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Extraction, Purification, Composition and Quality Deterioration of Fish Body Oil Extracted From *Sardinella Fimbriata* by Traditional Method

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Abstract:

The extraction, refining and evaluation of fish oil from Sardinella fimbriata by Traditional method has been conducted in this work. The total percentage oil yield was 13.5% and the yield after purification was 58%. The analytical properties of the crude and the refined oil were evaluated and it was observed that the crude oil consist from: specific gravity 0.9036, refractive index 1.461, moisture content 0.7%, free fatty acid value 2.9 mg/KOH, iodine value 188 I_2/100g, peroxide value 2.18 mEq/kg, saponification value 202.7 mgKOH/g and slight brownish colour.

The refined oil was also evaluated as follows: specific gravity 0.8987, refractive index 1.465, moisture content 0.32%,free fatty acid value 2.1 mg/KOH, iodine value 181 I_2 /100g, peroxide 1.21 meq/kg, saponification value 174.8 mg KOH/g, and bright yellow colour. The predominant fatty acids found in crude and refined oil were C14:0, C15:0, C16:0, C17:0, C18:0, C23:0, C14:1ω-4, C18:1ω-9, C18:2ω-6, C18:3ω-3, C20:4ω-6, C20:5ω-3, C22:5ω-3 and C22:6ω-3. The total n-3 fatty acid content of the purified catfish oil was 13.361 mg/g of oil. The purified oil contained 5.009 mg/g DHA and 1.173 mg/g EPA. All the essential amino acids were present in the crude fish oil ranged from 1.907 to 0.067µg/g. After purification the major amino acid present was Valine (2.07µg/g) and Threonine (1.26µg/g). The vitamins present were in minor quantities; 0.06, 0.005 and 0.002µg/g of vitamin A, D and E, respectively in crude fish oil. In refined fish oil; 0.04 and 0.003µg/g of vitamin A and D were present.

Keywords: ***Sardinella fimbriata, fatty acids, amino acid, vitamin.***

Introduction

Fish oil is produced by several methods, including physical fractionation [1], low temperature solvent fractionation [2] and supercritical fluid extraction [3], however, the conventional process compromises of cooking, pressing and centrifuging to recover the oil from miscella is the common method used to produce fish oil [4, 5]. The conventional method of extraction is so far considered as the finest extraction process due to its winsome quality such as yield, economically viable, less time consumption etc. The production of the fish oil deals with the separation of lipids from other constituents of the fish. Generally, the production involves a series of steps for the purification of the product. [6] According to American Oil Chemist's Society, (AOCS), the major steps in fish oil refining are degumming, neutralizing, bleaching and deodourizing. Both soluble and insoluble impurities are removed through degumming process, followed by neutralization of crude oil with Sodium hydroxide that removes Free Fatty Acids (FFAs). Bleaching aids in the removal of soap, trace metals, sulfurous compounds and part of the more stable pigments and pigment-breakdown products, aldehydes and ketones. The final step is deodourization that removes residual FFA, aldehydes and ketones, which are responsible for an unacceptable oil odour and flavor [7]-[9].

Apartfromfatty acids, amino acids are also present in fish oil, contributing its beneficial effect.Fish contain significant amounts of all essential amino acids, particularly lysine which is relatively poor in cereals [10]. Free amino acids in fish play essential role in metabolism, such as adjustment of osmotic pressure and also as an energy source. Even though amino acids are present in fish oil, they are not completed explored and there is no clear evidence up to date about the amino acid composition of fish oil. The fish oil contains reasonable quantities of amino acids and will be extracted along with the fatty acids during the conventional process of extraction. Marine fish oil is highly unsaturated and is susceptible to oxidation during exposure to oxygen, light, and heat [11]. Fish oil spoils in two major ways; oxidative spoilage and hydrolytic spoilage [12]. Due to its high content of polyunsaturated fatty acids, fish oil is highly susceptible to oxidative spoilage [13] and the rate of fish oil oxidation is significantly higher than that of other oil. The course of oxidation is often quite different between extracted fish oils and lipids in fish tissues [14]. The autoxidation of fish oil is the prime cause of deterioration of its quality [39]. Undesirable flavours and odours develop even at very low peroxide values (early stage of oxidation)

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during the induction period [15, 16]. Many species of marine fish have been studied for fish oil production, but little attention has been paid to the production of fish oil from low value fishes. Understanding the nutraceuticals properties of fish oil and its wide application, an attempt has been made in the present study to extract fish oil from low value fish. The objectives of the present study were to produce fish body oil from low value fish, Sardinella fimbriata (lesser sardine) by direct steaming method and to determine the effect of purification on the composition of fatty acid, amino acid, vitamins and the quality of oil.

Materials and Methods

Sardinella fimbriata (lesser sardine) were collected from fish landings of Muddasalodai and Parangipettai (lat. $11^0$29'N; long. $79^0$46'E). The fishes were washed thoroughly in running water for the removal of sand and external debris; scales, head, fins, spines, digestive system and excretory system were removed and the tissues alone were taken for extraction of oil. The tissues were subjected for extraction of oil by direct steaming method (conventional method). Thehomogenized fish tissues was taken in a muslin bag and kept in Steam Boiler at 70-80°C for 30minutes. The boiled fish tissues were then pressed using Fish Oil Extractor, so as to remove the liquid content from the tissues (containing oil and water). The oil was separated from the water by centrifuging at 2000rpm for 15minutes and by using separating funnel. The filtered oil was stored separately in opaque dark bottle and placed in deep freezer at -20°C.

Purification of oil

Purification/Refining is a series of process that includes 4 steps, namely degumming, neutralization, bleaching, deodourization and esterification.

Degumming: It was carried out following the method of [17] with modifications. A sample of crude oil (100g) placed in a 600ml beaker and heated to 70°C in oven for 1min. 3ml of 3% aqueous citric acid solution was added to the oil, and the mixture was thoroughly mixed at 70°C for 1minute. The oil was then cooled to room temperature and centrifuged at 2,560 × g for 10minutes to remove impurities.

Neutralization: The degummed oil was neutralized following to AOCS Official Method (1989). Sodium hydroxide (12.6g of 9.5% NaOH solution) was added to 100g of degummed oil and the mixture was heated at 65°C for 30minutes with constant stirring using a magnetic stirrer bar. The sample was then cooled to room temperature and kept undisturbed for 6hours. After centrifugation at 2,560 × g for 10minutes, the oil was decanted from the precipitated soap. 50ml of demineralized water was added to the centrifuged sample to wash out any remaining soap. This operation was repeated three times. The remaining water and impurities were removed by centrifugation at 2,560 × g for 10minutes.

Bleaching: The neutralized oil was bleached following to the method of [18]. The 100ml of neutralized oil was bleached with 1g of acid activated bentonite at 100°C for 20minutes with constant stirring with a magnetic stirrer bar. The activated bentonite with the absorbed impurities was removed immediately by filtration to avoid colour changes to the oil.

Deodorization: The purpose of deodorization was to remove FFAs, mono and diacylglycerols, pigment decomposition products, oxidation products and bad odour producing impurities. The bleached oil was deodorized following to the method of [19] with modifications. The bleached oil was deodorized using a laboratory distillation unit, consisting of a 500ml round-bottomed boiling flask with three outlets. One outlet was connected to a vacuum pump and outlet was connected to a glass distillation column, and the third outlet was sealed with a thermometer inserted. The flask was placed on a heating system. The bleached oil (100ml) was added to the flask and heated at 100°C for 30minutes under vacuum (5mm Hg). The temperature was controlled manually. Volatile products were condensed in a cooling system installed on the vacuum line and are removed.

Estimation of fatty acids: Fatty acid in the samples were identified following the procedure outlined by [20] and quantified as methyl esters in NEON II Gas chromatography instrument (ASHMACO) equipped with Flame Ionization Detector (FID) and DEGS (10%) column. The oven temperature was set at 180°C and flashed with carrier gas (N_2) at rate of 3 ml/min.

Estimation of Amino acids: The fish oil was precipitated by heating and the residues was dissolved in known volume of 10% iso-propanol and centrifuged at 1000rpm for 30minutes. The supernatant was taken for amino acid estimation. The HPLC conditions were adapted from [21] and [22]. Briefly, mobile phases were prepared by mixing ACN and water (1:5), both containing 5mM citric acid adjusted at pH 6.5 with sodium hydroxide. A gradient elution from 5 to 30 % ACN in 30 min, followed by an increase from 30 to 50% ACN in another 5min, was used. Detection was performed at 340nm (465nm as reference). In all cases, 20µl was injected at a flow rate of 1ml/min. Peak areas were measured with the Chem. Station for LC v.10.02 software (Agilent). The HPLC used was Agilent 1100 series with UV-vis variable multiwave length detector, quaternary pump and Kromasil C18 column (250 mm × 4 mm, 5µm particle size (thermostated column).

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Vitamin analysis: The fat soluble vitamins such as A, D, E & K and water soluble vitamins such as vitamins C, B6, B2, B1, and B12 in the fish oil from *S. fimbriata* was determined by HPLC with UV-visible diode array detector (Agilent 1100 Series) and Hypersil BDS (thermostatted column) described by [23].

Quality Assessment of Fish oil: The oil was characterized to determine the specific gravity by the method outlined by [24], Refractive index by Hollow prism Method, moisture content by ISI methods (1974), free fatty acids (FFA) by of [25], iodine value (IV) by [26], peroxide value (PV) by [25], saponification value (SV) by [26] and Observation of colour.

Results

The fish oil extracted from the tissues of *Sardinella fimbriata* employing direct steaming method produced an average of 130 ± 4.5 ml for every 1000g of *Sardinella fimbriata* fish tissues. The results of purification of fish oil obtained from *Sardinella fimbriata* by DS method is presented in Table 1. From 100ml of crude fish oil subjected to degumming produced 80±0.5ml, 100ml of degummed oil subjected to neutralization produced 88 ± 0.5ml, 100ml of neutralized oil produced 94 ± 1.0ml of bleached oil and from 100ml bleached oil subjected to deodorization yielded 89 ± 0.5 ml of purified oil. The percentage wise loss of oil removed as impurities in each step of purification was calculated. Nearly 19.9% of oil was removed as impurities in degumming, 11.88% in the neutralization process, 5.72% in the bleaching process and 10.77% in the deodorization process. The total recovery percentage of oil from the initial to the final stage of the purification process was 58% (Fig. 1) i.e. 58ml of refined oil was obtained from 100ml of crude oil. The weight of the oil progressively decreased through various steps of purification, might be due to the removal of FFA and other impurities. The volume weight conversion of fish oil during various stages of refining were worked out in Table 2 and graphical representation in figure 2, which proved that out of 100ml oil, the average weight was to the tune of 90.908g to 90.392g.

Table-1.Quantity of Sardine fish oil obtained after different steps of purification

S.No	Purification process	Oil used (ml)	Oil obtained (ml)
1	Degumming	100	80 ± 0.5
2	Neutralization	100	88 ± 0.5
3	Bleaching	100	94 ± 1.0
4	Deodorization	100	89 ± 0.5

Fig-1. Percentage of fish oil obtained after different steps of purification

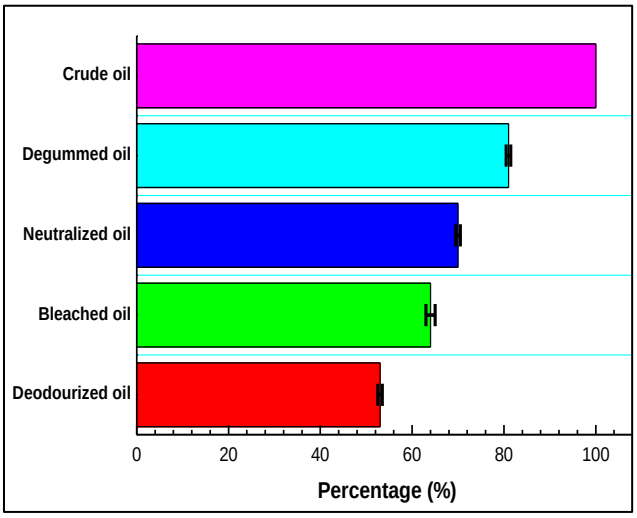
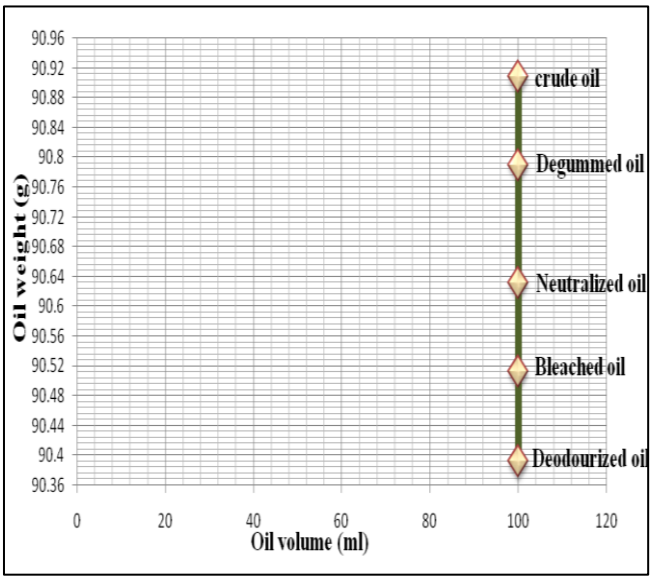


Table-2.Volume-Weight relationship of fish oil under various stages of Refining

S.No	Oil (at different stages of purification)	Oil volume (ml)	Oil weight (g)
1	Crude	100	90.908
2	Degumming	100	90.790
3	Neutralization	100	90.632
4	Bleaching	100	90.512
5	Deodorization	100	90.392

Fig-2. Decrease in Weight of oil through the stages of refining



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Analytical Properties of oil:

The analytical properties of the crude and the refined fish oil were evaluated separately for freshly prepared samples and for 30 days old samples stored in refrigerator at 0°C. The analytical properties of the crude and the refined fish oil were tabulated in table-3. These analytical values of the crude oil fall well within the acceptable standard values. The results showed that refining of fish oil brought notable improvement in the analytical properties of the oil, especially in the reduction of FFA, IV, PV and SV.

Table-3.Quality Analysis of fish oil

S.no	Analytical Parameters	Crude oil		Purified oil	
		Fresh (0 days)	Stock (30 days)	Fresh (0 days)	Stock (30 days)
1	Specific Gravity at RT	0.9036 ± 0.0009	0.9041 ± 0.0011	0.8985 ± 0.0006	0.8987 ± 0.0009
2	Refractive index	1.461 ± 0.006	1.485 ± 0.01	1.473 ± 0.004	1.465 ± 0.09
3	Moisture Content (%)	0.7 ± 0.04	0.4 ± 0.07	0.3 ± 0.05	0.32 ± 0.08
4	Free fatty acid (mg KOH/g)	2.9 ± 0.24	3.8 ± 0.38	1.74 ± 0.13	2.1 ±0.2
5	Iodine Value (I_2/100g)	188 ± 3	194 ± 5	174 ± 2	181 ± 5
6	Peroxide Value (mEq/Kg)	2.18 ± 0.027	2.73 ± 0.27	1.03 ± 0.06	1.21 ± 0.3
7	Saponification value (mg/KOH/g)	202.7 ± 1.6	207.39 ± 2.1	153.1 ± 2.05	174.8 ± 3.2
8	Colour	Slight Brownish yellow	Slight Brownish yellow	Bright yellow	Bright yellow

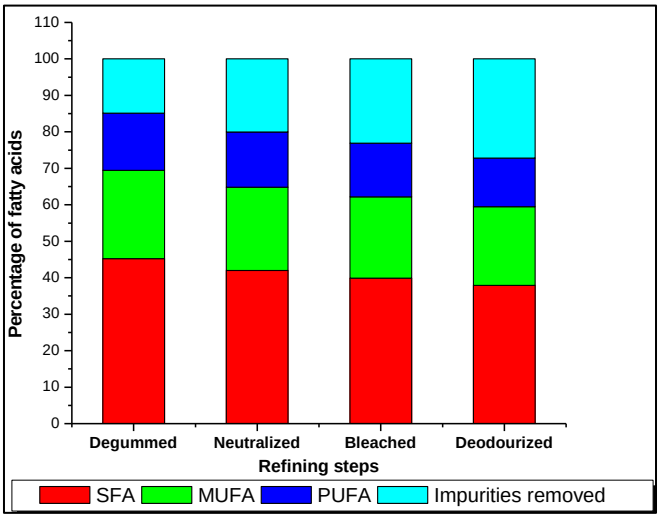
Proximate composition of the fishes:

Lipid content extracted from the fish muscles varied between species. The details of fatty acid composition of crude fish oil is arranged in Table 4 and graphically represented in figure 3. 50.726 % w/w of oil was extracted from the tissues of *Sardinella fimbriata* employing direct steaming method. The major fatty acids found in fish oil are Lauric acid (C12:0), Tri decyclic acid (C13.0), Myristic acid (C14:0), Physeteric acid (C14:1ω-5), Myristoleic acid (C14:1ω-4), Pentadecyclic acid (C15:0), Palmitic acid (C16:0), Palmitoleic acid (C16:1ω-7), Margaric acid (C17:0), Stearic acid (C18:0), Oleic acid (C18:1ω-9), Linoleic acid (C18:2ω-6), α-linolenic acid (C18:3ω-3), Nonadecyclic acid (C19:0), Arachidic acid (C20:0), Arachidonic acid (C20:4ω-6), Eicosapentaenoic acid (C20:5ω-3), Pehenic acid (C22:0), Docosapentaenoic acid (C22:5ω-3), Docosahexaenoic acid (C22:6ω-3), Tricosanic acid (C23:0) and Lignoceric acid (C24:0). The Palmitic acid was the predominant fatty acid in the saturated content of the fish oil, accounting for around 50% of all saturated fatty acids. The total unsaturated fatty acid content in the fish oil was almost equal or slightly above that of the total saturated fatty acid. The predominant fatty acids in the monounsaturated and polyunsaturated content of the fish oil were oleic acid (C18:1ω-9) and α-linolenic acid (C18:3ω-3) &docosahexaenoic acid (C22:6ω-3) respectively.

Table-4. Fatty acid composition of fish oil (*S. fimbriata*) at various steps of Refining (w/w %)

Carbon chain	Fatty acids	Crude	Degummed	Neutralized	Bleached	Deodorized
SFAs						
C12:0	Lauric	0.176	0.112	-	-	-
C14:0	Myristic	6.21	5.91	5.04	5.04	4.96
C15:0	Pentadecyclic	5.1	4.8	3.92	3.89	3.74
C16:0	Palmitic	11.45	10.87	10.48	10.46	10.45
C17:0	Margaric	10.56	9.06	8.92	8.85	7.51
C18:0	Stearic	9.67	6.61	6.58	6.11	6.11
C22:0	Pehenic	1.71	1.71	1.61	0.95	0.90
C23:0	Tricosanic	4.7	4.56	3.89	3.07	2.68
C24:0	Lignoceric	1.15	1.62	1.55	1.55	1.54
Sum of SFAs		**50.726**	**45.252**	**41.99**	**39.92**	**37.89**
MUFAs						
C14:1ω-4	Myristoleic	5.56	5.50	5.47	5.05	4.75
C16:1ω-7	Palmitoleic	1.50	1.05	0.87	0.84	0.728
C18:1ω-9	Oleic	18.08	17.67	16.47	16.37	16.11
Sum of MUFAs		**25.14**	**24.22**	**22.81**	**22.26**	**21.588**
PUFAs						
C18:2ω-6	Linoleic	1.845	1.808	1.603	1.595	1.487
C18:3ω-3	Alfa linolenic	3.659	3.059	3.011	2.931	2.789
C20:4ω-6	Arachidonic	1.67	0.907	0.855	0.840	0.736
C20:5ω-3	Eicosapentaenoic	1.808	1.80	1.713	1.68	1.173
C22:5ω-3	Docosapentaenoic	2.75	2.50	2.41	2.205	2.167
C22:6ω-3	Docosahexaenoic	5.89	5.59	5.551	5.447	5.009
Sum of PUFAs		**17.617**	**15.664**	**15.143**	**14.698**	**13.361**

Fig-3. Fatty acid composition of fish oil at various steps of refining



Fatty acid composition of refined fish oil:

The fatty acid compositions of sardine oil for different steps in purification are tabulated separately in Table 5. The major FA found in sardine oil during refining are C14:0, C15:0, C16:0, C17:0, C18:0, C22:0, C23:0, C24:0, C14:1ω-4,C16:1ω-7,C18:1ω-9,C18:2ω-6, C18:3ω-3, C20:4ω-6, C20:5ω-3, C22:5ω-3 andC22:6ω-3. The yield of fish oil after purification was 58%. The major yield loss took place during the degumming process. The fatty acid compositions of fish oil from each purification step are shown in Table 4. The fatty acid composition of the crude oil decreased from 93.693 to 72.839 % (w/w) in refined oil. The major FA loss was also found during the degumming process. There were minor losses of MUFA (25.25 to 21.588 % w/w) and PUFA (17.617 to 13.361% w/w); the reduction of saturated fatty

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acid was significant during purification steps resulting from 45.252 to 37.89 % (w/w).

Amino acid composition of fish oil:

This forms a baseline work for the analysis of amino acid from fish oil. Except Asparagine, Cysteine and Methionine, all other non-essential and essential amino acids were recorded in all the samples. The amino acid composition of the crude fish oil was summarized in table 6. The sardine oil extracted by DS method was purified and analyzed for variation in amino acid composition during each step of purification and are summarized in table 5. The values are graphically represented in Figure 4.The major non-essential amino acids present in the oil was Glutamic acid (2.8µg/g) in the crude oil; but became absent after deodorization. All the essential amino acids were more or less equally present in the crude fish oil ranging from 1.907 to 0.067µg/g. After purification the major amino acid present was Valine (2.07µg/g) and Threonine (1.26µg/g).

Fig-4. Amino acid composition of purified fish oil

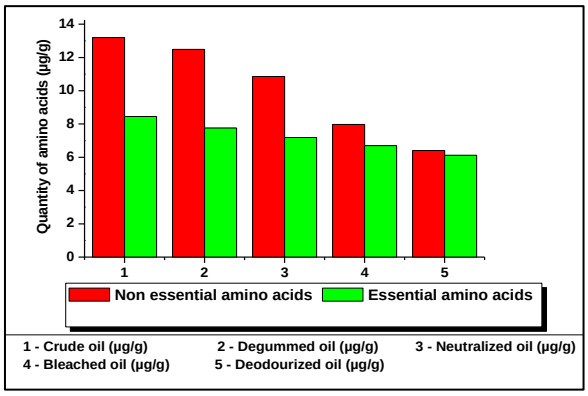

Table-5.Amino acid composition of Crude fish oil

Amino acids	Crude (µg/g)	Degummed (µg/g)	Neutralized (µg/g)	Bleached (µg/g)	Deodorized (µg/g)
Non-Essential Amino Acids (Non EAA)					
Alanine	1.06	1.03	1.02	1.01	0.945
Arginine	1.5	1.1	1.07	1.05	0.965
Asparagine	-	-	-	-	-
Aspartic acid	1.91	1.84	1.215	1.20	1.10
Cysteine	-	-	-	-	-
Glutamic acid	2. 8	2.07	1.89	0.94	Traces
Glutamine	3.11	1.45	1.267	0.95	0.90
Glycine	1.14	0.76	0.58	0.54	0.47
Proline	1.12	1.78	1.54	0.45	0.37
Serine	1.9	1.38	1.25	1.21	1.07
Tyrosine	1.6	1.07	1.02	0.624	0.59
Total Non EAA	**13.34**	**12.48**	**10.852**	**7.974**	**6.41**
Essential amino acids (EAA)					
Isoleucine	1.156	0.356	.32	0.29	0.21
Histidine	0.212	0.056	Traces	Traces	Traces
Leucine	0.990	0.135	0.13	0.13	0.12
Methionine	Traces	-	-	-	-
Lysine	1.398	1.123	1.015	0.833	0.817
Phenylalanine	1.56	1.51	1.216	1.06	0.801
Tryptophan	1.12	1.01	1.0	0.96	0.91
Threonine	1.74	1.565	136	1.33	**1.26**
Valine	2.78	2.0	2.15	2.10	**2.07**
Taurine	-	-	-	-	-
Total EAA	**10.956**	**7.755**	**7.191**	**6.703**	**6.188**

Vitamin analysis:

Vitamin analysis for the fish oil was performed by HPLC technique. The crude oil extracted from *Sardinella fimbriata* contained all fat soluble vitamins (A, D & E), but the refined oil lacked vitamin E, none of the water soluble vitamins were present in the fish oil. The fat soluble vitamins were also present only in minor quantities; 0.06, 0.005 and 0.002µg/g of vitamin A, D and E, respectively in crude fish oil. In refined fish oil; 0.04 and 0.003µg/g of vitamin A and D were present.

The data were statistically treated with the help of computer software packages such as MS-Excel (MS OFFICE 2007) and Origin (ver. 6.0).

Table-6. Vitamin Analysis of Crude and Refined fish oil from *S. fimbriata*

S.No	Vitamins	Crude (µg/g)	Purified(µg/g)
Fat-soluble vitamins			
1.	Vitamin A	0.06	0.04
2.	Vitamin D	0.005	0.003
3.	Vitamin E	0.002	-

Discussion

During last few years, Fish oil is being approved for human consumption as food supplement and as an ingredient in food. This warrants a great demand for fish oil. Several hygienic and scientific measures were employed, so as to improve the quality of fish oil, in conventional meal plants and in other commercial processes, where fish oil is a byproduct. Significant yield of oil extracted from *Sardinella fimbriata* samples from direct steaming was observed.Direct steaming is considered as a good old traditional and economic technique for extraction of oil. The present experiment supports the suggestions of [27] that oil extraction by direct steaming is easier, cheaper, quicker and is affordable to laymen and rural communities. [28] Emphasised that direct steaming at 80-85°C is a simple and economical technique that ensures viable results. The oil extracted from *Sardinella fimbriata* was 10-13%, which was almost double the quantity of oil extracted from *Sardinella lemuru* (>6%) by [36] in Malaysian waters.

Purification:

The major steps in purification are Degumming, Neutralization, Bleaching and Deodorization. From 100ml of crude fish oil, 58 ± 0.3ml of the deodorized sardine fish oil was produced. The major weight loss of oil was observed during the degumming process, which resulted in a loss of 19.9% followed by 11.88% in neutralization, 10.77% in deodorization and 5.72% in bleaching respectively. The results of purification were similar to the work carried out

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by [29] on oil production from catfish viscera, where there was 19% loss in degumming, 12% loss in neutralization and 4.2% in bleaching; except for deodorization where the loss was only 3.2%. This disparity in deodorization process might be due to the difference bleaching steps adopted, where activated bentonite was used in the present investigation instead of activated earth.

Volume – Weight relationship:

The weight of the oil exhibited a decreasing trend from initial to final steps of purification. The weight of 100 ml of crude fish oil was 45.454g which decreased to 45.196g after deodorization. The reduction in weight of the oil at a constant volume during the refining process was primarily due to the removal of impurities, minerals, other suspended solids, etc. Crude fish oil is expected to contain certain amount of minerals since phospholipids are reported to carry minerals into oil [30]. These particles are removed during the degumming step, resulted in decrease of weight as it progress through different steps of purification.

Analytical Properties of oil:

The standard values for fish oil have been emphasisedby [10]. In the present study, certain properties of fish oil were evaluated for fresh and age old fish oil so as to determine the deterioration of stability of oil during storage. The results of quality analyses for the fish oil are presented in Table 3. It was observed that the results obtained were similar to the standard values; nevertheless some values were pretty close to upper limit of standard range. [31]Emphasized that most of the standard values from the literature are dependent on geographical locations, seasons and purpose to which the oil will be used for.

The free fatty acid value ranged from 2.9 (fresh) & 3.8 (30days old) mg KOH/g for crude fish oil and 1.74 (fresh) & 2.1(30days old) mg KOH/g for purified fish oil. Iodine value was 188 (fresh) & 194 (30days old) I_2/100g for crude fish oil and 174 (fresh) & 181 (30days old) I_2/100g for purified fish oil. Peroxide value was 2.18 (fresh) & 2.73 (30days old) mEq/kg for crude fish oil and 1.03 (fresh) & 1.21 (30days old) mEq/kg for purified fish oil. The saponification value was 202.7 (fresh) & 207.39 (30days old) mg KOH/g for crude fish oil and 153.1 (fresh) & 174.8 (30days old) mg KOH/g for purified fish oil. A similar observation has been made by [32], who reported that the oil refined from heat rendering and gives a low free fatty acid. Results of the present study confirm the findings of [33] were purified oil produced showed very low percentage of free fatty acids and peroxides. The minor presence of oxidizing peroxides may be advantageous to the quality of the oil during long term storage. [32] Explained that oil obtained by heat rendering and could be stored for a longer period under tropical conditions without subsequent loss of quality.

The appreciably lower values in freshly purified oil could be attributed to the fact that the lesser the period of exposure of oil to atmosphere, the lesser the rate of the oxidation of the oil and consequently the lesser values of oil [12]. The results of Free Fatty acids, Iodine value, Peroxide value and Saponification value were increased from fresh to 30 days old samples; which are in line with the findings of [8].Due to its high content of unsaturated fatty acids, fish oil is highly susceptible to oxidative spoilage [13]. Fish oil also having high concentrations of phospholipids, containing unsaturated fatty acids, which make them even more sensitive than other oil [14, 15]. Moreover the course of oxidation is often quite different between extracted fish oils and lipids in fish tissues [14]. [34] Opined that autoxidation of fish oil is the most important cause of deterioration in quality. All parameters showed lower values in the refined oil than the crude, since it is subjected to removal of all impurities such as sterines, trace metals, phospholipids, sulphur, insoluble matter, free fatty acids, soap, colour producing pigments, oxidation products, mono & diacylglycerols and bad odour producing impurities [29]. The reduction in free fatty acids during the deodourization process has been due to the vaporizability of free fatty acids [9, 35]. The reduction in the Saponification value equals the calorific and weight loss by the oil. The Iodine value of the refined oil reduced to 174 (fresh) & 181 (30 days old) I_2/100g which implies that few of the double bonds in the oil has been saturated, as suggested my [36].

The specific gravity of the crude fish oil was found to be 0.9036 and 0.9041 for fresh and stock samples, respectively which is close to that of the commercially available standard Menhaden oil of 0.900 to 0.910. The specific gravity of the refined oil was 0.8985 and 0.8987, which is slightly lesser than that of the Menhaden oil. The refractive index of the crude fish oil was 1.461 and 1.485 (for fresh and stock, respectively) which falls between the standard values of 1.460 and 1.495.The refractive index of the refined oil was 1.473 and 1.465 which also falls between the values of commercially available standard Menhaden oil. Moisture Content of the crude fish oil was 0.7 and 0.4 % and for refined oil it was 0.3and 0.32 % (for fresh and stock correspondingly). The extracted oil from the sardines are high in lipid content, therefore it will be typically accompanied by moisture content in oil or vice versa [31].

The appearance of the crude fish oil was brownish yellow, which might be due to the prolonged heating period during, that often oxidizes the product (i.e. the oil) and imparts a brownish yellow colour [28]. After refining the oil turned bright yellow colour, which indicates the significant removal of pigments. [36] Suggested that bleaching contributed to the physical improvement of the refined oil. The aldehydes in crude fish oil (autoxidize), such as 2-hexenal and acedaldehyde, appear to react by aldol

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condensation and dehydration to form crotonaldehyde and 2-(1-butenyl)-octa-2, 4-dienal, during the early stage of browning [37]. Since crude fish oil is having comparatively higher Peroxide value, degradation products such as aldehydes, could easily be produced and these undergo browning reaction. As a result, a darker colour was obtained for the crude fish oil. The impurities were completely removed during the purification process, which results in bright yellow colouration to refined oil. [38] studied the mechanism of browning reaction on emulsions of menhaden oil in aqueous protein solutions; stated three parallel processes for browning, such as (1) oxidation of polyunsaturated fatty acids bound in the fish oil, (2) polymerisation of oxidation products into macromolecular compounds, and (3) formation of brown pigments by reactions between different active groups of oxidising lipids and amine groups of proteins. Among these, the process results in most intensive discolouration. [39] and [40] opined that dark brownish colouration in fish oil produced from boiled dry anchovy, dry mackerel, and salty trout might be due to reactions of proteins with autoxidising methyl linoleate.

Fatty acid composition:

Estimation of fatty acid profile is one of the major criteria to assess the quality of the oil produced. Similarly fatty acid profile of crude fish oil from *Sardinella fimbriata* subjected to different steps of purification was also analysed. The predominant saturated fatty acids in all the samples were palmitic acid (C16:0) followed by margaric acid (C17:0), stearic acid (C18:0) and tricosanic acid (C23:0). The results of the present investigation about the predominant SFAs in the oil samples were supported by the works of [41, 29, 42, 43] and [44].

The monounsaturated fatty acid levels in the sardine oil ranged from 23.072 to 26.45%. Similar levels of monounsaturated fatty acids were reported in sardine fish oil by [20], in which the estimated range was 26.22 to 32.30%. The dominant MUFAs in all samples were oleic acid (C18:1ω-9) and palmitoleic acid (C16:1ω-7). The abundance of oleic acid (C18:1ω-9) and palmitoleic acid (C16:1ω-7) in the fish oil samples were also reported [41, 29, 45, 42, 43] and [44].

According to [45] oil extracted from marine fishes, polyunsaturated fatty acid was dominated. [46] Reported that the polyunsaturated fatty acid ranged from 1.08 to 7.38% in liver oil extracted from two species of shark, namely, *Eusphyra blochii* and *Carcharhinus bleekeri*. In the present study, the level of total polyunsaturated fatty acids ranged from 10.192 to 17.717 % in oil samples. [44] Reported higher levels of polyunsaturated fatty acids ranging from 22.67 to 26.39% from liver samples of *Sardinella lemuru*. Among Polyunsaturated fatty acids, α-linolenic acid (C18:3ω-3), docosahexaenoic acid (C22:6ω-3), docosapentaenoic acid (C22:5ω-3) and eicosapentaenoic acid (C20:5ω-3) were the major components. Among these docosahexaenoic acid content (5.97%) being higher, followed by α-linolenic acid (4.21%), docosapentaenoic acid (2.75) and EPA (2.564%) in all the extracts. The composition of docosahexaenoic acid content in the present study was comparatively higher than that of eicosapentaenoic acid in the samples which is in line with works of [47, 48, 45, 43] and [44]. The EPA: DHA ratio has been suggested as a useful indicator for comparing relative nutritional values of fish oils. It was suggested that a ratio of EPA: DHA is 1:1–1.5 would constitute better for healthy human diet [49].

Fatty acid composition of refined fish oil:

The fatty acid compositions of sardine oil of each purification step are shown in Table 4. Palmiticacid (C16:0) was the predominant fatty acid accounting for about 24% of all SFAs. Among MUFA, oleic acid was the predominant FA, accounting around 42% of total unsaturated fatty acids. The EPA and DHA of the crude fish oil was 1.803 and 5.89%, respectively. EPA and DHA obtained finally after refining was 1.173 and 5.009%, respectively. That yields around 66 and 85% of EPA and DHA after purification. [50] Reported only 35.5% and 64.4% of recovery of EPA and DHA by fractionation of fatty esters using column chromatography. Urea inclusion method put forth by [51] and [52] for PUFA recovery and enhancement, yielded only 60 and 73% of EPA and DHA; which lies below the level of oil recovered in the present study. Currently, purification of n-3 PUFA from fish oil was accomplished through a three stage process: namely, oil saponification, PUFA concentration and fractional separation by HPLC [53]. These methods imparts more instrumentation skill and cost, which will yield only less significant results compared to those obtained from the present study. Nevertheless, traces of methyl carbamate may be formed during urea complexation for fractionation of fatty acids in those mentioned methods, which is a carcinogen [54 and 55] and is not advisable for human consumption.

Amino acid composition of fish oil:

The amino acid composition of the crude and refined oil was illustrated separately table 5. The present study is a baseline work for the analysis of amino acid from sardine oil. Except Asparagine, Cysteine and Methionine all other essential and non-essential amino acids were recorded in all the samples. There have been various studies on amino acid composition of the fishes such as those of [45, 42, 56, 57 and 58].

[59] Classified the vegetable oil according to their botanical origin using amino acid profiles by HPLC with UV-Vis detection, which is a pioneering study about amino acids for classifying their origin. A study of this kind will throw light

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on the need to research on amino acid profile in fishes so as to compile a database to classify the origin of fishes. The presence of amino acids though in low levels might be attributed to the occurrence of glycoproteins associated with fish oil. According to [38] brownish colouration in oil was obtained not only due to oxidation of PUFA and polymerisation of oxidation products into macromolecular compounds, but also due to the interaction of oxidizing lipids and amine groups of protein. Therefore, the brownish yellow colour of crude sardine oil was also due to the presence of amino acids, accounting 21.652µg/g whereas for the refined oil it was only 12.598µg/g. Major amino acid present was valine (2.07µg/g) and threonine (1.26µg/g).

Vitamin Analysis:

The results of the vitamins analysis by HPLC technique illustrated traces of vitamin A, D and E resulting less than 1µg/100g. Supporting our results (presence of only fat soluble vitamins), [60] Suggested that fish oil is an important source of fat soluble vitamins and water soluble vitamins are not abundant as the previous. According to [61], level of fat soluble vitamins in whole body is very low as it is more concentrated in the liver (preferring cod liver oil as a potential source for fat soluble vitamins). The results of the present investigation coincide with the studies carried out by [62], showing the presence of fat soluble vitamins in tuna body oil was only in µg/100g. Vitamin E is considered for its antioxidant property [63] was present in the crude fish oil and it might have been washed away with the impurities while refining, so further more trials are required for conforming the presence of vitamin E. Our present study concludes the presence of all fat soluble vitamins A, D and E but in very minor quantities since liver is the major source for these vitamins (which has been excluded from our extraction).

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