Studies on Characterization and Variation in Triglyceride Fatty Acids from *Puntius sarana* Body Lipids

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ABSTRACT

Body lipids of *P. sarana* of four different sizes were fractionated into phospholipids, neutral lipids, nonsaponifiables, total fatty acids, polyunsaturated, monounsaturated and saturated fatty acid fractions. Percentage composition of each fraction was determined. The triglyceride fatty acids were identified by thin layer and gas liquid chromatography. C_8 to C_{23} fatty acids including both odd numbered and branched chain acids were detected. The major constituents were C_{14}, C_{15}, C_{16}, $C_{16:1}$, C_{18}, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$; forty-three other acids were detected in lower proportions. Composition of each fatty acids and their variation with size have been discussed. *P. sarana* body lipids in general showed a behavior typical of fresh water fish by having a higher percentage of saturated C_{16} and unsaturated C_{18} acids and a lower percentage of unsaturated C_{20} acid.

INTRODUCTION

For centuries fish oils have been of interest and importance to man. Although a considerable information about fatty acid from fish oils was available (1, 2), recently it has been possible to expand the knowledge of the complexity of fish oil by gas liquid chromatography. The geographical location of catch, season of the year, sex, age and feeding habits of the fish are reported to have a bearing on the nature of the fatty acids found in the fish (3). Lovern (2) studied the variation in the chemical composition of herring oil obtained from the same species during different parts of the year. Changes in fatty acid composition with growth have also been reported in pilchard (*Sardina ocellata*) (4). The effect of habitat on the nature and composition of fatty acids has been studied by Lovern (2) and Hilditch (5). It has been reported by various workers (6, 7) that there is a direct correlation between the lipids of fish and the nature of food intake.

The present investigation was undertaken to determine the composition of triglyceride fatty acids and their variations with fish size in the body lipids of *Puntius sarana*, which is economically an important fresh water food fish in India. This species has not been investigated for the fatty acid composition. Jafri and Qasim (8) reported only the total liver fat content from *P. sarana* along with other carps.

EXPERIMENT

Materials

All the chemicals and the solvents were A.R. (BDH/E. Merck). The solvents were redistilled under nitrogen before use. Standards were procured from Sigma Chemicals Company (U.S.A.).

Sampling of fish

The fish were collected from river Jamuna at Delhi during a three week period in October from the same locale. Only male fish were selected and transported in ice. Fish were sorted out into four different groups on the basis of their average length (10.2 cm, 16.4 cm, 23.7 cm and 29.3 cm) and were stored at -20 C until used.

Extraction of lipids

The preweighed samples were extracted by homogenizing the tissues in a mechanical blender with a mixture of chloroform and methanol (9). After flushing with nitrogen the extract was dried over anhydrous sodium sulphate, filtered and stored in a glass stoppered volumetric flask at -20 C. The volumetric flask was kept filled to the neck with solvent until used for further investigation. For storage longer than a week, an antioxidant, BHT (2, 6-di-t-butyl-4-methyl-phenol) was added.

Separation of Neutral lipids from Phospholipids

The solvent from lipid extract was removed at 30 C under a mild vacuum in a Büchi rotary evaporator. The phospholipids were separated out by acetone precipitation (10). The fatty acids were recovered after saponification (11) of neutral lipids. The mixed fatty acids were separated into their polyunsaturated, monounsaturated and saturated fractions by lithium (12, 13) and lead-salt (1) methods. The percentage composition of each fraction was determined (10). Methyl esters of the constituent fatty acids were prepared by diazomethane method (11) for chromatographic analysis.

Thin Layer Chromatography

Methyl esters were separated and identified by argentation TLC using 10% silver nitrate impregnated 0.5 mm thick layer of Silica Gel G plates and developed with diethyl ether-hexane (15:85) (14), and benzene (15); reversed-phase TLC was also used, using 0.5 mm thick layers of Kieselguhr G impregnated with 10% v/v liquid paraffin in petroleum ether (60-80°) at 5 C, and developed in nitromethane/acetonitrile/acetic acid (75:10:10) (16).

Gas Liquid Chromatography

Methyl esters were analyzed on Perkin-Elmer 900 model dual column chromatograph, equipped with both automatic digital and disc integrator, using flame ionization detector and employing either polyester (polar) or silicone (non-polar) column. Each chromatogram was run for half an hour more after the emergence of the last peak. The conditions were as follows.

Polyester column: Stainless steel column (3.6 m x 2 mm i.d.) packed with chromosorb W (mesh 80-100) impregnated with 20% (w/w) diethylene glycol succinate (DEGS); column temperature 200 C; nitrogen flow rate 20 ml/minute; sample size 0.3 λ to 2 λ (0.2 to 0.4% in Hexane).

Silicone column: Stainless steel column (2.4 m x 2 mm i.d.) packed with gas-chrome Q (90-100 mesh) impregnated with 5% (w/w) silicone rubber gum (SE-30) programmed from 160 to 280 C at the rate of 6 C per min with the initial and final hold times of 3 and 6 min respectively; nitrogen flow rate, 20 ml/min; sample size 0.4 λ to 1.5 λ.

Gas chromatograms of authentic samples were regularly run under exactly identical conditions in all cases. Sample peaks were identified by $\log_{10}$ relative retention time (17) and $\log_{10}$ relative retention temperature (18) vs. carbon number plotting procedures.

Unsaturated acids were confirmed by hydrogenating the methyl esters in methanol in the presence of platinum oxide catalyst and rechromatographing on DEGS column under identical conditions. Absence of cyclopropane fatty

TABLE I

Percentage Composition of Various Fractions of Total Lipids and Triglyceride Fatty Acids from the Body of *Puntius sarana* of Various Size Groups[a]

Average length of fish (cm)	Number of fish	Total weight of fish (gm)	Total lipid (%)	Phospholipid (%)	Neutral lipid (%)	Nonsaponifiables (%)	Fatty acids from neutral lipids (%)	Polyunsaturated (%)	Monounsaturated (%)	Saturated (%)
10.2	43	1374.6	5.49	2.10	3.32	0.16	2.92	0.41	2.38	0.1
16.4	8	1019.8	5.69	1.67	3.78	0.1	2.65	0.35	2.11	0.1
23.7	2	782.5	7.16	3.96	3.12	0.24	2.55	0.60	1.73	0.15
29.3	1	818.5	10.75	4.85	5.6	0.15	4.85	0.74	3.55	0.36

[a]Percentage of body weight.

acids and presence of branched chain acids was established by rechromatographing the hydrogenated samples after bromination in diethyl ether. In SE-30 analysis, the identification was confirmed by determining their methylene unit values (19).

The area counts were printed out by automatic digital integrator. Known standards were run regularly to check the linear response of the instrument. The percentage composition of each component was determined by area normalization.

RESULTS AND DISCUSSION

The percentage of total lipids, phospholipids, neutral lipids and fatty acids (saturated, monounsaturated and polyunsaturated) found in each size group are listed in Table I. The data show an increase in oil content with size. The fish of average length of 10.2 cm. had only 5.49% lipids as compared with 10.75% found in fish of 29.3 cm average length. The range of percentage lipids found in *P. sarana* lies within that found by Ackman (20) for fresh water fish, sheepsheard (*Aplodinotus grunnieus*), tullibee (*Coregonus artedii*), and alewife (*Alosa pseudoharengus*), and their variation agrees with the report on rainbow trout (21), and rohu (*Labeo rohita*) (22), which show graduation with size.

The amount of nonsaponifiable was quite low in all the four groups, and the lowest level of phospholipids was found in 16.4 cm size and the highest in the 29.3 cm. size fish. Neutral lipids, nonsaponifiables and total triglyceride fatty acids did not show any set pattern of variation, although the highest level of neutral lipids was found in the largest size fish. The relative distribution of triglyceride fatty acids among saturated and unsaturated (mono, poly) acids showed that poly and monounsaturated acids did not follow any set pattern of variation, but percentage of saturated acids increased with size.

The fatty acid methyl esters were analyzed by GLC on DEGS as well as on SE-30 columns. The peaks were identified from the plot of $\log_{10}$, relative retention time vs. carbon chain length. A few peaks in the chromatograms could not be identified unambiguously; therefore, the samples were hydrogenated and rechromatographed on DEGS column under identical conditions. The hydrogenation confirmed the presence of $C_{12:1}$, $C_{12:2}$, $C_{16:1}$, $C_{16:2}$, $C_{16:1}$, $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ as their peaks shifted to the position normally occupied by the corresponding saturated fatty acid methyl esters. It was suspected from $\log_{10}$ plot that the peak for $C_{20:4}$ could be either $C_{22:1}$ or $C_{20:3}$ or $C_{20:4}$. Hydrogenation confirmed that it was not $C_{22:1}$. The possibility of $C_{20:3}$ was ruled out, and this peak was identified as $C_{20:4}$ by following Ackman's (23) view. The identity of this acid was further confirmed by TLC. A few peaks in the chromatograms did not shift their position after hydrogenation, and therefore were suspected to be either branched chain or cyclopropane acids. This doubt was clarified by brominating the hydrogenated sample. The bromination of hydrogenated methyl esters did not lead to disappearance of any of those peaks on rechromatographing the sample, and these peaks were identified from $\log_{10}$ plot as branched chain fatty acids: $IC_{10:0}$, $AIC_{15:0}$, $AIC_{16:0}$, $AIC_{17:0}$ and $AIC_{22:0}$ (I = Iso; AI = Anteiso).

The samples were also analyzed on an SE-30 column to check the data obtained in DEGS column and also to verify if any acid of chain length higher than C_{23} was missed in DEGS analysis. The acids were identified from the plot of $\log_{10}$ relative retention temperature vs. carbon chain length and by calculating the methylene unit values. The SE 30 data indicated no fatty acid longer than C_{23}. However, acids like $C_{8:0}$, $IC_{9:0}$, $C_{9:0}$, $C_{10:0}$, $C_{11:0}$, $AIC_{13:0}$,

$IC_{19:0}$, $AIC_{19:0}$, $IC_{20:0}$, $AIC_{20:0}$, $IC_{21:0}$, $C_{21:0}$, $IC_{22:0}$ and $C_{22:0}$, which were missed during GLC on DEGS column (Table II), were detected here.

The estimation of the fatty acid components were done from the DEGS analysis. It was found that some acids which did not appear in the total fatty acid fraction did appear in the polyunsaturated, monounsaturated or saturated acids fraction in low concentrations. Therefore, the percentage of each peak was recalculated from the following formula:

$$A = B \times \frac{100}{100 + X}$$

where

A = new percentage of the fatty acid
B = percentage calculated from total triglyceride fatty acids fraction (Table I)
X = total percentage of the peaks that appeared in fractions other than total fatty acids fraction.

The results are presented in Table II. The components which were not detected in DEGS column but appeared in the SE-30 column were present only in negligible amount. While comparing the fatty acids detected in different size fish body lipids, it was observed that $AIC_{8:0}$, $AIC_{14:0}$ could be detected in 16.4 cm. size fish only. Similarly, $AIC_{10:0}$, $IC_{15:0}$ and $IC_{23:0}$ in 29.3 cm. size; $C_{19:1}$ in 10.2 cm. and $IC_{21:0}$ and $C_{22:2}$ in 23.7 cm. size fish only. The $IC_{9:0}$, $AIC_{11:0}$, $IC_{12:0}$, $IC_{13:0}$, $AIC_{13:0}$ were present only in 16.4 cm. and 23.7 cm. size, while $AIC_{12:0}$ and $AIC_{21:0}$ in 16.4 cm. and 29.3 cm.; and $IC_{20:0}$ could be detected in 23.7 and 29.3 cm. size fish only. The $IC_{10:0}$, $IC_{19:0}$, $IC_{22:0}$ and $C_{22:0}$ were absent in the 10.2 cm. size while these were present in all the higher sizes. Similarly, $C_{20:0}$ and $C_{20:1}$ were absent in 23.7 cm. size and $IC_{11:0}$ was absent in 29.3 cm. size fish.

The variations in the total percentage composition of various fatty acids in the body of *P. sarana* of different sizes are complied in Table III. The amount of saturated, even-numbered fatty acids decreased with size; 36.45% being in 10.2 cm. size fish lipids against 21.95% in 29.3 cm. size fish lipids. The major constituent was $C_{16:0}$, which was as high as 25.92% in 10.2 cm. size fish but decreased to 14.52% in 29.3 cm. size fish. Hilditch and Williams (5) and also Lovern (2) independently found that fresh water fish oil contains more $C_{16:0}$ acid than marine fish oil.

The straight chain odd numbered fatty acids from $C_{9:0}$ to $C_{21:0}$ were found to be present in *P. sarana* body lipids in low percentage. The $C_{15:0}$ was the major constituent followed by $C_{11:0}$. The percentage of total odd-numbered acids decreased with size, being 9.48% of total fatty acids in 10.2 cm. size and 1.68% in 29.3 cm. size fish.

The total percentage of branched chain acids in *P. sarana* body lipids in smallest size (10.2 cm) was only 1.08, which increased in 16.4 cm. size fish, and then gradually decreased to 3.88% in 23.7 cm. and to 2.9% in 29.3 cm. size fish, respectively. The level of iso-acids was lower than anteiso-acids in all cases. Main iso-acids present were $C_{9:0}$ to $C_{13:0}$, $C_{15:0}$ and $C_{19:0}$ to $C_{23:0}$, the major constituent being $IC_{10:0}$, which was found to be the highest in 16.4 cm. fish.

The Anteiso-acids present were $C_{8:0}$ to $C_{17:0}$ and $C_{19:0}$ to $C_{22:0}$, $AIC_{17:0}$ being the major constituent. Its highest concentration (2.09%) was found in 23.7 cm. size

TABLE II

Estimation of Triglyceride Fatty Acids of Body Lipids of *Puntius sarana* of Various Size Groups as Determined by GLC

Fatty acids		10.2 cm	16.4 cm	23.7 cm	29.3 cm
AI	8:0[a]	—	Trace	—	—
	8:0[a]	Trace	Trace	Trace	Trace
I	9:0[a]	—	Trace	Trace	—
	9:0[a]	Trace	Trace	Trace	Trace
I	10:0	—	1.25	0.32	0.03
AI	10:0	—	—	—	Trace
	10:0	0.04	Trace	0.14	0.04
I	11:0[a]	Trace	Trace	Trace	—
AI	11:0	—	0.32	Trace	—
	11:0	1.88	0.42	0.13	0.37
I	12:0	—	0.39	0.12	—
AI	12:0	—	Trace	—	Trace
	12:0	1.79	0.46	0.18	0.20
	12:1	0.30	0.22	0.09	0.61
	12:2	0.24	0.27	0.06	0.18
I	13:0	—	Trace	0.12	—
AI	13:0[a]	—	Trace	Trace	—
	13:0	0.31	0.41	0.13	0.30
AI	14:0[a]	0.31	Trace	—	—
	14:0	3.59	2.97	2.45	2.97
I	15:0	—	—	—	0.03
AI	15:0	0.48	0.57	0.13	0.60
	15:0	5.84	1.12	0.72	0.27
AI	16:0	0.48	0.79	0.32	0.15
	16:0	25.92	15.25	18.73	14.52
	16:1	10.34	4.03	8.98	5.40
	16:2	0.58	1.78	0.15	0.52
AI	17:0	0.61	2.06	2.09	—
	17:0	0.99	1.02	1.22	0.15
	17:1	0.61	0.72	0.63	0.35
	18:0	3.61	4.60	3.45	1.82
	18:1	37.73	37.73	45.39	59.56
	18:2	1.33	16.07	1.90	5.47
	18:3	1.43	2.98	5.14	3.20
I	19:0[a]	—	Trace	Trace	Trace
AI	19:0[a]	Trace	Trace	Trace	Trace
	19:0	0.21	0.24	0.20	0.25
	19:1[a]	Trace	—	—	—
I	20:0[a]	—	—	Trace	Trace
AI	20:0[a]	Trace	Trace	Trace	Trace
	20:0	0.20	0.41	—	0.11
	20:1[a]	Trace	Trace	—	Trace
	20:4	1.17	2.75	0.07	1.07
I	21:0[a]	—	—	Trace	—
AI	21:0[a]	—	Trace	—	Trace
	21:0	0.21	0.14	0.21	0.34
I	22:0	—	0.36	0.26	0.23
AI	22:0	0.12	0.44	0.52	0.47
	22:0	—	0.22	0.06	0.39
	22:2	—	—	0.49	—
I	23:0	—	—	—	—

[a]Identified from SE 30 column. Rest identified from DEGS column.

TABLE III

Comparison of the Triglyceride Fatty Acids in the Body of *Puntius sarana* of Different Sizes

Size of fish (cm)	Saturated				Unsaturated			
	Straight chain		Brached chain					
	Even numbered (%)	Odd numbered (%)	Iso (%)	Anteiso (%)	Monoenoic (%)	Dienoic (%)	Trienoic (%)	Tetraenoic (%)
10.2	36.45	9.48	Trace	1.08	49.29	2.16	1.44	1.17
16.4	30.09	3.35	2.00	4.18	42.70	18.12	2.98	2.75
23.7	29.38	2.61	0.82	3.06	55.09	2.60	5.14	0.07
29.3	21.95	1.68	0.68	1.22	65.92	6.17	3.20	1.07

fish. The presence of odd-numbered fatty acids was first reported by Farquhar et al. (24) in menhaden oil. Morice and Shorland (25) first demonstrated the presence of brached-chain fatty acids in shark liver oil. Ackman et al. (26) provisionally identified saturated odd-numbered, straight chain fatty acids in seal oil and later confirmed them along with branched chain acids in marine mammals and fish (6).

Sen and Schlenk (27), while investigating fatty acids of mullet (*Mugil cephalus*), found that mullet oil contains more than 25% straight chain, odd-numbered saturated, monounsaturated and polyunsaturated fatty acids. They held the view that this might be a result of slow catabolism of the odd-numbered fatty acids which accumulated after ingestion from phytoplankton. However, Ackman (28) suggested that odd-numbered fatty acids arise out of dietary thetin (29), which on enzymatic degradation may give rise to acrylic acid or propionic acid, which act as a precursor for odd-numbered fatty acids (33). *P. sarana* is a surface feeder, feeding mainly on the phytoplankton of green algae and the zooplankton of crustacea and insects (31). Small amounts of odd-numbered acids have been found in fresh water algae (*Chlorella pyrenoidosa*) (32), and their occurrence may also be expected in other fresh water organism. Thus, the presence of odd-numbered straight chain and branched chain acids in *P. sarana* lipids could be dietary or biosynthesized by the fish; branched chain arising from the oxidative decarboxylation of branched chain amino acids like valine, leucine and isoleucine and odd-numbered straight chain from propionic or acrylic acid.

However, as the percentage of the branched chain as well as odd-numbered straight chain saturated fatty acids decreased with size, the accumulation of these acids cannot be due to slow catabolism of deposited fatty acids as proposed by Sen and Schlenk (27).

The unsaturated acids were the predominant fatty acids in the neutral lipids (Table III). It was found that 54% of the fatty acids present in lipids isolated from 10.2 cm. size of *P. sarana* were unsaturated as compared to 76.4% in 29.3 cm. size fish. Variation in their percentage composition in the intermediate sizes was small. These acids were predominantly straight chain and even-numbered. No branched chain, unsaturated fatty acid was detected. Among the odd-numbered unsaturated acids, $C_{17:1}$, and $C_{19:1}$ (trace) were detected. On the basis of relative retention time, these acids were assigned to be *cis* configuration.

Monoenoic acids were found to be the main fraction of the unsaturated acids; 49.29% of the total fatty acids from the 10.2 cm. size *P. sarana* were monoenic acids as compared to 42.7% in 16.4 cm., 55.01% in 23.7 cm., and 65.92% in 29.3 cm size fish. $C_{18:1}$ and $C_{16:1}$ were found to be present in the highest proportion. The $C_{18:1}$ remained constant in the young fish with 37.73% in 10.2 cm. and 16.4 cm., and then increased to 42.39% in 23.7 cm and 59.56% in 29.3 cm. size fish. The only other even-numbered monoenoic acid detected was $C_{12:1}$ in very low concentration.

Variations in the proportions of dienoic acids with size showed very wide range; for example, 10.2 cm and 23.7 cm had only 2.16% and 2.6%, respectively, of dienoic acids against 18.12% in 16.4 cm and 6.17% in 29.3 cm fish. Such wide fluctuation with size are difficult to explain in the absence of similar data from other fish. Linoleic acid was the main dienoic fatty acid present in *P. sarana*, being 16.07% in 16.4 cm and 5.47% in 29.3 cm. The $C_{16:2}$ acid was present only in small amounts, the highest being 1.78% in 16.4 cm fish. The $C_{22:2}$ appeared only in 23.7 cm fish (0.49%).

Among the trienoic acids, only $C_{18:3}$ was present, which varied from 1.44% to 5.14%, the highest being found in the 23.7 cm. fish. However, predominant polyunsaturated acids of marine lipids are linolenic type (33); thus, fresh water fish *P. sarana* differs from marine fish in this respect.

Among the tetraenoic acids, only $C_{20:4}$ was present, and its concentration varied from 1.07% (29.3 cm. size) to 2.75% (16.4 cm. size) and only in traces (0.07%) in 23.7 cm size fish. Polyenoic acids higher than $C_{20:4}$ could not be detected in *P. sarana*.

Composition of *P. sarana* body lipids in general shows a behavior typical of fresh water fish. Saturated C_{16} and unsaturated C_{18} acids were the dominant acids, and the only higher unsaturated acid present was $C_{20:4}$ as a minor constituent. This is different from marine fish oil where C_{20} and C_{22} polyenoic acids are the dominant constituents (2, 34, 35).

It is noticed that there is a variation of each fatty acid component (minor as well as major) with size. Whether this variation truly reflects the effect of size is difficult to say at present, as such studies with other fish are available only to a very limited extent (21, 22). The complexity of factors influencing the composition of fish oils such as sex, age, diet, temperature, physiological stress and strain makes it difficult to give a definite conclusion. Extensive studies on similar lines on other fish as well as a good control on all the parameters is essential before coming to a definite conclusion.

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