Long chain *n*-3 polyunsaturated fatty acid enriched oil emulsion from sardine oil

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Dietary fats are used to build every cell in the body and cell membranes are made of a variety of individual fatty acids which are carboxylic acids with long hydrocarbon chains (usually C_{12-22}). The essential fatty acids from marine fish have protective mechanisms against coronary heart disease, which became apparent in the investigations of the health status of Greenland Eskimos who consumed diets very high in fat from seals,whales, fish etc, and yet had a low rate of coronary heart disease. This paradox was explained by the fact that the Eskimos diet contained large quantities of the very-long-chain and highly polyunsaturated fatty acids with C_{20-22} carbons and 5-6 olefinic bonds, which are abundant in marine fish, but scarce or absent in terrestrial animals and plants. Fatty acids with two or more double bonds are termed as polyunsaturated fatty acids (PUFAs) which are broadly divided into two major families, *n*-3 and *n*-6 PUFAs. The long chain C_{20-22} *n*-3 fatty acids are found abundantly in marine fish and phytoplankton. These affect many physiological processes including cognitive function, visual acuity, immunosuppressive and anti-thrombic activities along with a major role in glucose and lipid metabolism. Research on exploring sources for long-chain C_{20-22} PUFAs (LC-PUFAs) such as Eicosapentaenoic acid (EPA, 20:5 *n*-3) and Docosahexaenoic acid (DHA, 22:6*n*-3) for nutrition have received considerable attention (Fig. 1). Since these PUFAs are usually low in abundance in humans, but regarded as essential they have to be supplied in the diet. The importance of PUFAs in human nutrition has been extensively investigated during the past 20 years. DHA one of the important PUFAs maintains structural and functional integrity in larval cell membranes in addition to the neural development and function, while Arachidonic acid (AA, 20:4*n*-6) and EPA are involved in the production and modulation of eicosanoids respectively. DHA is a vital component of the phospholipids of cellular membranes, especially in the brain and retina, and necessary for their proper functioning. An imbalance in *n*-3/*n*-6 ratio can accentuate *n*-3 fatty acid deficiency state, as shown by earlier studies. The ratio is found to have increased in industrialized societies because of increased consumption of vegetable oils rich in *n*-6 fatty acids, *ie*, linoleic acid (18:2*n*-6) and reduced consumption of foods rich in *n*-3 fatty acids. Another important feature of *n*-3 fatty acids is their role in the prevention and modulation of certain diseases that are common. A partial list of diseases that may be prevented or ameliorated with *n*-3 fatty acids is given below.

- Coronary heart disease and stroke
- Diabetes
- Cancers of the breast, colon, and prostate
- Hypertension

The LC - PUFAs are also recognized to have beneficial therapeutic, physiological and nutritional effects on human health.

Imported products (PUFA supplement) include Seven Seas by a UK healthcare company producing PUFA rich Cod Liver Oil by Ocean Gold™ technology. A value-added PUFA concentrate named "fish oil-1000 natural omega 3®" containing 100 capsules manufactured by Healtheries of New Zealand Ltd. priced at about ₹ 1150 per pack, is currently being marketed in India by Perma Healthcare, Bangalore. "EPAX 1050 TG®", a marine omega 3 formula produced from selected marine oils and marketed by EPAXAS, Norway is an imported product containing

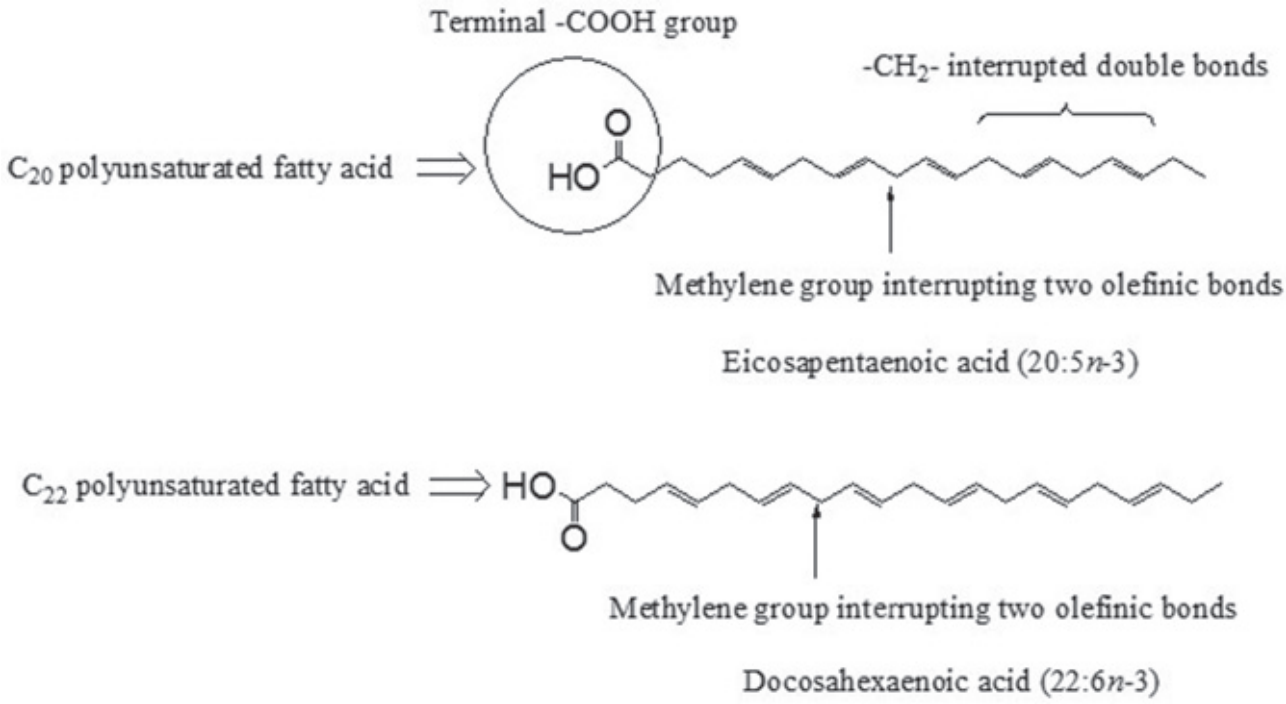


Fig. 1. C_{20-22} polyunsaturated fatty acids (Eicosapentaenoic and Docosahexaenoic acids)

17% EPA for use as fish feed supplement. OMEGA XL® is marketed by DeColores in a bottle containing 60 capsules of refined combination of omega-3's costing about $50 per pack, as a nutraceutical. Great Health Works manufactures a concentrate of PUFA from Blue Grenadier Fish that is also a costly PUFA supplement. DSM Nutritional Products, Switzerland manufactures and sells PUFA concentrate under the trade name ROPUFA® produced from refined vegetable and marine oils. These products are currently imported to meet the domestic demand of PUFA concentrate formulation for fish feed supplements and nutraceutical purposes. This underlied the need to develop an indigenous *n*-3 (PUFA) supplement for which the Marine Bioprospecting section of Marine Biotechnology Division screened locally available low-value fish for *n*-3 PUFAs, concentrating these essential fatty acids therefrom by chemical and/or enzymatic process.The aim was to develop an indigenous *n*-3 PUFA enriched formulation(s) comprising fatty acid concentrate and individual or combination of additives with potential antioxidant properties to form a stabilized and concentrated form of long chained *n*-3 PUFAs which may be a cheaper alternative to the imported PUFA supplements, and will be useful as nutraceuticals and in mariculture operations for fish larval nutrition.

It is believed that the optimal formulations for the first-feeding fish larvae should simulate the yolk composition and to some extent reflect the nutrient requirements and metabolic capacities of the pre-feeding fish. Dietary long chain PUFAs play an important role as vital sources of essential fatty acids, needed for normal growth and suvival. Larvae of many marine fishes require highly unsaturated fatty acids of the n-3 series, such as, DHA due to the absence of essential enzymes required for biosynthesis of C_{20-22} LC-PUFAs from their short chain analogues (Fig. 2). Some investigations have shown that DHA is superior to EPA for larval fish suggesting a different physiological function.

The important natural sources of *n*-3 LC-PUFAs are fishes such as mackerel, sardines, sharks; microalgae, polychaetes, etc. Among these, sardines are inexpensively available and contain considerable amount of PUFAs, particularly 20:5*n*-3. Hence sardine oil was preferred as the raw material to formulate

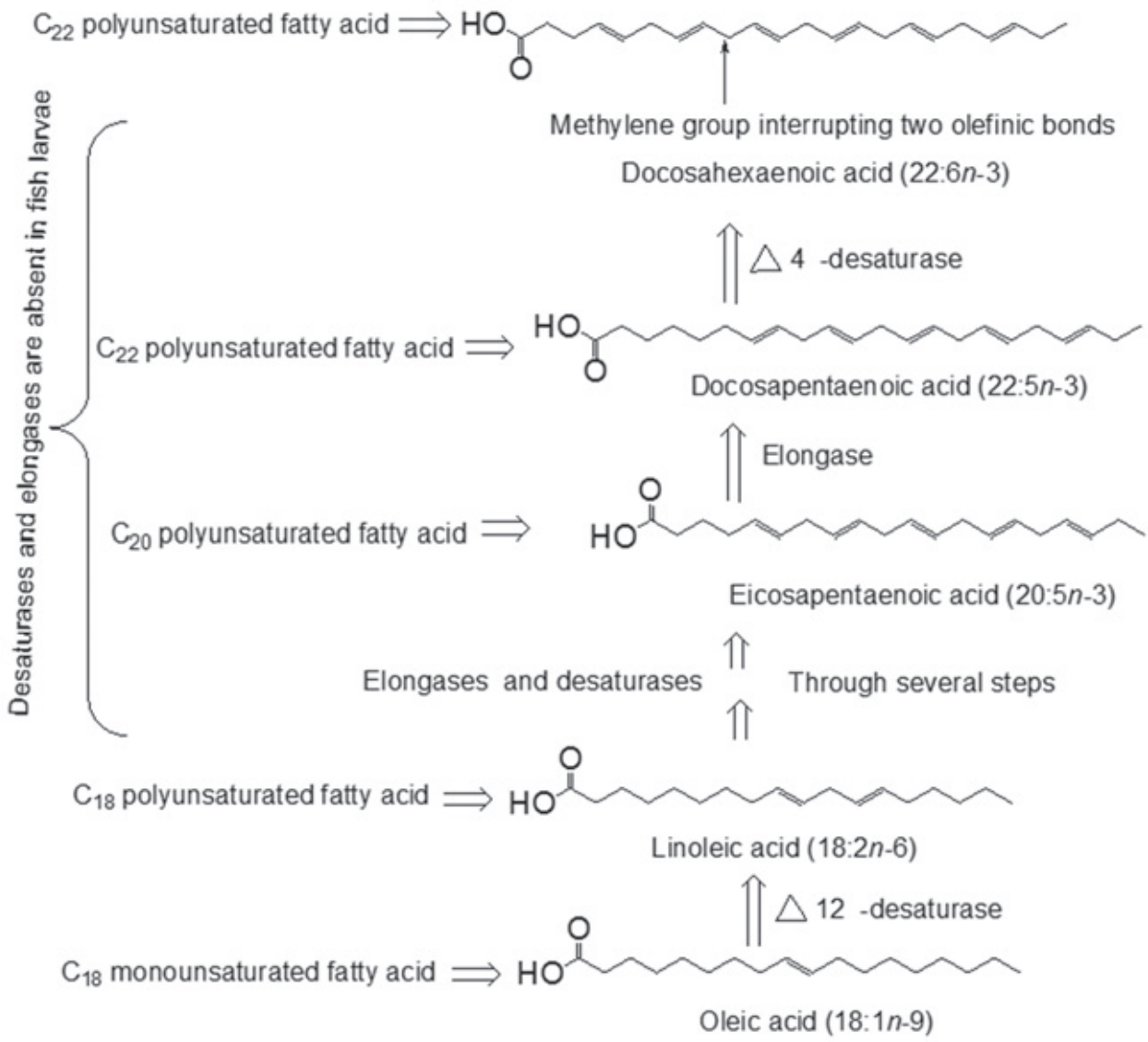


Figure 2. Biosynthetic pathways of the long chain polyunsaturated fatty acids (LC-PUFA)

the *n*-3 C_{20-22} LC- PUFA concentrates based on different physicochemical properties associated with the olefinic bonds in fatty acids and/or acyl chain length. The objective was to purify EPA and DHA from sardine oil by saponification of fish oil to derive free fatty acids, enrichment of PUFA content from the mixed fatty acid concentrate by amide fractionation and chromatography by utilizing the silica gel complexed with a d-block element.The unique substrate specificity of microbial triacylglycerol acyl hydrolases was utilized for the enhancement of PUFA content in triglycerides to further enrich the C_{20-22} LC PUFAs. Triacylglycerol acyl hydrolases specifically hydrolyse carboxyl esters of triglycerides into free fatty acids and partial acylglycerols. The unique characteristics of this group of enzymes such as positional and stereospecificity were utilized to selectively concentrate targeted fatty acids in triglycerides that can be readily absorbed into plasma triglycerides.

PUFA enrichment by different physicochemical procedures

The crude sardine oil was clarified by a sequential process of degumming, decolorization, and

deodorization, and was found to contain LC-PUFAs, particularly 20:5 *n*-3 or EPA (17.80 ± 1.57%) of total fatty acids, TFA) and 22:6 *n*-3 or DHA (7.67 ± 1.50% of TFA) along with other *n*-3 and *n*-6 PUFAs like Linolenic acid (LA or 18:3 *n*-3; 4.47 ± 0.84% TFA), Linoleic acid (18:2 *n*-6; 0.71 ± 0.23% TFA), and Docosapentaenoic acid (DPA or 22:5 *n*-3; 1.14 ± 0.08% TFA) (Fig. 3). The *n*-6 fatty acids have a minor share of the total fatty acid content of sardine oil (0.81% TFA). The PUFAs containing C_{18}- C_{22} acyl chain length contributed a major share of the total fatty acids of the sardine oil (>30% TFA). Among the saturated fatty acids (SFAs), 14:0 was found to be predominant (7.04 ± 0.22% TFA), while 16:1 n-7 contributed the major share (31.56 ± 2.59% TFA) among the monounsaturated fatty acids (MUFAs). When fatty acids are required in free form for further analyses, lipids were hydrolyzed in alkaline medium for extracting the unsaponifiable material. Sardine oil was saponified with NaOH/Na_2EDTA to yield free fatty acids. Na_2EDTA appeared to form complex with traces of metal ions (Cu, Fe), which catalyze oxidation of unsaturated fatty acids during saponification, and subsequently removed by extraction with water thus hindering the interferences of metal ions during the course of further purification process. Relatively large volumes of *n*-hexane were added to the aliquot of the salt of fatty acid mixtures for better phase separation, thus removing the unsaponificable materials. Among saturated fatty acids (SFAs), 14:0 was found to be predominant (7.04% TFA), while 16:1*n*-7 contributed the major share among all individual fatty acids in the crude sardine oil (>31% TFA). EPA and DHA were found to be the major *n*-3 PUFAs contributing to 17.8% and 7.67% of TFA, respectively. The *n*-6 fatty acids have minor share in the total fatty acid content of sardine oil. Solvent extraction resulted in marginal increase of unsaturation (0.85%) in the fatty acid profile. The PUFA exhibited an increase of 6.49%, while MUFA and SFA reduced by 2.96% and 6.91% respectively. The *n*-3 fatty acids exhibited an increase of 5.73% in the solvent extract of fatty acids.

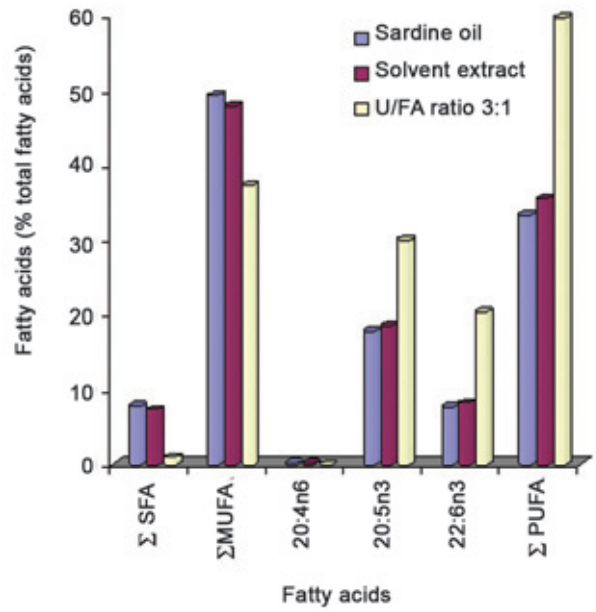


Fig. 3. Fatty acid composition of crude and solvent extracted sardine oil, and PUFA concentrate obtained by the amide fractionation (U/FA: amide/fatty acid ratio)

The free fatty acids derived from sardine oil were subjected to amide fractionation using methanol as solvent at three different temperatures and urea-fatty acid ratios to obtain PUFAs of high purity. The interfering SFAs and most of the MUFAs were removed in the form of amide inclusion compound. Further, as oxidized products do not form amide adducts, the peroxidation of *n*-3 PUFAs could be avoided during the extraction of free acids from fish oil triglycerides. Amide fractionation resulted in total reduction of SFAs (>95%) (14:0, 16:0, and 17:0), moderate reduction of MUFAs (>65%). The U/FA ratio of 4:1 (w/w) was found to be optimal for getting high-purity EPA (47.8%) while that at 3:1 (at 2^0 C) yielded higher content of DHA (>20 %) when crystallized at 2^0C. Based on these results, DHA obtained from sardine oil at 2°C temperature of amide-crystallization by using a U/FA ratio of 3:1 was selected for subsequent purification of C22 fatty acid DHA. It is likely that at the lower temperature (2^0C), the reaction kinetics to form amide-inclusion complex with SFAs and MUFAs was relatively lower resulting in higher DHA in the extract.

Change in fatty acid composition as a function of microbial triacylglycerol acyl hydrolase-catalyzed hydrolysis of fatty acids from sardine oil

The free fatty acids were esterified by using a mixture of ethyl alcohol and 0.1 (N) H_2SO_4 resulting in the ethyl ester of the fatty acids. An extracellular triacylglycerol acyl hydrolase derived from *Bacillus subtilis* isolated from marine macroalga, *Turbinaria conoides*, was used to prepare C_{22} *n*-3 polyunsaturated fatty acid concentrates from the ester fraction. The enzyme was purified 132-fold with specific activity of 386 triacylglycerol acyl hydrolase units/mg. The urea fractionated fatty acyl esters were hydrolyzed with enzyme purified from the bacterium *Bacillus subtilis* and the TFA content of fatty acyl esters after lipase hydrolysis were analyzed. SFA levels showed a reduction to 0.05% after 3 hours (h) of hydrolysis. The decrease in the content of SFAs and MUFAs in the fatty acyl ester mixture with the progress of hydrolysis suggested that SFAs and MUFAs were more easily hydrolyzed by the lipase than those in esterified fatty acids that contain DHA, resulting in the enrichment of the latter in the ester fraction.

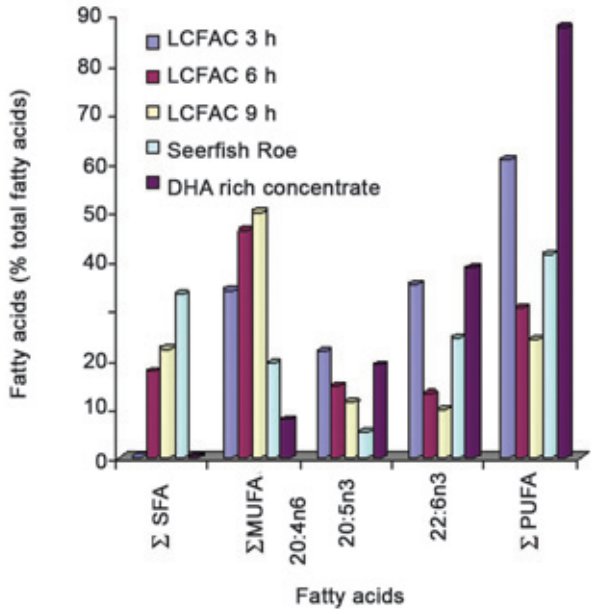


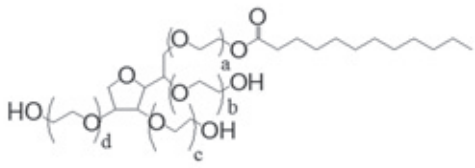
Fig. 4. Fatty acid composition of urea concentrated fatty acids, triacylglycerol acyl hydrolase-catalyzed fatty acid concentrate, seer fish roe, and DHA rich concentrate

The variations of PUFA content of sardine oil triglycerides as a function of time during the microbial triacylglycerol acyl hydrolase-catalyzed hydrolysis are illustrated (Fig. 4). The total DHA of fatty acyl ester fraction increased with time up to 3 h of enzyme-catalyzed hydrolysis (35.27% TFA), beyond which it slowly decreased (9.81% TFA after 9 h). The purified triacylglycerol acyl hydrolase was able to enrich DHA with 35.27% 22:6 *n*-3 after 3 h of hydrolysis. The results also suggested that the esteritic bonds of C_{22} acyl chain lengthened *n*-3 PUFAs are resistant to hydrolysis by the lipase. However, after prolonged hydrolysis (>9 h), when only a few target fatty acid ester bonds (*n*-6 fatty acyl ester bonds and esters other than C_{22} *n*-3 fatty acids) were available in the enzyme hydrolysate that were susceptible to hydrolysis by the bacterial hydrolase, the microbial triacylglycerol acyl hydrolase could cleave bonds highly resistant to hydrolysis, i.e., DHA. It can therefore be concluded that it is possible to separate and concentrate C_{22} PUFAs with *n*-3 double bonds like DHA using lipase from seaweed associated bacteria like *Bacillus subtilis*.

Separation of phospholipid fraction from the seer fish roe and preparation of DHA rich oil emulsion was also done.The total lipids of seer fish roe were separated into different lipid classes by silicic acid column chromatography. The lipid fractions were qualitatively analyzed by Thin Layer Chromatography (TLC) for identifying triglycerides, glycolipids and phospholipid components. Fatty acid methyl esters (FAMEs) of the total lipid and the individual lipid classes were prepared by transesterification process. The DHA enriched fatty acid concentrate was enriched through biochemical and microbiological procedures to formulate enrichment emulsions which contained roughly, 90% DHA enriched fatty acid concentrate and 10% phospholipids fraction extracted from seer fish roe. The aggregate content of DHA in fish roe phospholipidic fraction was recorded as 24.3% DHA along with 5.2% EPA. The polyunsaturated fatty acid concentrate of the fish body oil after adding fish roe was found to contain greater than 35% DHA with significantly lesser content of saturated fatty acids (0.22%) and monounsaturated fatty acids (~7% of total fatty acids).

Use of emulsifiers to increase the stability of enriched PUFA emulsion

An emulsion is a mixture of two or more liquids that are normally immiscible and an emulsifier is a substance that stabilizes an emulsion by increasing its kinetic stability. Emulsion should be used when both the dispersed and the continuous phase are liquids. An experiment was conducted to understand the effect of emulsifier to stabilize the DHA concentrate. The emulsifier used was Tween 20 (or Polysorbate 20), which was able to contain the stability of the preparation for an extended time period (Figs. 5 & 6). Longer chain TAGs such as polysorbate 20 are more hydrophobic and therefore have higher oil/water interfacial tension than shorter chain ones. The stability of the PUFA concentrate (as such without Polysorbate 20) decreased after 15 minutes whereas the same appended with Polysorbate 20 was able to maintain the stability for an extended period of time due to increased kinetics (increased Brownian movement)



Tween 20: Cn (a, b, c, and d) = a + b + c + d = 20

Fig. 5. Polyoxyethylene derivative of sorbitan monolaurate (or Polysorbate 20)

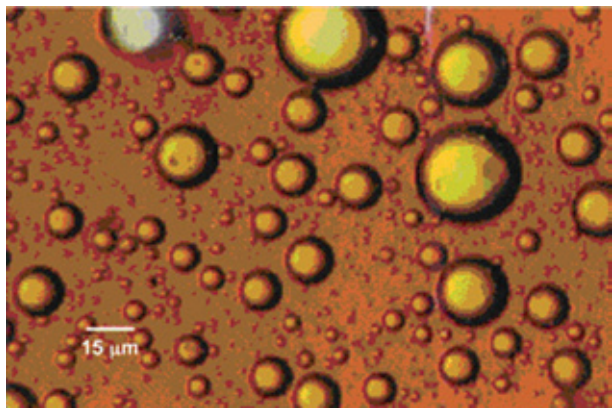


Fig. 6. Photomicrograph of a water-in-oil emulsion by using Tween 20

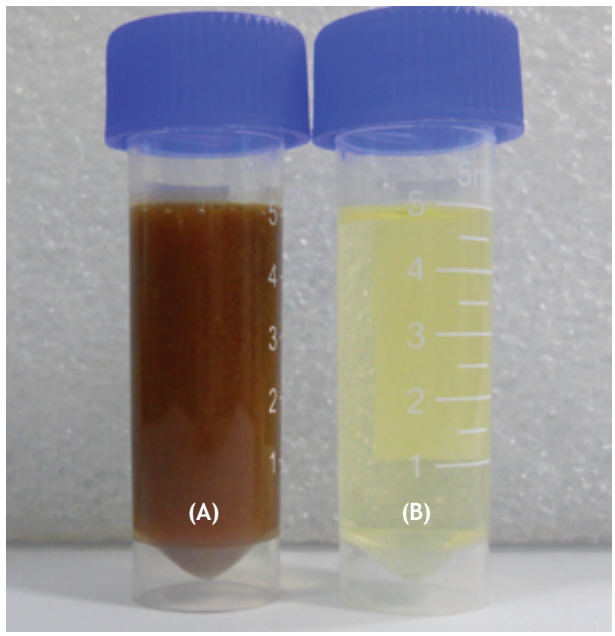


Fig. 7. Polyunsaturated fatty acid concentrate prepared from sardine oil. (A) Crude sardine oil (B) PUFA concentrate

Conclusions

A fatty oil from the livers of various fishes (as cod, halibut or sharks) used chiefly as a source of fatty acids, vitamin A and also of vitamin D is called fish liver oil. Fish oil is short for "fish body oil" and not the same thing as "cod liver oil" available in the market. Cod liver oil contains greater concentrations of vitamin A. Taking cod liver oil in the same amounts that are recommended for fish oil can be toxic, and even more so in people who have chronic renal failure (because vitamin A can build up to toxic levels). The $C_{20\text{-}22}$ *n*-3 polyunsaturated fatty acid concentrate prepared from the inexpensively available marine sources, such as sardine oil can be a potential substitute of the imported PUFA supplements as functional food product.The PUFA enriched formulation (Fig. 7) from low value "fish body oils" will also overcome the risks associated with hypervitaminosis (A, D) and exposure to environmental toxins (mercury, PCBs, dioxins etc.) associated with "liver oils" available in market. The indigenous *n*-3 polyunsaturatated fatty acids emulsion developed from the locally available low-value fish may also serve as a cheaper alternative to the imported fatty acid emulsions used in fish larval nutrition during mariculture operations.