Isolation, Characterization and Fatty acid investigation of oil from *Mugil parsia* fish.

Abstract: This paper reports the isolation, characterization and GC-MS analysis of oil of *Mugil parsia* fish collected from the Rupsha River in Khulna. The oil was extracted from the dried fish with n-hexane using Soxhlet apparatus with an oil yield of 17± 4.0 %. The moisture, ash and protein content of the raw fish were found to be 75.45 %, 1.258 % and 20.00 % respectively. The physico-chemical properties of the extracted oil like color, specific gravity, peroxide value, saponification value, free fatty acid (FFA), acid value and iodine value were determined and were found to be yellowish brown, 0.913±0.31, 9.9±0.1 meq/kg, 295.4±mg KOH/g, 11.9±2.0%, 5.7±0.11mg KOH/g, 5.7± 0.11 and 150.3±0.12 mg/100g respectively. Identification of fatty acid composition by GC-MS analysis of the *Mugil parsia* fish oil, showed the Phthalic acid, mono-(2-ethylhexyl) ester, palmitic acid, oleic acid, stearic acid, icosanoic anhydride, glycidyl stearate were identified as the major constituents.

Key words: *Mugil parsia*, Soxhlet apparatus, peroxide value, iodine value, GC-MS analysis.

1. Introduction

Fishes are generally defined as an aquatic vertebrate animal that are typically cold blooded covered with scales equipped with two paired fins and several unpaired fins [1] that use gills to obtain oxygen using gills put together and thus various kinds of fish vary greatly in size, shape and color [2].

Mugil parsia (Synonym *Liza parsia* Hamilton 1822), English name 'Gold spot mullet' occurs in the area, along the coasts of Sri Lanka, west India, Pakistan, Bangladesh and the Andaman Islands. In shallow coastal waters, estuaries, lagoons and sometimes entering tidal rivers, there occurs a schooling species. In ponds with 87% salinity, they can survive, Spawning takes place at sea.

Many studies have been conducted on fish flesh and its oil. Fish oil is currently under intensive scientific research due to its numerous health benefits. This fish oil is receiving a lot of attention because of its health benefits associated with the high levels of the long chain omega-3 polyunsaturated fatty acid (PUFA) (Wu and Peter, 2008). Fish flesh is composed of high-quality proteins and lipids (oils) that are high in monounsaturated and polyunsaturated fatty acids [3]. The nutritional benefits of fish oil consumption are due to the presence of protein of high biological value, unsaturated essential fatty acid, minerals and vitamins namely vitamin B3 (niacin), vitamin B6 (pyridoxine), vitamin B_{12} (cobalamine), vitamin E (tocopherol) and vitamin D (cholecalciferol) in fish tissues [4]. Fish oil has been used to relieve muscular pain as well as arthritis [5]. Various reports on the study and identification of fish oil having high pharmacological activity potential as a hypoglycemic [6], hypolipidemic agent [7−9], antiarthritic agent [10−12] and preventing an agent renal damage [13−15]. The most important health benefit of taking fish oil is that it is good for the functional development of infant's nervous system (nerve and brain) as well as the retina particularly in premature infants [16,17].

These uses inspired us to undertake the present study. This investigation deals with the fatty acid and lipid characterization of fish oils from marine fish *Mugil parsia* available in the coastal waters of Bangladesh.

2. Materials and methods

2.1 Sample Preparation

Mugil parsia (Synonym *Liza parsia* Hamilton 1822), samples were collected from the Rupsha River in Khulna at Rajshahi University in May, 2016. The average weights of Gold spot mullets were1.5 kg. The head, scales, bones, fins, viscera and gills were removed and cut the meat in to small pieces and dried for an hour to reduce the moisture content and then crushed well to almost paste form by a hand crusher. All fish samples were frozen and stored at -20^{o} C until use.

2.2 Physico-chemical analyses of fish

2.2.1 Determination of moisture content

Moisture was determined by drying the sample at +105^{0} C in an oven [18]. By subtraction the moisture was calculated. For determining moisture content, the weight of the aluminium dish was taken. Differences between two weights, of the sample was ascertained. Then the dish with sample was put in a controlled oven and was dried at 105^{0}C until a constant weight was achieved. The percentage moisture content was calculated using the following equation: Moisture (%) = $W_2 – W_3 /W_2 –W_1 \times 100$

Where,

W_1=initial weight of empty crucible

W_2= weight of crucible and sample before drying

W_3= Final weight of crucible and sample after drying

2.2.2 Determination of ash content

Ash was determined by muffling the sample at 6000- 7000^0 C to dry ash. [18] First, clean porcelain crucibles were heated in a muffle furnace at 6000^{0}C and the crucibles were then weighed until a constant weight was obtained. The sample with the crucible was weighed and recorded. The sample was ignited at 600^0 C for about 6 hours until the residue was uniformly grayish to white. Afterwards crucibles were transferred to a desiccator to cool them at room temperature for few minutes. Final weights of the crucible were recorded. The following equation was used to determine the ash content of the dry fish samples:

Ash (%) = weight of ash /weight of sample ×100 W_3-W_1/W_2-W_1 × 100

Where: W_1= weight of empty crucible

W_2= weight of crucible + sample before ashing

W_3= weight of crucible + ash

2.2.3 Determination of protein content

Water-soluble protein was determined spectrophotometrically by Lowry method [19]. To a conical flask 0.2g of sample was added, 20ml of H2SO4 (concentrated) was also added. To the mixture, 5ml of CUSO4 (0.2N) was added. This was allowed to digest for thirty minutes and greenish blue colour was observed. This was diluted by adding 10ml of water, and to the mixture 5ml of 2% boric acid was added, and 2 drops of phenalpthalein indicator was added to the mixture. From the digested material, 10ml was obtained and to that was added 20ml of NaOH (40%) and titrated against (0.1N) HCl. The following equation was used to obtain amount of protein

N (%) = (Volume of HCl ×normality HCl × 0.014 × 100) /weight of sample Protein (%) = % Nitrogen × 6.2

2.3 Extraction of oil

100g of dried *Mugil parsia* fish was then weighed into a thimble and plugged with fat-free cotton and then placed into Soxhlet apparatus for approximately 16 h. Anhydrous n-hexane was used for the extraction of fish oil. Then the oil was freeze-dried by freeze dryer and stored at -80^{o} C until use.

2.3.1 Physico-chemical analyses of extracted fish oil

2.3.1.1 Determination of fish oil color

Colour of absolute oil was noted from physical appearance.

2.3.1.2 Determination of Specific gravity

Specific gravity of the oil was determined by using the specific gravity bottle. A clean and dry bottle of 25 mL capacity was weighed (W0) and then filled with the oil, stopper inserted and reweighed to give (W1). The oil was substituted with water after washing and drying the bottle and weighed to give (W2). The specific gravity (Sp.gr) was obtained from the expression [20]:

Specific gravity = $W_1 – W_0 /W_2 –W_0$ = Mass of the substance / Mass of an equal volume of water.

2.3.1.3 Determination of Peroxide values:

The Peroxide values (PV) of fish oil were determined according to AOAC method [21]. Oilsample (5 g) was weighed into a 200 ml conical flask and mixed with 300 ml of glacial acetic acid and chloroform (3:1) and mixed thoroughly by swirling the flask. Saturated potassium iodide (0.5 ml) was then added and the mixture was left in the dark for 1 min with occasional swirling, followed with further addition of 30 ml distilled water. The mixture was titrated with0.1 N sodium thiosulphate solution with 1 ml of 1.0 % soluble starch as indicator until the blue color disappeared. A blank sample titration was also carried out in the same manner but with no oil added.

2.3.1.4 Determination of saponification value :

The saponification value (SV) of the fish oil was determined according to the procedures described in AOCS method [20]. Oil sample (1 g) was dissolved in 12.5 ml of 0.5 N ethanolic potassium hydroxide. The mixture was refluxed for 30 min until oil droplets disappeared and was left tocool to room temperature. Phenolphthalein indicator was then added and the hot soap solution was titrated with 0.5 N HCl until the pink color disappeared. A blank titration was also carried out in the same manner except no oil was added.

2.3.1.5 Determination of FFA value and acid value:

Into a 250 milliliters conical flask, 2 grams of oil was weighed and added, 100 milliliters of 95% ethanol previously neutralized with 2 millimeters of phenolphthalein indicator was also added. The conical flask was placed on a hot water bath until the oil was completely dissolved in the solvent. The hot solution was titrated with 0.1 mole KOH until a pink color appeared which persisted for about 10 seconds. The acid value was calculated using:

Acid value (milligram) = $56.1 \times M \times V / W$

Where V= titre value

M= molarity of KOH used

W= weight in grams, of sample

From the FFA value, acid value (AV) was also calculated.

2.3.1.6 Determination of Iodine value:

Into a 250 milliliters conical flask, 1 gram of oil was weighed and added, 10millitres of carbon tetrachloride was also added. To the mixture, 13 milliliters of Wij's solution was added. The flask was stoppered, shaked and allowed to stand in the dark for one hour after which 10 milliliters of 15% potassium iodide solution together with 50 millimeters of water were added to the flask. The liberated iodide solution was titrated with standard thiosulphate solution and was shaken vigorously. Starch was used as indicator until the blue color disappears. A blank test was carried out without the oil under the same conditions. Iodine value was calculated using:

Iodine value (mg) = $12.69 \times M \times (V_b - V_a)/W$

Where,

V_b = Volume of standard $Na_2S_2O_3$ solution used for blank test

V_a = Volume of standard $Na_2S_2O_3$ solution used for the test sample

M = Molarity of $Na_2S_2O_3$

W = Weight of oil sample (gram)

2.3.1.7 Gas chromatography–mass spectrometry (GC-MS) analysis of *Mugil parsia* fish oil

Gas chromatography–mass spectrometry (GC-MS) is an analytical method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample. GC-Mass spectroscopy was conducted (GCMSQP5050, Shimadzu, Japan) to characterize bioactive compound. A large number of constituents have been identified by GC-MS analysis of the crude ethanol extract. The quantification and the identification of compounds in the crude extract and active bands isolated by preparative TLC were accomplished using GC-MS analysis.

2.3.1.8 GC-MS analysis of fatty acid composition of *Mugil parsia* fish

The chemical compositions of fish oil were analyzed by using GC–MS technique and the fragmentation analysis was performed. Fatty acid Compositions of the fish oil were separated and identified by gas chromatography–mass spectrometry (GC–MS) agilent 6890N gas chromatography hooked to agilent 5973N mass selective detector. They equipped with a flame ionization detector and capillary column with HP-5MS (30 m×0.25 mm×0.25μm). The GC settings were as follows: the initial oven temperature was held at 60 °C for 1 min and ramped at 10° C min $^{-1}$to 180° C for 1 min and then ramped at 20° C min $^{-1}$ to 280 °C for 15 min. The injector temperature was maintained at 270° C. The samples (1μL) were injected neat, with a split ratio of 1: 10. The carrier gas was helium at flow rate of 1.0mL min $^{-1}$. Spectra were scanned from 20 to 550 *m/z* at 2 scans s $^{-1}$. Most constituents were identified by gas chromatography by comparison of their retention indices with those of the literature or with those of authentic compounds available in database.

3. Statistical Analysis: All the experiments were carried out in triplicate and the data is reported as mean ± standard deviation (SD) [22].

4. Result and Discussion

Physico-chemical properties of Mugil parsia fish oil extracts

In order to determine the stability and quality of fish oil extracts, some quality assessment was conducted. These results are shown in Table 1, Table 2 and Table 3. In Table 1, moisture, ash and protein content were 56.45± 4.0 %, 1.258± 0.2 % and 20± 0.23 g% respectively. The estimated oil in *Mugil parsia* fish was 17± 4.0 %. The oil obtained from *Mugil parsia* fish had a yellowish-brown color. The oil was showed the specific gravity 0.913±0.31. As shown in table 2, PV content was 9.9 ± 0.1 meq/kg, Young [23] had reported that peroxide value (PV) of crude fish oil was between 3 and 20 meq/kg. In this study, the PV content was 9.9 ± 0.1 meq/kg, which is well below acceptable limit of 20 meq /kg oil. This indicated that the fish oil extracted had low lipidoxidation rate [24]. The values of acid value (AV) and free fatty acids (FFA) in extracted fish oil were found to be 5.7 ± 0.11 mg KOH/g and 11.9 ± 2.0 %, respectively. The acceptable limit for AV was reported to be 7-8 mg KOH/g [25]. Chantachum [26] had reported that high heating temperature during oil extraction deactivated the enzyme and the release of free fatty acids by the lipase activity thus lowered the FFA value. Ashraduzzaman et al [27] had reported that acid value of seed oil was between 1.3-1.6 mg KOH/g. Due to low temperature used during the extraction of oil in this study, enzyme lipase present may not have been deactivated and thus more free fatty acids could be release by lipase activity. Thus, caused high fatty acid value in this study which was also probably due to enhanced oxygen transfer which led to increased lipid oxidation, as propounded by Dauksas et al. [28]. Saponification is the process of breaking down a neutral fat into glycerol and fatty acids by alkali treatment. The SV of fish oil obtained in this study was higher (295.4 ± 3.0 mg KOH/g) than standard value for fish oil (180-200 mg KOH/g), given by AOCS [18]. Bimbo &Crowther [25] reported that crude oil contains minor amount of non-triglyceride substances. Thus, it is possible that high SV was due to impurities present in crude fish oil. Additionally, higher saponification value may be contributed by the unsaponifiable matter present in the leaching wastes materials such as sterols, glyceryl ethers, hydrocarbons, fatty alcohols and some minor quantities of pigments and vitamins. [25]

GC-MS analysis:

The chemical composition of fish oil identified the aid of gas chromatography and decomposition products were characterized by mass analyzer detector GC/MS. The present study was carried out to identify fatty acid composition by GC-MS analysis of *Mugil parsia* fish oil (Fig 1). Phthalic acid, mono-(2-ethylhexyl) ester, palmitic acid, Oleic acid, Stearic acid, Icosanoic anhydride, Glycidyl stearate were identified as the major constituents of *Mugil parsia* fish oil by GC-MS analysis. Oleic acid, a monounsaturated fatty acid. It decreased LDL cholesterol and increased HDL cholesterol [29]. It may also be responsible for the hypotensive (blood pressure reducing) effects. [30]. In previous study, it is revealed that stearic acid lowers LDL cholesterol [31]. It is reported that icosanoic acid (arachidonic acid) should be noted that for persons with chronic inflammatory disorder such as rheumatoid arthritis or inflammatory bowel disease. It is possible that icosanoic acid can exacerbate joint inflammation and pain [32].

5. Conclusion: The results of present study indicated that *Mugil parsia* fish contained significant amount of oil and protein. The presence of appreciable level of essential fatty acids and other favorable physiochemical characteristic make the *Mugil parsia* fish oil nutritionally viable for human health. Unsaturated fatty acids content in fish oils may reduce the risk of chronic diseases.

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Table 1: Proximate composition of *Mugil parsia* fish.

Analysis	Fish oil
Moisture (%)	75.45± 4.0
Ash (%)	1.258± 0.2
Protein (g%)	20± 0.23
Oil (%)	17± 4.0
Color	yellowish brown
Specific gravity	0.913±0.31

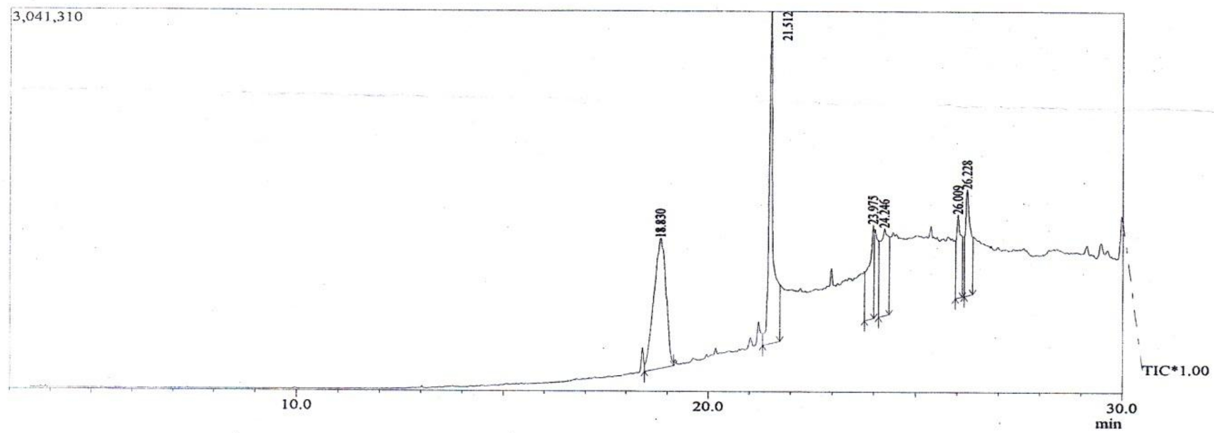
Table 2: Physico-chemical analysis of extracted *Mugil parsia* fish oil.

Analysis	Fish oil
Peroxide value (meq/kg)	9.9 ± 0.1
Saponification value (mg KOH/g)	295.4 ± 3.0
Free fatty acid (%)	11.9± 2.0
Acid value (mg KOH/g)	5.7± 0.11
Iodine value(mg/100g)	150.3±0.12

Table 3: Following compound are identified by GC-MS from *Mugil parsia* fish oil,

SI No	Name of the compound	Molecular weight	Concentration (Area %)
1	Phthalic acid, mono-(2-ethylhexyl) ester	278	30.42
2	Palmitic acid	256	27.24
3	Oleic acid,	282	9.72
4	Stearic acid	284	14.10
5	Icosanoic anhydride	606	7.67
6	Glycidyl stearate	340	10.85

Fig 1: Chromatogram of *Mugil parsia* Fish oil



Peak Report TIC

Peak#	Name	R.Time	Height	Area	Area%
1	Phthalic acid, mono-(2-ethylhexyl) ester	18.830	1024097	21047781	30.42
2	Palmitinic acid	21.512	2660523	18848497	27.24
3	Oleic acid	23.975	741328	6726926	9.72
4	Stearic acid	24.246	690768	9754487	14.10
5	Icosanoic anhydride	26.009	661952	5307467	7.67
6	Glycidyl stearate	26.228	845740	7511237	10.85
			6624408	69196395	100.00