Distribution and Identification of the Fatty Acids from the Coho Salmon, *Oncorhynchus kisutch* (Walbaum)[1]

JAMES B. SADDLER, ROBERT R. LOWRY, HUGO M. KRUEGER, and IAN J. TINSLEY,
Departments of Fisheries and Wildlife and of Agricultural Chemistry,
Oregon State University, Corvallis, Oregon

Abstract

To study the fatty acids of the coho salmon, entire fish were homogenized and the total lipids extracted with methanol-chloroform. The fish ranged in size from 75 to 85 mm total length and contained from 2.1%–6.9% lipid in the tissues. Methyl esters of the fatty acids were produced with anhydrous methanol and HCl. Qualitative identification of the fatty acid methyl esters was accomplished by gas-liquid chromatography.

Thin layer silver nitrate-silicic acid plates were used to separate the component methyl esters according to the number of double bonds. Location of the ethylenic groups of the unsaturated fatty acid methyl esters was established by reductive ozonolysis and identification of the aldehydes and aldehyde-esters produced. The number of carbons in the unsaturated fatty acid methyl esters was determined by hydrogenation of each of the fractions.

Fatty acids found in the highest concentrations were: 16:0, 16:1, 18:0, 18:1, 18:4, and 22:6. Fatty acids 16:0, 18:1, 18:2, 20:5, and 22:6, differed markedly from concentrations found in tubificid worms, the exclusive diet of the fish during the experiment.

Introduction

For centuries, fish oils have been of interest and importance to man. Thus there is considerable information concerning fish lipids; but only recently has it been possible to obtain quantitative data on fatty acid composition through gas-liquid chromatography (GLC). This investigation was undertaken to identify the fatty acids in the coho salmon, *Oncorhynchus kisutch* (Walbaum), and to establish their relative concentrations.

In other studies of fish lipids (1,8) retention times have been used to identify the component fatty acids. In many cases, however, this procedure is not conclusive. Thus to complement this information a number of confirmatory procedures have been employed. The chain length of different fatty acids has been confirmed by hydrogenation and the number of double bonds in each fatty acid by chromatography on silver nitrate silicic acid thin-layer plates (TLC). The location of the double bonds has been determined by reductive ozonolysis and identification of the aldehydes and aldehyde-esters produced. A synthesis of the information from these analyses characterizes the different fatty acids with a high degree of certainty.

Materials and Methods

Sampling of the Fish

All fish used in this investigation were coho salmon, *Oncorhynchus kisutch* (Walbaum), taken from the Yaquina River near the town of Nashville, Oregon. The total lengths of the fish when analyzed were between 75 and 85 mm. The fish were collected between June and September of 1964 and were kept at the Oregon State University Oak Creek Fisheries Laboratory from two to six weeks. During the time the fish were retained in the laboratory, they were fed ad libitum exclusively on small aquatic worms of the genus *Tubifex*. The temperature of the water in the aquaria was maintained at 18C with an average dissolved oxygen content of 9.60 ppm.

Extraction of the Lipids

The entire fish used for the lipid analysis was kept frozen after removal from the aquaria until the time of the lipid extraction. The fish samples were placed in a Serval Omni-mixer for 2 min for comminution. The extraction procedure of Bligh and Dyer (3) utilized a mixture of chloroform and methanol. The extracted lipids were protected from oxygen by dry nitrogen gas and stored in solvent in a freezer at 0C until preparation of the methyl esters. An aliquot of the lipid extract was used to determine total lipid content in the fish. The remainder of the lipid extract was used for the preparation of the methyl esters.

Preparation of the Fatty Acid Methyl Esters

After the extraction of the lipids, methyl esters were prepared by adding 4% HCl in methanol to the lipids dissolved in hexane. Total methylation required 3 hr in a water bath maintained at 80C in a sealed tube. After cooling, the upper layer containing the methyl esters was removed. The fatty acid methyl ester mixture was purified by passage of the hexane solution through silicic acid columns prior to GLC analyses (13).

Gas Liquid Chromatography

Methyl esters of the fish oil fatty acids were analyzed with a Beckman GC-2 chromatograph equipped with a hydrogen flame detector. The column used was ⅛ in. by 6 ft aluminum and packed with 15% ethylene glycol succinate on Chromosorb P mesh size 35–80. The operating conditions of the chromatograph were as follows: Oven temperature 190C; helium flow rate 60 ml/minute; sample size 0.2 mg/10 μl and solid sample injection (9).

The relative concentrations of the fatty acids have been shown to be proportional to the peak areas (2). The peak areas were determined by multiplying the retention time of a given fatty acid methyl ester by the peak height (5).

Thin-Layer Chromatography

Thin-layer silver nitrate-silicic acid plates were used to separate the component methyl esters into bands according to the number of double bonds present in the fatty acids (11,12). Detection of the fatty acid bands was accomplished using 2,7-dichlorofluorescein as a spray and then viewing the plate under ultraviolet light.

---

[1] Technical Paper No. 2059, Oregon Agricultural Experiment Station.
[2] Work supported in part by research Grant No. EF105, U.S.P.H.S.

The fractions obtained on the silicic acid plates were carefully removed and extracted with chloroform. These fractions were analyzed by gas chromatography in order to verify previous tentative identification of the peaks obtained on the original GLC chromatograms. The best separations were obtained using 20% ether in hexane and developing until the solvent reached 10 cm above the point of spotting. The amount of lipid used per plate was between 10 and 20 mg of the fatty acid methyl ester mixture. By comparing the retention time of the chromatographed fractions from the TLC plates with the chromatograph of the original TLC sample it was possible to evaluate quantitatively the accuracy of the separation. If the original separation was poor, by respotting the previously separated fraction on another TLC plate, the purity of the separation was improved. After improvement poor separations were analyzed a second time by GLC.

Hydrogenation

The unsaturated fractions removed from the TLC plates were subsequently hydrogenated (7). The hydrogenation procedure was accomplished using 15 mg of the fatty acid methyl ester mixture in hexane and 20 mg of Adams platinum oxide, catalyst. After 30 min hydrogenation was complete and the sample was filtered to remove the catalyst. The hydrogenated samples were injected into the gas chromatograph for identification of the fatty acids present after hydrogenation. Hydrogenation converted the unsaturated acids to saturated acids and thus the chain length of the various acids was confirmed.

Ozonolysis

Location of the ethylenic groups of the unsaturated fatty acid methyl esters was established by reductive ozonolysis. An apparatus modified after Bonner (4) was used for ozonolysis of sample fractions separated by silver nitrate thin-layer plates and of standard fatty acid methyl esters of known purity. The reaction was conducted in a dry ice-acetone bath at −65C. The ozonide formed yielded on reduction an aldehyde and an aldehyde ester. Polyunsaturated acids give rise to one or more moles of malonaldehyde depending on the number of double bonds in the molecule (14). It was possible to separate the aldehyde and aldehyde-ester portions with silicic acid columns. The aldehydes were eluted with dichloromethane and the aldehyde-esters were eluted with ethyl formate. The aldehyde and aldehyde-ester portions were separately subjected to GLC anlysis at oven temperatures of 100C and 160C, respectively.

Identification of Fatty Acids

Comparison of the retention times of the fatty acid methyl esters obtained from the fish lipids with the retention times of fatty acid methyl esters of known purity from the Hormel Institute aided in the direct identification of peaks on the chromatographic record. The fatty acid methyl esters obtained from the Hormel Institute were: 8:0, 10:0, 12:0, 14:0, 16:0, 18:0, 20:0, 16:1, 18:1, 18:2, 20:4, and 22:6.

Identification of some saturated and unsaturated fatty acids was accomplished by plotting the log of the retention times versus the number of carbons in the chain (6). For fatty acids with the same number of double bonds, the graph of log retention time against the number of carbon atoms gives a straight line. This procedure allowed the tentative establishment of the number of carbons and the number of double bonds for the fatty acids contributing peaks to the chromatographic record. However, errors can be made by relying simply upon retention time as a means of fatty acid identification, since retention time varies not only with the number of double bonds but also with the location of the double bonds in the molecule.

Further identification of the unsaturated fatty acids was obtained by the hydrogenation and ozonolysis of the fractions obtained from the silver nitrate silicic acid thin layer plates. By hydrogenation, the number of carbons in the chain was established. Ozonolysis of the TLC fractions allowed the position of the double bonds to be determined.

Results

The average value for total lipid as a percentage of the total wet weight of 42 coho salmon was 4.4 ± 1.2%. The average lipid percentage varied from 3.65% after two weeks in the laboratory to 6.09% at the end of six weeks. Twenty-seven fatty acids were found in the coho salmon, ranging in quantity from trace amount (22:4) to an average of 19.3% (18:1) of the total fatty acids (Table I). There did not appear to be any simple relationship between the percentages of individual fatty acids and the total lipid content of the salmon. In general, salmon containing less than 3% total lipid (measured as a percentage of the wet weight of the fish), had lower percentages of 16:0, 16:1, and 20:1 and higher percentages of 20:4, 20:5, and 22:6 than fish containing more than 3% total lipid.

A sample chromatogram of the methyl esters of the fatty acids from a coho salmon is given in Figure 1. The average fatty acid percentage contribution of individual fatty acids to the total fatty acids are given in the upper part of Table I. Standard deviations of the percentage of individual fatty acids are given in the lower part of Table I. The saturated fatty acids comprised 26.5% of the total fatty acids. Fatty acids with one double bond comprised 34.8%, two double bonds 15.4%, three double bonds 2.1%, four double bonds 8.3%, five double bonds 5.6%, and six double bonds 7.4% of the total fatty acids present. The ratio of saturated to unsaturated fatty acids was 1:2.8.

Graphic representation of the relations between log

TABLE I

Mean Percentage Contribution of Individual Fatty Acids to Total Fatty Acids of *Oncorhynchus kisutch*

Number of Carbons	Doube bonds						
	0	1	2	3	4	5	6
	Mean percentage contribution						
8	0.1						
10	0.1						
12	0.5						
14	4.6	1.1					
15	0.5	0.4					
16	14.7	9.0	1.4				
17		0.6					
18	6.1	19.3	11.7	1.0	4.3		
20		3.0	2.4	4.0	3.8		
22		0.5	Tr[a]	Tr[a]	Tr[a]	1.8	7.4
	Standard deviation of percent contribution						
8	0.03						
10	0.02						
12	0.20						
14	1.23	0.28					
15	0.09	0.13					
16	1.46	1.33	0.23				
17		0.08					
18	0.62	1.59	1.59	0.25	1.74		
20		0.73	0.81	0.36	0.68	0.57	
22		0.13				0.40	1.79

[a] Tr indicates trace or less than 0.05%.

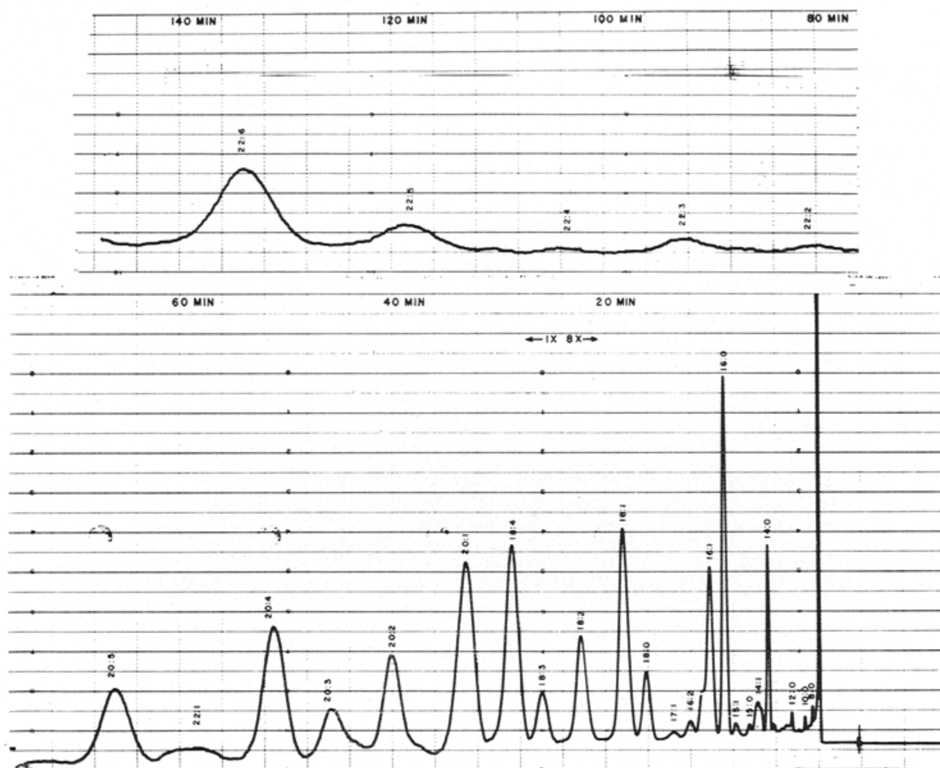

FIG. 1. Chromatographic record of the fatty acid methyl esters found in the coho salmon, *Oncorhynchus kisutch*.

retention time and the number of carbons in the chains of the aldehydes and aldehyde-esters, obtained on ozonolysis of fractions removed from TLC plates is given in Figure 2. The products resulting from the ozonolysis of the unsaturated fatty acids and the classification of the fatty acids, based on the terminal chain of the unsaturated fragments, are given in Table II.

Table III gives the fatty acid concentrations found in the tubificid worms used in feeding the salmon.

Discussion

The fatty acids of salmon differ markedly from the fatty acids of the tubificid worms (Table III). The salmon is not indiscriminately impounding the lipid received in the diet of worms. Specific fatty acids may have been selected for deposition and there are sufficient quantities of most acids in the diet to account for the fatty acids of the salmon. It is also possible that the fish is metabolizing specific fatty acids according to a pattern that will allow the incorporation of fatty acids of more importance to the fish. In the tubificid worms, fatty acids 14:0, 18:2, 20:4, and 20:5 occurred in greater amounts than in salmon; however, fatty acids 16:0, 18:1, and 22:6 oc-

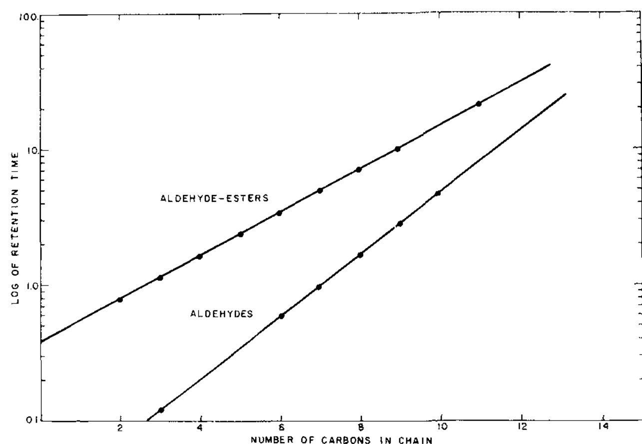

FIG. 2. Semilog plot of the retention times of the aldehydes and aldehyde-esters obtained from the ozonolysis of the unsaturated fractions separated by silver nitrate-silicic acid plates versus the number of carbons in the chain.

TABLE II

Products Resulting from Ozonolysis of Unsaturated Fatty Acids

Number of Carbons in the Chain of:

Number of Double Bonds	Aldehydes	Aldehyde-esters
1	6,7, and 9	8,9,11, and 13
2	6 and 7	6,9, and 11
3	3 and 6	8 and 9
4	3 and 6	5 and 6
5	3	5 and 7
6	3	4

Classification of Unsaturated Fatty Acids Based on the Terminal Chain of the Unsaturated Fragments

ω3 [a]	ω6	ω7	ω9
18:3	14:1	16:1	17:1
18:4	15:1	16:2	18:1
20:5	18:2		20:1
22:5	20:2		22:1
22:6	20:3		
	20:4		

[a] Symbol ω, indicating the aldehyde chain resulting from the ozonolysis of an unsaturated fatty acid (Mohrhauer and Holman, 1963).

TABLE III

Distribution of the Fatty Acids in the Total Lipids of Tubificid Worms

Number of Carbons	Double bonds 0	1	2	3	4	5	6
8	0.07						
10	0.23						
12	0.4						
14	6.2	3.3					
15	1.0	1.0					
16	7.8	7.5	1.7				
17		0.8					
18	4.8	11.5	17.5	0.1	3.3		
20		5.7	5.6	2.3	6.3	8.5	
22		Tr[a]	Tr[a]	Tr[a]	Tr[a]	1.4	3.0

[a] Tr indicates trace or less than 0.05%.

curred in greater amounts in salmon than in the tubificid worms.

Fatty acid 18:2 was 11.7% of the total fatty acids of the salmon under study, and this percentage is considerably higher than the figure of 1.2% reported by Gruger et al. for the fatty acid percentage in the fatty acids of coho salmon steak samples taken ahead of the dorsal fin (8).

A sample of worms comprised many individual animals whereas a sample of fish comprised only one animal. The weight of the worms and fish samples used per extraction of the lipids was about 3 g. Total lipid content was 3.33%, 3.19%, and 3.17% in separate masses of tubificid worms. The total lipid content of 42 coho salmon averaged 4.39% with a standard deviation of 1.17%.

Unsaturated fatty acids are sometimes named from the Δ or COOH end and sometimes from the ω or CH_3 end. The unsaturated fatty acids can be divided into series based on the length of the carbon chain beyond the double bond most distal from the acid group (10). The fatty acids comprising the ω3 series, ω6 series, ω7 series, and ω9 series contain 16%, 36%, 9% and 18%, of the total fatty acids found in the worms; and in salmon they are 18%, 21%, 10%, and 23%, respectively.

It is of interest that in the salmon the amounts of the ω3, ω6, and ω9 series are similar. The total percentage of saturates found in the worms is 20% whereas in the fish the total percentage is 27%.

The gas chromatographic tracing of the fractions collected from a TLC plate show some carry-over from one separated group into other groups. By spotting a plate with the fractions collected from the first separation, the fractionation of the fatty acids was improved. This procedure, however, reduced the amount of fatty acid that could be recovered from the plate for gas chromatographic analysis. Therefore, fatty acids occurring in higher percentages of the total were clearly visible on the chromatographic record while those occurring in small percentages were invisible. In this study, approximately 15 mg of fatty acid methyl ester mixture in hexane was used per 8 in. plate. This gave good separation on the plate and showed high resolution between components on the chromatogram obtained from GLC.

The identity of the different acids obtained by TLC on silver nitrate silicic acid plates could be established without question when one considered the relative amounts of aldehyde and aldehyde-ester obtained by ozonolysis. The most complex mixture was obtained in the one double bond group and the only two acids whose structure could not be established unequivocally were 15:1 and 17:1. These two acids were present only in very small quantities and in this study it was not possible to separate the components of 15:1 and 17:1 from the other acids with one double bond.

ACKNOWLEDGMENT

Work supported in part by research grant No. EF105, USPHS.

REFERENCES

1. Ackman, R. G., JAOCS *40*, 558 (1963).
2. Bartlet, J. C., and D. M. Smith, Can J. Chem. *38*, 2057 (1960).
3. Bligh, E. G., and W. J. Dyer, Can J. Biochem. Physiol. *37*, 911 (1959).
4. Bonner, W. A., J. Chem Ed. *30*, 452 (1953).
5. Carroll, K. K., Nature *191*, 965 (1961).
6. Evans, C. D., P. M. Cooney and E. J. Panek, JAOCS *39*, 210 (1962).
7. Farquhar, J. W., W. Insull, P. Rosen, W. Stoffel and E. H. Ahrens Jr. Nutr. Rev., Suppl. *17*, 29 (1959).
8. Gruger, E. H. Jr., R. W. Nelson and M. E. Stansby, JAOCS *41*, 662 (1964).
9. Lowry, R. R., Anal. Chem. *36*, 1407 (1964).
10. Mohrhauer, H., and R. T. Holman, J. Nutr. *81*, 67 (1963).
11. Morris, L. J., Chem. Ind. *27*, 1238 (1962).
12. Privett, O. S., and C. Nickell, JAOCS *39*, 414 (1962).
13. Smith, L. M., and R. R. Lowry, J. Dairy Sci. *45*, 481 (1962).
14. Stein, R. A., and N. Nicolaides, J. Lipid Res., *3*, 476 (1962).

[Received November 11, 1965]