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Physico-Chemical Behaviour of the Fish Lipid from *Lepidocephalus guntea* (Hamilton) and Variation of Lipid Profile with Size

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Abstract: The fish lipid was extracted from the body muscle (edible portion) of fresh *Lepidocephalus guntea* (Ham.) fish and the lipid content of the fish in size-1 (below 8.5 cm), size-2 (8.5-9.5 cm) and size-3 (above 9.5 cm) were found to be 2.515, 3.013 and 3.455%, respectively. The specific gravity and refractive index of the lipid were found to be 0.93 at 30°C and 1.467 at 31.5°C, respectively. The saponification value, saponification equivalent, iodine value, peroxide value and acetyl value of the lipid were found to be 220.325, 254.624, 96.05, 1.993 and 11.32, respectively. The acid value, percentage of free fatty acid as oleic and unsaponifiable matter present in the lipid were found to be 2.005, 1.008 and 0.593, respectively. The fatty acid composition of the lipid was determined qualitatively and quantitatively by TLC and GLC. The analysis revealed that the fatty acid composition of the lipid lies between C_{14} to C_{20}. The fish lipid of *Lepidocephalus guntea* (Ham.) was found to contain (average value) myristic acid (3.17%), palmitoleic acid (7.45%), palmitic acid (29.16%), linolenic acid (7.13%), linoleic acid (5.57%), oleic acid (22.93%), stearic acid (17.42%), arachidonic acid (7.17%) and arachidic acid in trace amount.

Key words: *Lepidocephalus guntea*, lipid, physico-chemical behaviour, variation of lipid profile with size

INTRODUCTION

The fish *Lepidocephalus guntea* (Ham.) belongs to the genus *Lepidocephalus* of the family Cobitidae (Nelson, 1976) is one of our natural wealth of fresh water. It is greatly preferred by the people of Indian sub continent for its therapeutic value and easy availability. Its vernacular name is gutea, Pui or Poa. The polyunsaturated fatty acids (PUFA) especially ω-3 and ω-6 present in fish lipid might protect against coronary heart disease (Daviglus *et al*., 1997; Kromhout *et al*., 1995) by inhibiting the biosynthesis of cholesterol in the liver. It also plays important roles in the metabolic processes. From the literature, it appears that the effectiveness of fish oil to reduce the cardiovascular problem has attracted the investigators extensively to analyze the fish oil and their nature of action as well (Oliver, 1981; Metcalf *et al*., 2007). The present study has been undertaken with a view to recognize the usefulness of this fish in oral administration for the protection against coronary heart disease and cardiovascular problem in association with the expression of the importance and function of fish lipid in reducing serum cholesterol level.

Our present investigation deals with the extraction, purification and hydrolysis of fish lipid; esterification of lipid hydrolysates to methylesters; separation, estimation and identification of methylesters mixture by gas liquid chromatography and thin layer chromatography; studies on the physico-chemical behaviour of lipid from *Lepidocephalus guntea* and variation of its lipid profile with size.

Nutritional studies along with the supplementary effects of fish lipid and fish protein concentrate on young albino rats for feeding for a period of 42 consecutive days with formulated cereal at different levels will form a separate study.

MATERIALS AND METHODS

The fish *Lepidocephalus guntea* (Ham.) of three different sizes were collected from various canals and local ponds in the North-Bangal of Bangladesh during the period March, 2006 to November, 2006.

Extraction of lipid from *Lepidocephalus guntea* (Ham.) fish: The lipid was extracted from the body muscle (edible portion) of *Lepidocephalus guntea* (Ham.) fish by the method cited in the literature (David *et al*., 2001). About 20 g of wet fish was ground well in a homogenizer (model AM-5, Ogawa Seiki Co. Ltd., Japan) with about 200 mL

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distilled water to make pulp. The pulp was transferred to a volumetric flask and 600 mL of chloroform-methanol (2:1v/v) mixture was added and shook well for about 3 h. For complete extraction, it was kept overnight at room temperature, preferably in the dark. The resulting suspension was subjected to centrifugation (12×103 rpm) where three layers were found. Chloroform layer with lipid was separated. To ensure complete extraction, the process was repeated and the combined extract was dried with a flow of nitrogen gas. Lipid thus obtained was purified by removing non lipid substances.

Characterization of the lipid of *Lepidocephalus guntea* (Ham.): Physical properties of the lipid i.e., specific gravity, refractive index, were determined by the standard method (Molla *et al.*, 1994). Chemical characteristics of the lipid i.e., iodine value, acid value, saponification value etc were also determined using the standard method (Molla *et al.*, 1987) cited in the literature.

Qualitative and quantitative analysis of lipid: The fish lipid was saponified and the fatty acid mixture of the lipid was converted to the corresponding methyl ester by BF_3-methanol complex according to the standard method of AOAC (1990). The methyl ester mixtures thus obtained were subjected to Thin Layer Chromatography (TLC) and Gas-liquid Chromatography (GLC) for the identification and estimation of the individual fatty acid components.

Thin layer chromatographic examination of methyl esters: Thin layer chromatography is an excellent tool for micro-preparative separation of mixtures. The methyl ester mixtures were charged on thin layer (20×20 cm×0.25) of silica gel-GF_{254} and the plate were developed by ascending technique using the following solvent system (Bobbit, 1996).

- Petroleum ether (40-60°C):ether (60:40)
- Petroleum ether (40-60°C):ether (80:20)
- Petroleum ether (40-60°C):ether:acetic acid (80:20:1)
- Petroleum ether (40-60°C):ether:acetic acid (85:15:1)
- Hexane:ether (80:20)

After development, the plates were dried at room temperature and sprayed with 2,7-dichlorofluroescein (0.2 g 2,7-dichlorofluroescein in 100 mL ethanol). The fatty acid methyl ester gave yellow coloured spot with the reagent under UV-lamp. The coloured spots were marked and the R_f values of the spots were calculated.

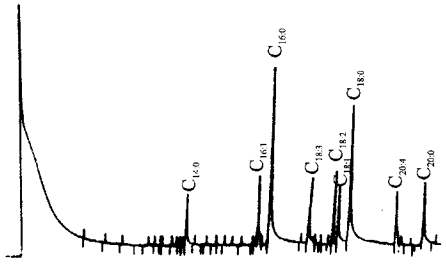

Fig. 1: Gas liquid chromatogram of the fatty acid methyl ester mixture derived from the fish lipid ($C_{14:0}$ = myristic, $C_{16:1}$ = palmitoleic, $C_{16:0}$ = palmitic, $C_{18:3}$ = linolenic, $C_{18:2}$ = linoleic, $C_{18:1}$ = oleic, $C_{18:0}$ = stearic, $C_{20:4}$ = arachidonic and $C_{20:0}$ = arachidic acid)

Gas liquid chromatographic examination of methyl esters: The experiment was carried out with a PYE UNICAM 4500 U model capillary chromatograph (Philips, England) fitted with a flame-ionization detector containing SE-54 as a packing material. The temperature was programmed from 120-220°C at a rising rate of 4°C per minute and the temperature of the injection port, detector and oven were maintained at 220, 230 and 190°C, respectively. Hydrogen was used as the carrier gas at a flow rate of 5 mL min^{-1} (Connor, 2001). The GLC graph (Fig. 1) represents some peaks of individual fatty acids. The identities of the individual fatty acids were achieved by co-chromatography with standard reference compounds. The peaks were tentatively identified by comparing their relative retention times with known values (Park and Goins, 1994) and by plotting the logarithm of the relative retention time curve against carbon number.

Determination of the average molar mass (M) of the lipid: The average molar mass (M) of the lipid was calculated by the following:

$$M/g\ mol^{-1} = 3(\sum x_i M_i) + 38.05\ g\ mol^{-1}$$

Where:

x_i = The mole fraction of fatty acids

M_i = The molar mass of the group CHCCH (Gonzalez *et al.*, 1997)

RESULTS AND DISCUSSION

The lipids from the body muscle of *Lepidocephalus guntea* (Ham.) were extracted from the three different size groups. The highest amount of lipid was found to be 3.455% in size-3 (above 9.5) whereas size-1 contained the lowest i.e., 2.515%. It was found that the lipid content

Table 1: Physical and chemical constants of the lipid from *Lepidocephalus guntea* (Ham.)

Physical and chemical constants	Lipid of *Lepidocephalus guntea* (Ham.) fish	Lipid of *Anguilla bengaleusis* Eel	Lipid of *G. centropus sineusis* Sinensis	Olive oil	Soybean oil	Linseed oil
R.I	1.467	1.462	1.464	1.467	1.472	1.479
Sp.Gr.	0.931	0.914	0.927	0.915	0.922	0.930
I.V.	96.050	96.000	98.037	88.000	129.000	175.000
A.V.	2.005	3.680	1.870	1.050	1.405	4.000
F.F.A.	1.008	1.850	0.940	0.527	0.705	2.001
S.V.	220.325	183.000	189.430	196.000	190.500	189.000
S.E.	254.624	305.000	296.140	291.400	295.430	296.800
P.V.	1.993	7.450	10.580	-	-	-
U.S.M.	0.593	8.370	1.120	1.2	0.900	1.250
Ac.V.	11.320	8.740	-	-	-	-

(R.I. = Refractive Index, Sp.Gr. = Specific Gravity, S.V. = Saponification value, S.E. = Saponification Equivalent, I.V. = Iodine Value, P.V. = Peroxide Value, A.V. = Acid Value, F.F.A. = Percentage of Free Fatty Acid, U.S.M. = Unsaponifiable Matter, Ac.V. = Acetyl Value)

Table 2: Thin layer chromatographic examination of methyl esters mixture obtained from the lipid of *Lepidocephalus guntea* (Ham.)

		R_f value obtained from the spots									
Name of the sample	Developing solvent system	Methyl palmitate	Methyl stearate	Methyl oleate	Methyl linoliate	Methyl myristate	Methyl arachidonate	Methyl ester mixture			
Lipid of *Lepidocephalus guntea* (Ham.) fish	P:E (80:20)	0.230	0.285	0.221	0.280	0.100	0.185	0.282	-	0.220	0.090
	P:E (60:40)	0.250	0.500	0.286	0.429	0.100	0.397	0.107	0.283	0.432	-
	P:E:A(85:15:1)	0.330	0.325	0.300	0.315	0.105	0.443	0.101	0.305	0.311	0.375
	P:E:A(80:20:1)	0.275	0.290	0.313	0.390	0.101	0.443	0.100	0.320	0.400	0.490
	H:E (80:20)	0.301	0.328	0.400	0.503	0.091	0.450	0.100	0.393	0.500	0.325

(P: E = Petroleum Ether (40-60°C): Ether, P: E: A = Petroleum Ether (40-60°C): Ether: Acetic acid, H: E = Hexane: Ether)

gradually increased with the changes in maturity of the fish. A number of physical and chemical tests were employed to identify the nature of oils and fats. The chemical constants are more important to characterize an oil or fat, yet the physical constants are also often capable of expressing valuable informations. These constants of the lipid of *Lepidocephalus guntea* (Ham.) are given in the Table 1. along with those of some standard oils and fats.

The refractive index of fats oils depends to some extant on their unsaturation (Peach and Tracy, 1995) and the higher refractive index represents higher unsaturation. The present result (1.467 at 31°C) indicates that the fish lipid contains large amount of unsaturated fatty acids. The specific gravity of practically all fats and oils lies between 0.90-0.95. The specific gravity obtained in the present studies (0.931 at 30°C) has close similarity to that of *Anguilla bengalensis* Eel (0.914 at 30°C, Molla, 1991).

The iodine value of the lipid of *Lepidocephalus guntea* (Ham.) was found to be 96.05 which is very close to that of *Anguilla bengalensis* Eel (96.0, Molla, 1991). It gives an estimate of the degree of unsaturation of fatty acids. Hence, it may be concluded that the lipid under investigation contains unsaturated fatty acids at higher concentrations. The acid value and the percentage of free fatty acid as oleic were found to be 2.005 and 1.008%, respectively. Since a low acid value indicates a lower tendency to become rancid and thus a lower percentage of free fatty acid (below 1.15%) is a determination or indication of suitability of the lipid for edible purpose. So the lipid under investigation might be suitable for edible purposes.

Saponification equivalent is directly proportional to the average chain length of fatty acids present. Fats and oils consists largely of C_{18} fatty acids with some palmitic acid, a little unsaponifiable matter and a low free acidity generally gave a saponification equivalent around 254.624; higher value indicates the presence of appreciable quantity of higher acids. The present result (220.325) indicates that the fish lipid contained mainly fatty acids of C_{18} molecular weight along with some palmitic acid (Molla *et al*., 1994). The peroxide value (1.993) of fish lipid indicates that the lipid contained a large amount of unsaturated fatty acids. The unsaponifiable matter amounting to 0.5-2.0% represents a mixture of several lipid classes, viz., sterols, tocopherols, hydrocarbons, higher aliphatic and terpenoid alcohol. The unsaponifiable matter in the lipid was found to be 0.593% which indicates that fish lipid also contained sterols, tocopherols, hydrocarbons etc. The acetyl value of the fish lipid was found to be 11.32. The low acetyl value indicates that the number of free hydroxyl group in the lipid is low. By column chromatography it was found that lipid contained 43.21% neutral-lipid, 5.44% glyco-lipid (with free fatty acid) and 41.97% phospho-lipid, respectively.

The fatty acid methyl esters mixture obtained from the lipid of *Lepidocephalus guntea* (Ham.) was subjected to TLC examination and their fatty acid compositions were identified by comparing with the R_f values of methyl esters of standard fatty acids compositions in five different solvent system (Table 2).

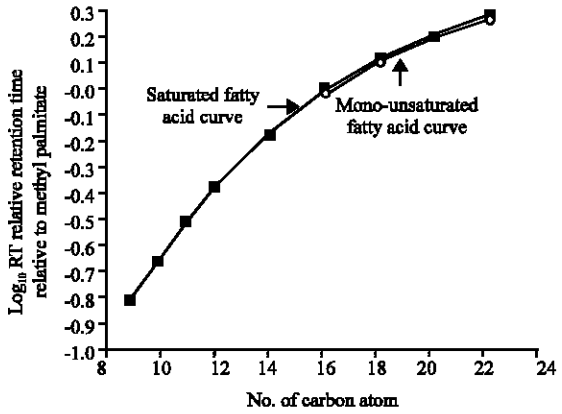

Fig. 2: Relationship between $\log_{10}$ relative retention times, relative to methyl palmitate and the number of carbon atoms of fatty acid methyl esters

It was found from the TLC chromatograms that the lipid gave 4 spots. Among the spots, three were identified as myristic acid ($C_{14:0}$), oleic acid ($C_{18:1}$) and linoleic acid ($C_{18:2}$) in all solvent systems. Other spot was identified as stearic acid ($C_{18:0}$) in n-Hexane:Ether (80:20) solvent system. Fatty acid analysis of the fish lipid was also carried out by gas liquid chromatography after trans-esterification of the glycerides to their methyl esters and their separation pattern has been presented in Fig. 1. It is evident from Fig. 1 that the substances have been smoothly separated on polar column. The identities of the individual fatty acids were achieved by co-chromatography with standard reference compounds. The peaks were tentatively identified by comparing their relative retention times with known values (Park and Goins, 1994) and by plotting the logarithm of the relative retention time curve against carbon number (Fig. 2). The analytical data were summarized in the Table 3.

Table 3: Gas Liquid Chromatographic analysis of the fatty acid composition of the fish lipid from *Lepidocephalus guntea* (Ham.)

Name of sample	Peak No.	Retention time	Relative retention time with respect to standard palmitic acid (RT)	$Log_{10}RT$	Name of the fatty acid present in the sample	Area under each peak	Relative percentage of fatty acid
Standard	1	2.51	0.154	-0.814	Nonanoic acid ($C_{9:0}$)		
	2	3.50	0.214	-0.669	Capric acid ($C_{10:0}$)		
	3	4.93	0.302	-0.520	Undecanoic acid ($C_{11:0}$)		
	4	6.79	0.416	-0.381	Lauric acid ($C_{12:0}$)		
	5	11.35	0.695	-0.158	Myristic acid ($C_{14:0}$)		
	6	15.83	0.969	-0.014	Palmitoleic acid ($C_{16:1}$)		
	7	16.34	1.000	0.000	Palmitic acid ($C_{16:0}$)		
	8	18.74	1.147	0.060	Linolenic acid ($C_{18:3}$)		
	9	20.33	1.244	0.094	Linoleic acid ($C_{18:2}$)		
	10	20.47	1.253	0.098	Oleic acid ($C_{18:1}$)		
	11	21.14	1.294	0.112	Stearic acid ($C_{18:0}$)		
	12	24.02	1.470	0.167	Arachidonic acid ($C_{20:4}$)		
	13	25.62	1.568	0.196	Arachidic acid ($C_{20:0}$)		
	14	29.91	1.830	0.263	Erucic acid ($C_{22:1}$)		
	15	30.96	1.895	0.278	Behenic acid ($C_{22:0}$)		
Size-1	1	11.38	0.696	-0.157	Myristic acid ($C_{14:0}$)	1560	3.08
(below-	2	15.83	0.696	-0.014	Palmitoleic acid ($C_{16:1}$)	3581	7.08
8.5 cm)	3	16.39	1.003	0.001	Palmitic acid ($C_{16:0}$)	13470	26.63
	4	18.81	1.151	0.061	Linolenic acid ($C_{18:3}$)	4076	8.06
	5	20.41	1.249	0.097	Linoleic acid ($C_{18:2}$)	3557	7.03
	6	20.57	1.259	0.100	Oleic acid ($C_{18:1}$)	10131	20.03
	7	21.20	1.297	0.113	Stearic acid ($C_{18:0}$)	8867	17.53
	8	24.15	1.478	0.170	Arachidonic acid ($C_{20:4}$)	5341	10.56
	9	25.70	1.573	0.197	Arachidic acid ($C_{20:0}$)	Trace	Trace
Size-2	1	11.32	0.693	-0.159	Myristic acid ($C_{14:0}$)	1690	2.61
(from	2	15.74	0.963	-0.016	Palmitoleic acid ($C_{16:1}$)	4458	6.90
8.5 -9.5 cm)	3	16.29	0.997	0.001	Palmitic acid ($C_{16:0}$)	20413	31.59
	4	18.70	1.144	0.059	Linolenic acid ($C_{18:3}$)	5451	8.44
	5	20.29	1.242	0.094	Linoleic acid ($C_{18:2}$)	2602	4.03
	6	20.46	1.252	0.098	Oleic acid ($C_{18:1}$)	13969	21.62
	7	21.09	1.291	0.111	Stearic acid ($C_{18:0}$)	13334	20.63
	8	24.02	1.470	0.167	Arachidonic acid ($C_{20:4}$)	2701	4.18
	9	25.58	1.565	0.195	Arachidic acid ($C_{20:0}$)	Trace	Trace
Size-3	1	11.38	0.696	-0.157	Myristic acid ($C_{14:0}$)	2758	3.82
(above	2	15.79	0.966	-0.015	Palmitoleic acid ($C_{16:1}$)	6039	8.37
9.5 cm)	3	16.34	1.000	0.000	Palmitic acid ($C_{16:0}$)	21111	29.27
	4	18.74	1.147	0.060	Linolenic acid ($C_{18:3}$)	3519	4.88
	5	20.33	1.244	0.095	Linoleic acid ($C_{18:2}$)	4071	5.64
	6	20.50	1.255	0.099	Oleic acid ($C_{18:1}$)	19567	27.14
	7	21.12	1.293	0.111	Stearic acid ($C_{18:0}$)	10176	14.11
	8	24.05	1.472	0.168	Arachidonic acid ($C_{20:4}$)	4885	6.77
	9	25.60	1.567	0.195	Arachidic acid ($C_{20:0}$)	Trace	Trace

It is evident from the Table 3 that the fish of *Lepidocephalus guntea* (Ham.) was found to contain (average value) myristic acid (3.17%), palmitoleic acid (7.45%), palmitic acid (17.42%), linolenic acid (7.13%), linoleic acid (5.57%), oleic acid (22.93%), stearic acid (17.42%), arachidonic acid (7.17%) and arachidic acid in trace amount.

The GLC analytical data also revealed that the lipid of *Lepidocephalus guntea* (Ham.) in size-1 contained saturated fatty acid 47.24%, monounsaturated fatty acid 27.11% and polyunsaturated fatty acid 25.65%. The size-2 contained saturated fatty acid 54.83%, monounsaturated fatty acid 28.52% and polyunsaturated fatty acid 16.65% whereas size-3 was found to contain saturated fatty acid 47.20%, monounsaturated fatty acid 35.51% and polyunsaturated fatty acid 17.29%. The results indicate that the fatty acid content of lipid changes with the change of fish length size. The highest amount of unsaturated fatty acid was found to contain in size-3 (52.80%) whereas size-1 contained the lowest (45.17%). Experimental studies with animals had suggested that polyunsaturated fatty acids must play an important role in the normal transport of cholesterol (Ascherio *et al*., 1995). It is evident from the GLC analysis that fish lipid contained polyunsaturated fatty acid namely oleic acid, linolenic acid, linoleic acid and arachidonic acid. Among these linoleic acid and arachidonic acid have been designated as essential fatty acid for the human. Fish lipid containing high level of PUFA is found to inhibit the activity of HMG-CoA reductase (Ide *et al*., 1978; Siscovick *et al*., 1995) which is the regulatory enzyme in cholesterol biosynthesis. Due to the inhibition of the biosynthesis of cholesterol in liver, PUFA plays an important role in maintaining the blood cholesterol level normal (Nestel, 1990). Several hypothesis have been advanced to explain the effect including the stimulation of cholesterol excretion into the intestine and inhibition of biosynthesis of cholesterol in the liver. In addition of cholesterol lowering effect of the PUFA, it is also now clear that the polyunsaturated fatty acids also have anti aggregating effect in the platelets, thus preventing thrombosis and reducing the risk of heart attack (Ide *et al*., 1978; Siscovick *et al*., 1995). The average molar mass of fish lipid was found 856.03 g mol^{-1} which is about the same of some standard oil such as olive oil (875.84 g mol^{-1}), corn oil (874.18 g mol^{-1}), grape pip oil (876.77 g mol^{-1}) etc. (Gonzalez *et al*., 1997). Hence we may conclude that the analysis of lipid by GLC method was authentic.

CONCLUSION

From the foregoing evidences, it may be concluded that due to the presence of appreciable amount of mono and polyunsaturated fatty acids in the fish lipid under investigation, it is suitable for edible purposes owing to the important role it plays in the transport of cholesterol and thus preventing atherosclerosis, thrombosis and effectively involved in the transport of cholesterol from blood.

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