fatty acids, the proportion in the lipid extract was too low to detect the $C_{n-2:1}$ and $C_{n-1:1}$ irradiation products. The extremely large peak of the C_{n-1} hydrocarbon 6,9-17:2 from linoleic acid is explained by the fact that the $C_{n-1:1}$ hydrocarbon 1,8-17:2 was generated as well, as shown by the analysis of the radiolytic products of the single fatty acids. These two isomers cannot be separated under the chromatographic conditions applied (see also Figure 2).

Prawns. In the gas chromatogram of the first fraction of non-irradiated prawns (Figure 5, left), a series of saturated hydrocarbons was detected but also the

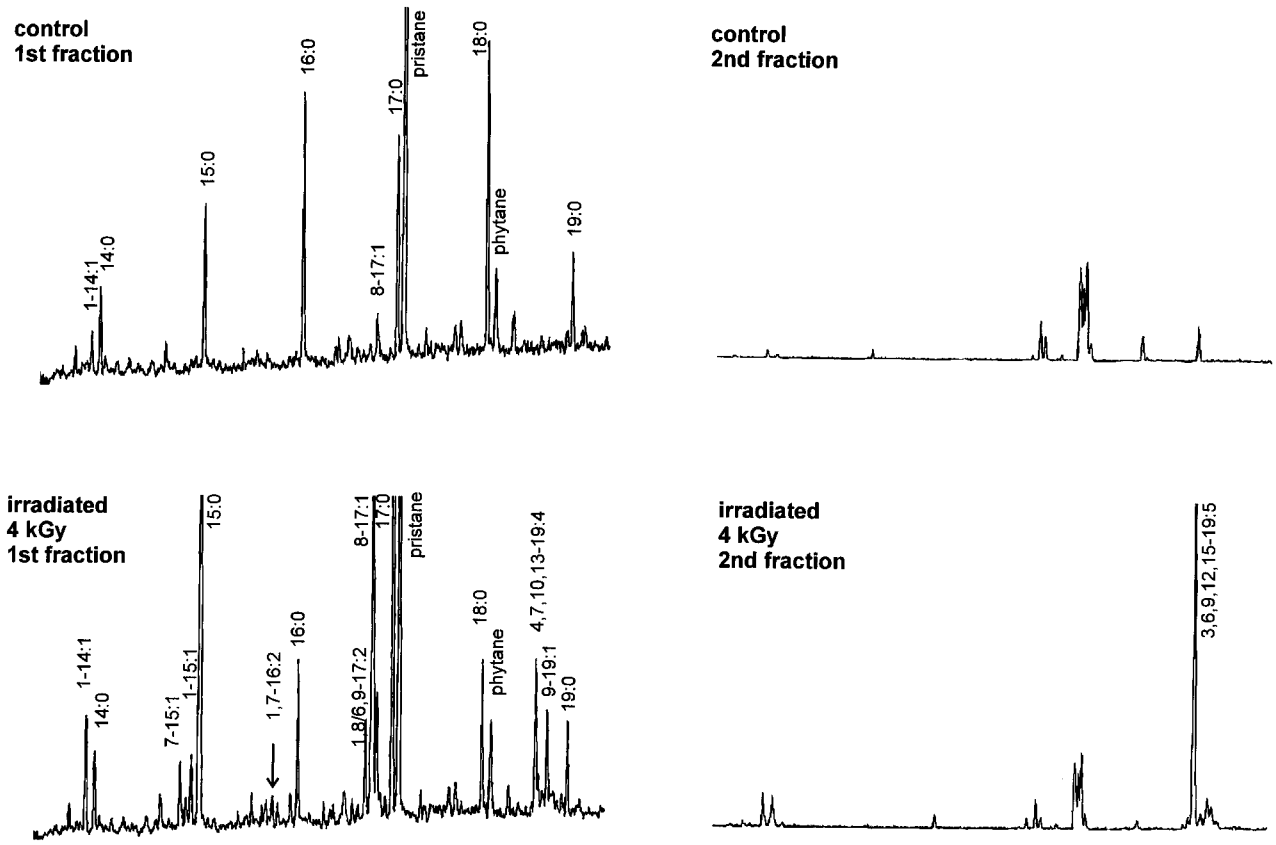

Figure 4. Gas chromatograms of the hydrocarbons from cod. Left, first fraction (6:10−8:40 min) containing alkanes to alkatetraenes; right, second fraction (8:40−11:10 min) containing alkapentaenes and higher unsaturated hydrocarbons; top, non-irradiated sample; bottom, irradiated at 4 kGy.

unsaturated ones, 1-14:1 and 1-16:1. The corresponding fraction of the irradiated samples contained the following C_{n-1} and $C_{n-2:1}$ products:

15:0 and 1-14:1 from 16:0 fatty acid

7-15:1 from 16:1ω7 fatty acid

8-17:1 and 1,7-16:2 from 18:1ω9 fatty acid

6,9-17:2 from 18:2ω6 fatty acid

3,6,9-17:3 from 18:3ω3 fatty acid

4,7,10,13-19:4 from 20:4ω6 fatty acid

Similar to the findings in cod, 1-15:1 was detected as the $C_{n-1:1}$ hydrocarbon from palmitic acid.

In the second fraction (Figure 5, right), 3,6,9,12,15-19:5 was identified as radiation-induced C_{n-1} hydrocarbon from 20:5ω3 fatty acid. The C_{n-1} hydrocarbon 3,6,9-17:3 from linolenic acid coeluted from the GC column with 8-17:1. Both were detected separately by the fractionated transfer of alkanes/alkenes and alkadienes/alkatrienes as had been carried out for the mixture of irradiated fatty acids (see Figure 2B). As in cod, the hydrocarbon from 22:6ω3 fatty acid was not detected.

The C_{n-1} hydrocarbons were also generated in considerably higher amounts (5-fold) than the $C_{n-2:1}$ and $C_{n-1:1}$ hydrocarbons (Table 3) whereas the C_{n-1} yields in prawns were less by one-half compared to cod. Nevertheless, C_{n-1} hydrocarbons from fatty acids with very low concentrations were clearly identified: 7-15:1 from 16:1ω7 fatty acid with a proportion of 1% of the total lipid and 3,6,9-17:3 from 18:3ω3 fatty acid, with 0.9%. As had been established for cod, most of the fatty acids were present in proportions too low to detect the corresponding $C_{n-2:1}$ and $C_{n-1:1}$ irradiation products. Again the isomers 6,9-17:2 and 1,8-17:2 coeluted as one peak.

CONCLUSIONS

For the first time, the present investigations have enabled the identification of radiation-induced hydrocarbons with more than four double bonds generated from polyunsaturated fatty acids (20:4ω6 and 20:5ω3). This was achieved by application of the on-line coupled LC−GC technique which facilitated hydrocarbon identification by fractional transfer to the gas chromatograph.

The exemplary analysis of a fatty fish, a lean fish, and a prawn species has revealed that there are differences in the hydrocarbon pattern of irradiated fatty foods depending on the lipid composition. In fatty fish species like halibut, both hydrocarbon types, C_{n-1} and $C_{n-2:1}$, were generated in similar amounts. In lean species like cod and prawns, the generation of C_{n-1} hydrocarbons was increased significantly and additional $C_{n-1:1}$ hydrocarbons were found. Comparable results were established for samples from other lean species like shrimps and frogs (Schulzki, 1996; Schulzki et al., 1997). We assume that the differences in the hydrocarbon yields are due to the fact that in lean species, most of the lipids are phospholipids which occur as structural lipids in the membranes of cells whereas in fatty species the fat is composed mainly of triglycerides.

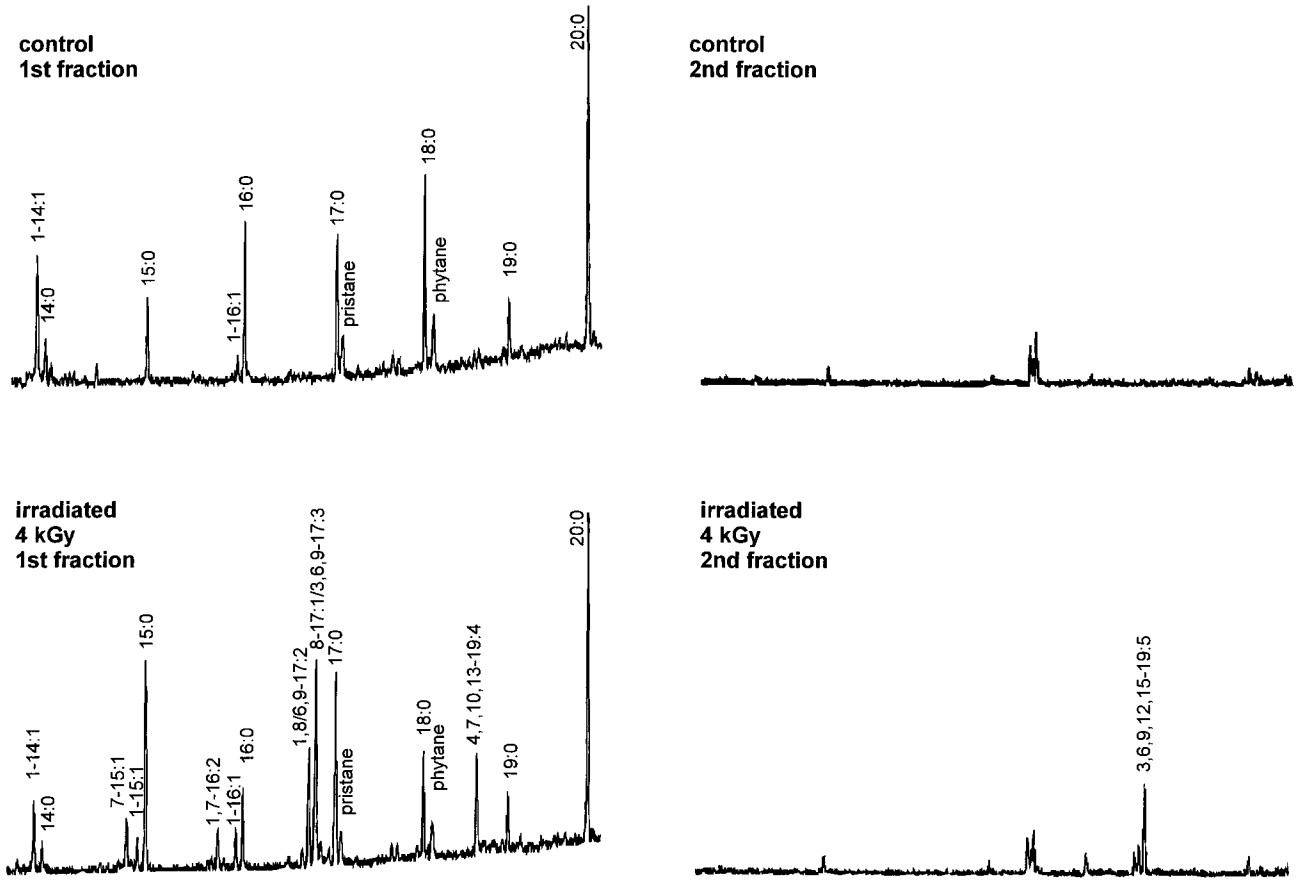

Figure 5. Gas chromatograms of the hydrocarbons from prawns. Left, first fraction (6:10−8:40 min) containing alkanes to alkatetraenes; right, second fraction (8:40−11:10 min) containing alkapentaenes and higher unsaturated hydrocarbons; top, non-irradiated sample; bottom, irradiated at 4 kGy.

$C_{n-2:1}$ hydrocarbons were generated in each of the examined samples in the same order of magnitude. The yields were also comparable to those achieved in terrestrial animals and plants. However, due to the low concentrations of the precursor fatty acids in the lean species, most of the $C_{n-2:1}$ hydrocarbons were not detectable at the dose absorbed (4 kGy).

On the basis of the present results, the following strategy for an identification of unknown fish or prawn samples should be followed: Fatty fish can only be identified as irradiated if all expected hydrocarbons with respect to the precursor fatty acid concentrations are determined. In lean species, the identification of every $C_{n-2:1}$ hydrocarbon might not be possible due to low triglyceride concentrations. In that case, the C_{n-1} products and especially those from higher unsaturated fatty acids are of major importance. They may be detectable down to precursor fatty acid concentrations of about 1% in the total lipid extract. The presence of only some saturated or monounsaturated hydrocarbons is not a conclusive argument to prove irradiation, since they can be already present in non-irradiated controls. The example of non-irradiated cod has shown that even in the fraction of higher unsaturated hydrocarbons, peaks with the retention time of radiation-induced hydrocarbons may appear. The fractionated transfer of hydrocarbons by on-line coupled LC−GC combined with mass spectrometry is, therefore, a very effective technique to clearly identify higher unsaturated hydrocarbons and to eliminate interfering substances in difficult food matrices such as fish and prawns which is necessary to draw a clear conclusion on the irradiation status of an unknown sample.

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Received for review April 3, 1997. Revised manuscript received June 30, 1997. Accepted July 1, 1997.[⊗]

JF970270+

[⊗] Abstract published in *Advance ACS Abstracts*, August 15, 1997.