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A New Strategy to Refine Crude Indian Sardine Oil

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Abstract: Current work aims to develop a refining process for removing phospholipids, free fatty acids (FFA), and metal ions without affecting n-3 polyunsaturated fatty acid (n-3 PUFA) esters present in the crude Indian sardine oil. Sardine oil was subjected to degumming with various acids (orthophosphoric acid, acetic acid, and lactic acid), conventional and membrane assisted deacidification using various solvents (methanol, ethanol, propanol and butanol) and bleaching with bleaching agents (GAC, activated earth and bentonite) and all the process parameters were further optimized. Degumming with 5%(w/w) ortho phosphoric acid, two stage solvent extraction with methanol at 1:1 (w/w) in each stage and bleaching with 3% (w/w) activated charcoal loading, at 80ºC for 10 minutes resulted in the reduction of phospholipid content to 5.66 ppm from 612.66 ppm, FFA to 0.56% from 5.64% with the complete removal of iron and mercury. Under these conditions, the obtained bleached oil showed an enhancement of n-3 PUFA from 16.39 % (11.19 Eicosapentaenoic acid (EPA) + 5.20 Docosahexaenoic acid (DHA)) to 17.91 % (11.81 EPA + 6.1 DHA). Replacing conventional solvent extraction with membrane deacidification using microporous, hydrophobic polytetrafluoroethylene membrane (PTFE), resulted in a lesser solvent residue (0.25% (w/w)) in the deacidified oil. In view of lack of reports on refining of n-3 PUFA rich marine oils without concomitant loss of n-3 PUFA, this report is significant.

Key words: bleaching, deacidification, degumming, n-3 PUFA, refining, *Sardinella longiceps*

1 Introduction

Indian oil sardine (*Sardinella longiceps*) is a chief pelagic fishery resource of India and ranks as a very valuable commercial fish owing to its food value and industrial use. Oil sardines are one of the richest and cheapest sources of n-3 polyunsaturated fatty acids (n-3 PUFA) such as Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA)[1]. The beneficial effects of n-3 PUFA in the prevention and treatment of coronary, neuromuscular, immunological disorders and allergic conditions are well documented[2]. The crude oil extracted from Sardines by physical methods consists primarily of triacylglycerides, which contain fatty acids of various chain lengths (including EPA and DHA) in addition to free fatty acid (FFA), primary oxidation products, minerals, pigments, moisture, phospholipids, and insoluble impurities[3]. Hence, the crude oil extracted from the Sardines need to be refined to eliminate the undesirable FFA, phospholipids, metal ions, pigments, moisture and insoluble materials, while retaining valuable triglycerides having n-3 PUFA. Thus, refining process is an important and inevitable step required to produce high quality oils with acceptable shelf life and suitable for human consumption.

Normally, sardine processing is carried out by heating the fish in large, continuous cookers at 95–100℃ for 15–30 min[4]. FFA are produced from lipids by cleavage of ester bonds due to enzyme (lipase) action, heat and moisture[5], before and after oil extraction. FFA in oils can act as pro-oxidants, which initiate the oxidation mechanism in lipids. Phospholipids, which are derived from the tissues of the fish, readily undergo hydrolysis to liberate FFA during storage of the oil[6] and imparts dark colour to the oil on exposure to air or sunshine. The speed of oxidation of the oils further increased due to the presence of metal ions (like copper and iron) and also depends on the concentration, type and valency of the metal ions[7].

The extracted oil is generally refined making use of either physical or chemical treatments. Conventional physical refining of fish oil consists of three steps, namely degumming, bleaching and deodorization. Degumming step aims at the removal of phospholipids, whereas pigments and FFA are removed during bleaching and deodorization, respectively. Deodorization process decreases the nutritional value of the fish oil, since the process is generally carried out at about 200℃, results in the release of trans isomers of n-3 PUFA and FFA due to the decomposition of

oil[8]. The chemical refining includes an additional neutralization step to neutralize the excess acid. Chemical refining has many disadvantages such as high-energy demand, nutrient loss, need for large quantities of water and chemicals, and disposal of polluted effluents. Enzymatic treatment employed to refine the crude oil is effective and eco-friendly, but seldom applied for industrial purposes due to cost constraints[9].

Solvent extraction is a potential alternative to the conventional neutralization process for the removal of FFA. Reports on successful extraction of FFA from crude olive oil using ethanol[10], cottonseed oil using methanol[11], rice bran oil by two stage ethanol extractions[12] corn oil using ethanol[13] are available. The decisive advantage of solvent extraction over other conventional deacidification is in the yield of neutral oil. Since the alkali is not added for FFA neutralization in solvent extraction process, the hydrolysis of the triglycerides due to the excess alkali is avoided and thus the oil loss is reduced[10]. However, reports on the use of solvent extraction for FFA removal in marine oils are rather scarce.

Although membranes could be used in principle, for all the stages of refining of oils, not much work has been reported[9]. Couple of reports are available on solvent extraction coupled with membranes for FFA removal from synthetic mixture of FFA and triglycerides[14] and groundnut oil[15]. With the expectation of preserving the greater part of nutrients, the membrane process carried out at low temperatures and with the elimination of a substantial number of unit operations as compared to conventional processes, appears highly viable, principally due to the total adequateness of this new technology with respect to environmental questions[16].

While several reports available on the refining of edible oil[15–18] the techniques and conditions adopted for vegetable oils cannot be extended to fish oil due to the profound dissimilarities in the impurities, valuable nutrients (n-3 PUFA) and their concentrations in comparison to vegetable oils. Moreover, very limited numbers of reports are available on the refining of n-3 PUFA rich marine oils, focussing on the removal of impurities without causing decomposition of valuable n-3 PUFA glycerides. In view of this, the current study was conducted with an objective to find a suitable refining strategy and optimize the conditions of refining process for crude Indian Sardine oil.

2 Materials and methods
2.1 Raw materials
Crude fish oil was obtained from Mukka Fish Oil Industries (Mangaluru, India) and centrifuged at 6000 x g for 20 minutes and stored at 4℃ in the dark. Orthophosphoric acid (OPA), lactic acid (LA), acetic acid (AC), methanol, ethanol, propanol, butanol, activated charcoal, bentonite, activated earth, iso-octane, glacial acetic acid, p-anisidine, potassium iodide, sodium thiosulphate, potassium hydroxide, phenolphthalein indicator, wijis solution, chloroform was purchased from Merck, India. Membranes were purchased from Axivia, India. All the reagents (analytical grade) and solvents (chromatographic grade) were used without further purification.

2.2 Refining methodology
The fish oil was refined through the steps, namely degumming, deacidification by conventional solvent extraction and membrane deacidification, and bleaching at various conditions. At each stage, the quality of the fish oil was carefully monitored by analyzing the parameters like acid value (FFA), phospholipid content, iodine value, moisture content, totox value and fatty acid composition. Degumming process was carried out by mixing 200 g of crude Sardine oil with different concentrations (% wt. basis) of OPA, LA, and AC as per procedure[19] with some modifications. The effectiveness of the individual acids and optimum conditions were established by studying various physicochemical characteristics of oil before and after the degumming process.

Deacidification was employed to remove the FFA from fish oils through solvent extraction. The oil obtained after degumming (154 g) was subjected to solvent extraction using various solvents such as methanol, ethanol, propanol and butanol. The solvent to oil mixtures of 1:1, 1.5:1, 2:1, 2.5:1, 3:1, 3.5:1, 4:1 (w/w) were subjected to further processing in accordance with the method by Kale *et al.*[20]. The solvent, which was capable of stripping out the maximum FFA content from the oil, was chosen for further studies. This oil was further subjected to the second stage of solvent extraction to see if there was any further decrease in the FFA content in the oil under the same conditions with the selected solvent. The requirement of multiple stages of deacidification of the oil depends on the acidity of the oil[21].

Membrane deacidification was carried out with polytetrafluoroethylene (PTFE) membrane (10 cm dia, Axivia, India) of pore size, 0.45 μm, under various pressures (0.5 bar, 1 bar, 2 bar, 3 bar). The stainless steel membrane unit with a working volume of 500 ml and able to withstand up to a pressure of 3 bar, was used for the present study. The phospholipid free oil was continuously stirred with methanol using a magnetic stirrer for an hour and the mixture was then subjected to separation at the required pressure by using a nitrogen cylinder. The solvent free oil was obtained as permeate with the rejection of lipid solution comprising FFA, glycerides and solvent. The permeate was analyzed for FFA and solvent content. The membrane performance indicators like FFA rejection in a membrane (Eq.1) and flux (Eq. 2) were calculated as;

2

$$\text{FFA rejection in membrane} = \frac{(CR - CP)}{CR} \times 100 \qquad (1)$$

$$\text{Flux}(LMH) = \frac{\text{volume of perm eate}(L)}{\text{membrane area}(\text{square meter}) \times \text{time}(\text{hours})} \qquad (2)$$

where CR and CP are concentrations of FFA in retentate and permeate respectively.

The bleaching process is aimed to remove the impurities, oxidized products and colour that are present in the oil. The oil obtained after deacidification (152 g) was subjected to bleaching using Granulated Activated Charcoal (GAC) (granulated, Spectrum Chemical Mfg. Corp, India), activated earth (powdered, Spectrum Chemical Mfg Corp, India) and bentonite (powdered, Spectrum Chemical Mfg. Corp, India). The required amount of bleaching agent was mixed with the deacidified oil in a closed vessel by a magnetic stirrer and the process was carried out under vacuum. Various parameters like temperature (50℃, 60℃, 70℃, 80℃, 90℃), concentration of bleaching agents (1%, 2%, 3%, 5%, 7%, 9%, 11%, 13%) (w/w) and duration of bleaching (10 min, 20 min, 30 min, 40 min, 50 min, 60 min) were optimized for the lower FFA content and higher iodine value to choose the best bleaching agent and operating conditions. Flow chart of refining process followed in this work is shown in **Fig. 1**.

2.3 Characterization of the oils

The phospholipid content of the oil during the degumming process was determined by a method adopted by Hundrieser *et al.*[22] with minor modifications. FFA content

CRUDE INDIAN SARDINE OIL

↓

ACID DEGUMMING USING 5% ORTHOPHOSPHORIC ACID

↓

DEACIDIFICATION

↓

SOLVENT EXTRACTION USING METHANOL (2 stages) {(1:1 w/w) at room temperature}

MEMBRANE ASSISTED DEACIDIFICATION (using hydrophobic membrane) WITH METHANOL (2 stages) {(1:1 w/w), room temperature, 3 bars }

↓

BLEACHING USING GRANULATED ACTIVATED CHARCOAL {3% (w/w), 80℃, 10 minutes}
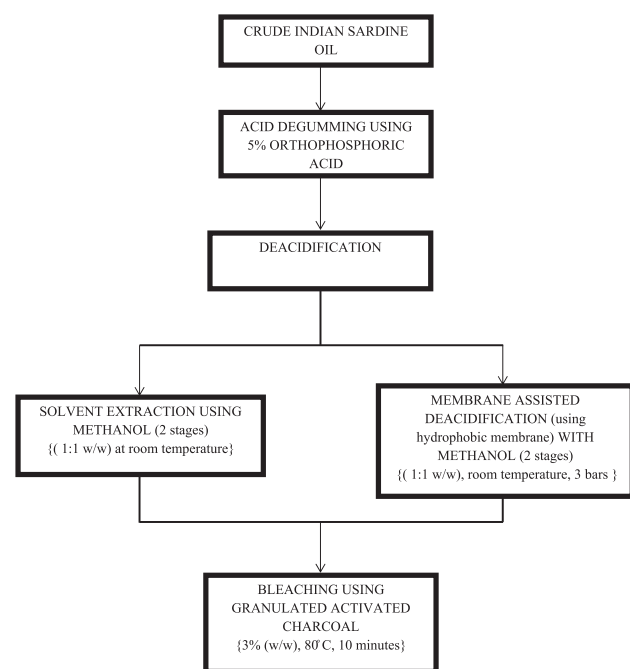
Fig. 1 Scheme followed for refining crude Indian Sardine oil.

in the oil was determined according to the official method of American Oil Chemists' Society (AOCS Cd 3d-63, 2009)[23]. Metal ion concentrations in the oil was determined by atomic absorption spectrometer (GBC scientific equipment, 932 plus) in accordance with the method followed by Aluyor *et al.*[24]. The peroxide value (PV) of the oil was determined according to AOCS Cd 8b-90[23]. The p-Anisidine value (pAV) of the oil was determined according to AOCS Cd 18-90[23]. TOTOX value was calculated as;

$$\text{TOTOX} = (2 \times PV) + pAV \qquad (3)$$

where PV is peroxide value and pAV is the p-anisidine value.

The fatty acid composition was estimated, after converting the fatty acids to their respective esters through tranesterification process[25] in a GC (Trace 3330 GC Ultra, Thermoelectron Corporation) with a flame ionization detector (FID) equipped with a split/splitless injector and DB-5 column (30 m × 0.25 mm × 0.2 μm). Oil samples (50 mg) were collected from each stage of refining step, and were taken for FAME preparation. FAME analysis was performed with an oven temperature of 160℃ and maintained at this temperature for 1 min, after which the temperature was increased at the rate of 5℃/min until it reached 185℃. This temperature was maintained for 10 min with a further increase of temperature to 240℃ at the rate of 8℃/min. The third ramp of GC was conditioned for 10 min at a temperature of 240℃. The right inlet and the detector temperatures were 280℃ and 300℃, respectively. The samples were prepared and analysed in triplicates. FAMEs peaks were identified by comparing the retention time of the samples with FAME standards from Sigma Aldrich. Quantification of fatty acids was done by integration of peak area. Chromatograms were analyzed using Chrom Cad software.

2.4 Statistical Analysis

Statistical analysis of the data was performed by SPSS (16.0) computer program. All the samples were analyzed in triplicates and the means were reported. The data were tested by analysis of variance (ANOVA) and the means were compared using Tukey's test. The significance of the means was measured at $p < 0.05$.

3 Results and Discussions

The sardine oil is an important source of n-3 PUFA, as EPA and DHA together represent one third of the total fatty acids[26]. The crude oil was analyzed for its properties and was found to contain substantial amounts of FFA (5.56%), phospholipids (612.66 ppm), heavy metals (copper, iron and mercury) with a high totox value (**Table 1**). The presence of such a high concentration of impurities and n-3 PUFA causes the oil to oxidize rapidly and hence

3

Table 1 Characterization of Indian Sardine oil during various stages of refining.

samples	% FFA	Phospholipid (ppm)	Totox value	Metal ions (ppm)		
				Copper	Iron	Mercury
CO	5.64 ± 0.01	612.66 ± 0.94	107.36 ± 0.1	0.606 ± 0.05	2.062 ± 0.11	1.179 ± 0.04
DO	4.5 ± 0.02	261.5 ± 2.12	19.14 ± 0.01	0.445 ± 0.01	1.266 ± 0.02	0.120 ± 0.01
S1	2.26 ± 0.11	156.5 ± 4.47	15.6 ± 0.07	0.194 ± 0.12	0.044 ± 0.07	0.188 ± 0.01
S2	1.13 ± 0.2	79.66 ± 3.95	72.3 ± 0.08	0.192 ± 0.2	0.128 ± 0.3	BDL
SM1	2.25 ± 0.04	NA	NA	NA	NA	NA
SM2	1.09 ± 0.03	NA	NA	NA	NA	NA
BO	0.56 ± 0.03	5.66 ± 0.57	26.1 ± 0.01	0.1 ± 0.05	BDL	BDL

Mean ± SD, n=3. CO, crude oil, DO, degummed oil, S1, first stage solvent extracted oil, S2, second stage solvent extracted oil, SM1, first stage membrane mediated solvent extracted oil, SM2, second stage membrane mediated solvent extracted oil and BO, bleached oil. NA, Not available; BDL, below detection limit.

refining is mandatory. However, the employed refining process must preserve the nutritionally important n-3 PUFA without releasing unwanted intermediary refining products. Consequently, it becomes important to control and monitor each refining stage/process to obtain good quality oil. Elucidation of the crude sardine oil composition revealed that the oil has a marked difference with that of commonly studied vegetable oils, specifically in the phospholipids, FFA contents, and fatty acid compositions. Hence, the refining strategy was tailor made in the present work with the objective of the removal of impurities and concomitant enhancement of EPA and DHA.

3.1 Degumming

Degumming is the initial step in edible oil refining which removes phospholipids and some portion of trace metals and mucilaginous substances[9]. Sardine oil was degummed through an acid degumming process by considering various acids like OPA, LA and AC. In general, hydratable and non-hydratable phospholipids present in the oil become hydrophilic at low pH, and forms sludge, which is easily separated by centrifugation[27]. The efficiency of each of these acids (OPA, LA, AC) in the removal of phospholipids was assessed and it was found that the OPA removes the phospholipids better than LA and AC (Fig. 2). It was observed that there was a significant reduction in the phospholipid content to 261.5 ppm from 612.66 ppm with 5% (w/w) OPA ($p<0.05$). However, higher strength of acid beyond 5% OPA, did not show a significant reduction in the phospholipid content ($p>0.05$) and tends to make the oil darker in colour by decomposing non-hydratable phospholipids to form brown sludge[28]. Hence, 5% OPA was chosen for degumming of crude sardine oil. The results were consistent with the findings of De and Patel[29] pertaining to degumming of rice bran oil. Degumming process using OPA reduced copper, iron and mercury content by 92.65%, 38.6%, and 89.82%, respectively (Table 1). It has been ob-
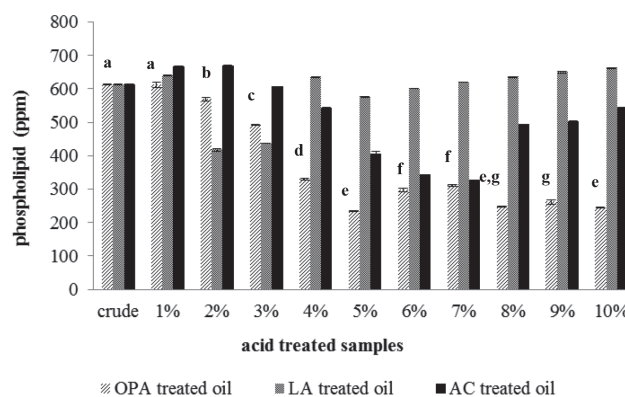

Fig. 2 Effect of various acids and its concentrations on degumming of sardine oil. Statistical comparison was made among different concentrations of OPA (%) and means with the same letters are not significantly different at 0.05 probability level.

served that the metal ions tend to bind to phospholipids under low pH and gets precipitated along with phospholipids after getting hydrated. A similar trend was observed during the development of "Total degumming process" for soybean oil[27].

3.2 Deacidification by solvent extraction

The solvent extraction of FFA is an alternative to the neutralization step used in conventional refining thereby reducing the heavy loss of oil due to soap formation. Studies suggest that the short chain alcohols are the most suitable solvents for the deacidification of palm oil and enhance flavor and aroma with reduced amounts of diacylglycerols and FFA in the palm oil[10]. Hamm[21], suggested in his patent that solvent deacidification was effective and was able to enrich the EPA and DHA in the fish oil with short chain alcohols. Hence, the short chain alcohols like methanol, ethanol, propanol and butanol were considered for the present work. Figure 3 depicts the performance of

4

various solvents on the removal of FFA from the oil. It was observed that the highest removal of FFA was achieved by methanol $(p < 0.05)$ when compared to the other three alcohols. As the chain length of alcohol increases, the hydrophobicity of the alcohol increases, and results in an incomplete separation of solvents from the oil phase. The lowest chain length alcohol like methanol hence provides a better phase separation along with the higher extraction selectivity for FFA[30]. A similar finding for the deacidification of rice bran oil with some short chain alcohols namely methanol, ethanol and iso propanol was reported[10].

The amount of methanol required for the removal of FFA was studied by varying the solvent to oil ratio (w/w) (**Fig. 4**). The maximum reduction of 2.26% FFA from 5.64% (crude) was observed at the ratio of 1:1 in the first stage of extraction $(p < 0.05)$. Also, high quantities of methanol
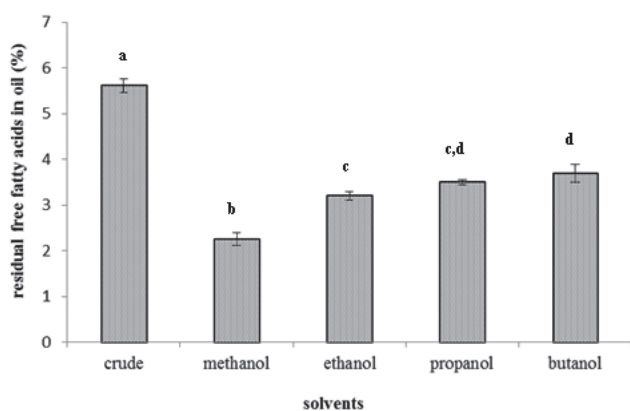
Fig. 3 Performance of various solvents on deacidification of oil at 1:1 solvent to oil ratio. Statistical comparison was made among different solvents and means with the same letters are not significantly different at 0.05 probability level.
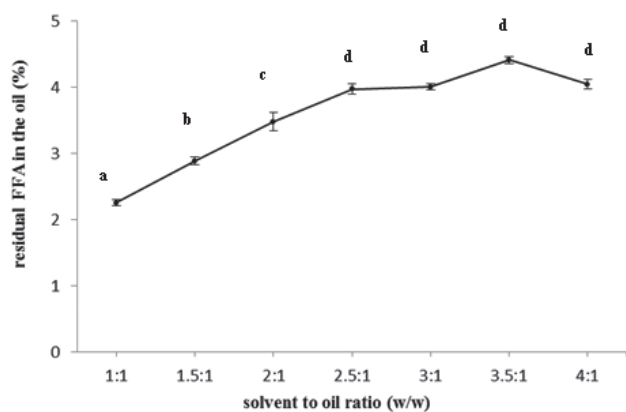
Fig. 4 Effect of solvent to oil ratio on deacidification during the first stage solvent extraction. Statistical comparison was made among different solvent to oil ratios and means with the same letters are not significantly different at 0.05 probability level.

usage for extraction could be avoided. To reduce the FFA content further, second stage solvent extraction was employed by keeping the same solvent to oil ratio. FFA content was further reduced to 1.13% in the second stage (data not shown). Kale *et al.*[20] conducted two-stage solvent deacidification with methanol at a solvent to oil ratio (w/w) of 1.8:1 in the first stage and 1:1 in the second stage of crude rice bran oil and found a reduction of FFA from 16.5% to 3.7% which is in accordance with the present study. At higher ratios beyond 1:1 led to an increase in FFA in the oil, perhaps due to hydrolysis of the triglycerides at a higher methanol concentration. Higher oil loss was also observed as the number of extraction stage increases with the simultaneous reduction in FFA. Mariano *et al.*[31] reported that the partitioning of FFA from macauba pulp oil required two stages of extraction with ethanol as solvent and higher oil loss was reported at higher number of extraction stages. Two stage solvent extraction processes for deacidification was studied by many researchers for the vegetable oils like rapeseed oil (with 12% FFA with ethanol), coconut oil (with 15% FFA with methanol)[10]. However, no such study has been reported for fish oil, specifically Indian Sardine oil, and this work demonstrate the reduction of FFA to 1.13% from the initial 5.64%.

3.3 Membrane assisted solvent extraction

The objective of deacidification was to remove FFA from the degummed oil with minimal loss of oil and n-3 PUFA content. In solvent extraction process, FFA and other impurities were removed based on the difference in the solubility of FFA and glycerides in solvents[10]. The solvent having dissolved FFA (extract) can be efficiently separated from glycerides using microporous hydrophobic membranes[32]. Since the differences in the molecular weights of FFA and glycerides are too small, the complete removal of FFA from oil is not possible by the use of membranes alone[33]. Membrane assisted solvent extraction of FFA from soya bean oil has been reported by Raman *et al.*[30].
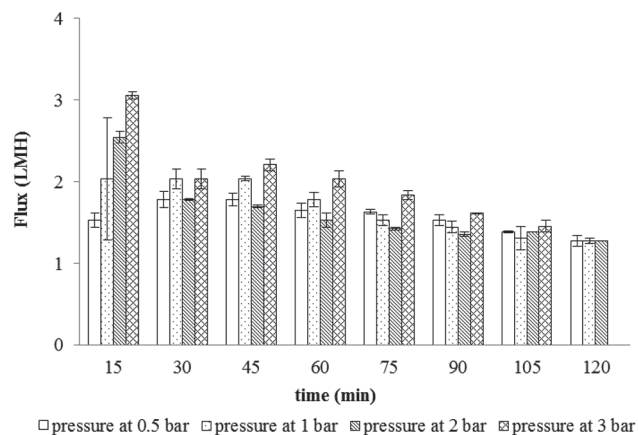
The conventional solvent extraction developed in the present work was further tested with the aid of membranes. When oil and methanol are brought in contact , due to the differences in the polarities of oil (nonpolar) and methanol (polar), they form micelles on agitation for an hour[36]. Since methanol has a very high selectivity for the FFA as compared to glycerides and due to the similar polarity[30], the FFA in the oil is entrapped within the micelles of polar methanol[34]. A hydrophobic membrane, polytetrafluoroethylene (PTFE), was used in a flat sheet membrane separation module to study the separation of micelle phase (extract) from the useful oil comprising glycerides (Raffinate). The hydrophobic nature of the membrane did not allow the micelles, which is polar in nature, to pass through. The increased strength of the carbon and fluorine bonds present in PTFE makes it non-reactive to any sol-

5

vents. In addition, the polar solvents like methanol have a lower flux through hydrophobic membranes as compared to that of hydrophilic membranes[35]. The experiments were conducted to study the flux at the pressure ranging from 0.5 bar to 3 bar, being the driving force. It is evident that the maximum FFA reduction was obtained at a transmembrane pressure of 3 bar $(p < 0.05)$ (Fig. 5 and Table 1) with a maximum permeate flux (Fig. 6). These results are concurring with the observation of Rao *et al.*[36].

A comparison between membrane deacidification and conventional solvent extraction was made and it was found that membrane based solvent extraction could be a better option for the FFA removal. The FFA rejection in the first and second stage of solvent extraction was 49.88% and 50.02%, respectively, which was almost similar to 50% in the first stage and 51.25% in the second stage of membrane assisted solvent extraction. However, the loss of oil in the first stage (25%) and second stage (5%) of solvent

extraction was found to be higher than the oil loss in membrane assisted solvent extraction process (4.5% and 2.5% in the first and second stage of extractions, respectively). Further, the residual solvent content in the solvent extracted sardine oil (0.51 and 0.45% (w/w) in first and second stages, respectively) was also found to be higher than the membrane assisted solvent extraction process (0.32 and 0.25% (w/w) in first and second stages, respectively). Thus, the membrane assisted solvent extraction leads to a lower oil loss with the presence of lower methanol traces against the conventional solvent extraction. The oil loss was found to be more in the conventional solvent extraction due to the incomplete separation of micelles, which forms a stable emulsion in oil-methanol mixture. The repulsive behaviour of the hydrophobic membrane from methanol further helps to reduce the methanol content in the permeate. Moreover, in membrane deacidification, the separation of oil from the micelles formed occurs at 3 bars due to the intervention of hydrophobic membrane.
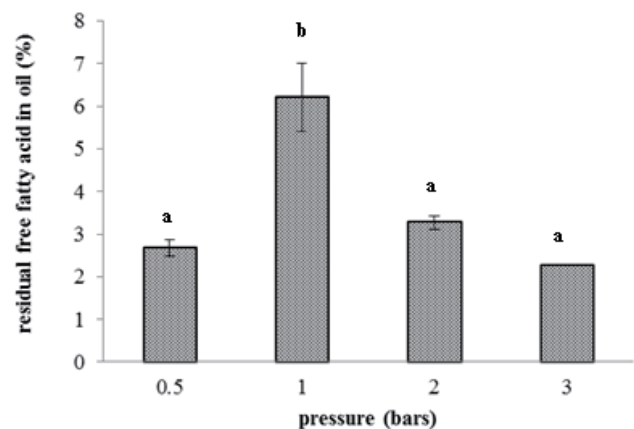
3.4 Bleaching

The success of a bleaching process depends on the ability of the bleaching agents to remove the color impurities, metal ions and result in minimum oil retention. Therefore, a proper selection of bleaching agents becomes essential.

In this work, the oil deacidified at room temperature was used for bleaching at five temperatures (50℃, 60℃, 70℃, 80℃ and 90℃) under vacuum in the presence of various concentrations of adsorbents (1%, 2%, 3%, 5%, 7%, 9%, 11%, 13%) for various durations of time (10, 20, 30, 40, 50, 60 min). As FFA is known to increase the susceptibility of oils to oxidative degeneration, the efficiency of bleaching using different adsorbents, for various concentrations and durations of bleaching was selected based on the FFA content and iodine value of the oil as indicators.

The degummed and deacidified fish oil was treated with Granulated Activated Charcoal (GAC), bentonite, and activated earth and resulted in oil with similar iodine value (169.85), however, the oil loss was found to be 12.68, 37 and 26 (% w/w), respectively. Moreover, removal of the bleaching agents by filtration /centrifugation was difficult with powdered activated earth and bentonite compared to GAC. The use of GAC as bleaching agent for the vegetable oils like soybean, corn, palm, sunflower, rapeseed, cottonseed, rice bran oil, linseed was reported in the literature[37−39]. Hence, GAC was chosen as the bleaching agent for all the further studies.

The deacidified oil was bleached under vacuum at five different temperatures (50℃, 60℃, 70℃, 80℃ and 90℃). The FFA content was observed to be the considerably low in the oil treated at 80℃ $(p < 0.05)$ as compared to the oil treated at other temperatures (Fig. 7). The reduced viscosity of the oil at high temperature helps to disperse the GAC

Fig. 5　Effect of trans-membrane pressure on FFA removal from oil. Statistical comparison was made among different trans-membrane pressures and means with the same letters are not significantly different at 0.05 probability level.

□ pressure at 0.5 bar　□ pressure at 1 bar　⊠ pressure at 2 bar　⊠ pressure at 3 bar

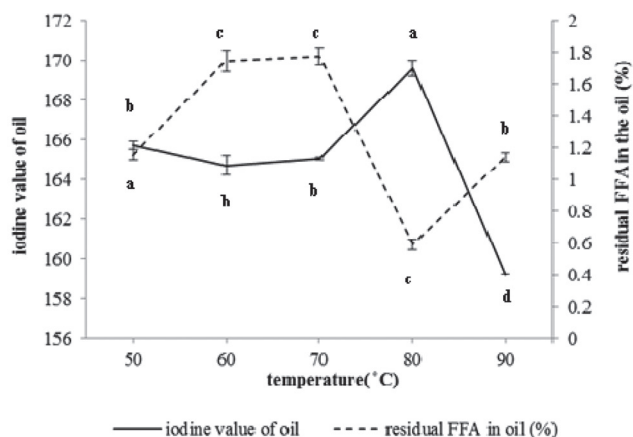Fig. 6　Effect of trans-membrane pressure on flux of oil at room temperature.

Fig. 7 Effect of temperature on bleaching of oil in vacuum for 10 minutes. Statistical comparison of iodine value and FFA content were made among different temperatures and means with the same letters are not significantly different at 0.05 probability level.
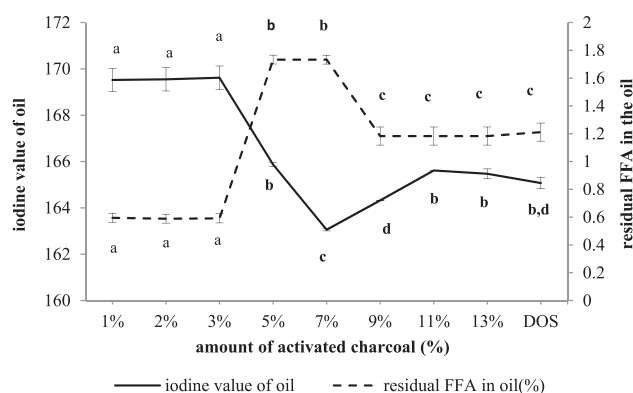

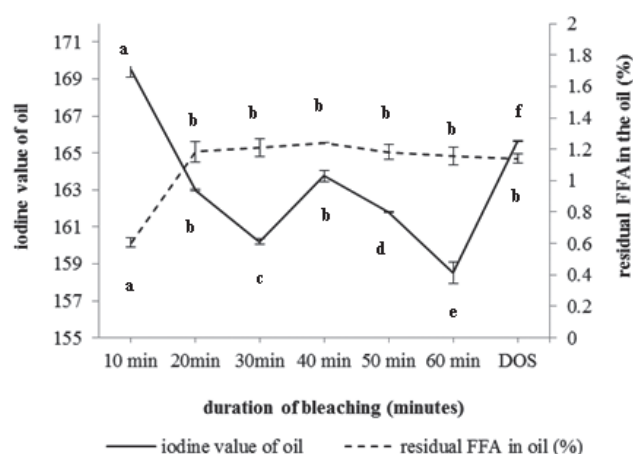Fig. 8 Effect of concentration of GAC on bleaching of oil at 80℃ for 10 minutes in vacuum (DOS-Deacidified oil sample). Statistical comparison of iodine value and FFA content were made among different concentrations of GAC and means with the same letters are not significantly different at 0.05 probability level.

Fig. 9 Effect of duration of bleaching on oil carried out at 80℃ with 3% (w/w) of GAC (DOS- Deacidified oil sample). Statistical comparison of iodine value and FFA content were made among different time intervals and means with the same letters are not significantly different at 0.05 probability level.

uniformly throughout the oil, which enhances the adsorption of impurities to the GAC. The adsorption characteristic was found to increase at higher temperatures due to the improved adsorbent oil interactions and flowability[40]. A sudden increase in FFA was noticed (Fig. 7) at 90℃, could be due to the decomposition of glycerides. Further, iodine value was observed to be the highest at 80℃ after which there was a drastic decrease in iodine value, perhaps due to cyclization and polymerization of long chain n-3 PUFA leading to the release of polymers and dimers of glycerides as degradation products[41]. Thus, to maintain the efficiency of the bleaching process, it is safer to maintain an upper limit of 80℃ as it will minimize the probability of chemical or physical changes in the oil.

The bleaching was carried out at 80℃ with various GAC loading to determine its optimum loading. Bleaching with 3% (w/w) GAC resulted in the oil having least FFA and high iodine value compared to others ($p < 0.05$) (Fig. 8). Though 1% and 2% of GAC were statistically similar to the results shown by 3% GAC, it was seen that bleaching with 3% of GAC resulted in the reduction in the intensity of color in the oil when visually observed. An increase in the quantity of GAC resulted not only the increase in FFA, but also the color of the oil when seen visually. This could be attributed due to the mineral acid leaching out of the activated charcoal and hence hydrolysis of oil[42]. Hence 3% (w/w) GACis used for further bleaching experiments. Published literature suggests that darker oils require as much as 2- 4% (w/w) bleaching agents to get satisfactory results[43].

The bleaching was then carried out for different durations at 80℃ with 3% (w/w) GAC. It was observed (Fig. 9) that the FFA content was the least in the oil bleached for 10 minutes beyond which there was a sharp increase in FFA at 20 minutes. The iodine value was high for the oil

that was bleached for 10 minutes ($p < 0.05$). The increase in contact time of GAC in oil resulted in the reduction of iodine value, which signifies the reduction in the unsaturation of the oil. An increase in the duration of bleaching causes an increase in the conjugation of the double bonds of fatty acids in the oil, rendering the oil prone to oxidation[8]. Fournier *et al.*[41] found that residence time and temperature has a maximum impact on trans-fatty acid formation in fish oils. Hence bleaching was performed for 10 minutes at 80℃ in the presence of 3% (w/w) of GAC. The obtained results concur with Berbesi's[40] observation that contact time for effective bleaching typically ranges from 10 to 45 minutes for palm oil and 20 to 30 minutes being

7

the most common.

3.5 Characteristics of oil at different stages of refining.

Table 1 shows the characteristics of the oil at each refining stage. It gives a basic trend of variation in the quantities of the FFA, phospholipids, and metal ions during the refining steps. The oil obtained after the complete refining of fish oil was analyzed for its physical quality and chemical composition (Table 2). Oil had the specific gravity of 0.92, a density of 0.91 g/mL and a viscosity of 24 mPa.s, which was well within the range of food grade fish oils as per the quality guidelines issued by International fish meal and oil manufacturers association[44]. The yield after degumming the crude oil was 77%. The subsequent yields of oil for the 1st, 2nd stages of solvent extraction and bleaching processes were 99.7%, 99.56% and 99.34% respectively. The overall yield of oil and oil loss (by weight) during refining was 76% and 24% respectively. The oil loss was maximum during the degumming stage due to high phospholipid content in the crude oil while in the subsequent stages of solvent extractions and bleaching, the oil loss was less (high yield of oil), due to the use of methanol, which reduces the neutral oil loss[10] and the use of GAC (acidic in nature) completely saturated with FFA in the active sites thereby reducing the neutral oil loss to a large extent[40].

From Table 1, it is clear that the effective stage for the removal of FFA content was solvent extraction where the FFA was reduced from 5.64% in crude oil to 1.13% and 1.09% in deacidified oil. Further reduction of FFA to 0.56% was noticed during bleaching, probably due to the adsorption of FFA on GAC. The degumming stage, making use of OPA brought a substantial reduction in phospholipid content. A further reduction of phospholipid content during solvent extraction was noticed possibly due to the inclusion of phospholipids in the micelles along with FFA. Additional removal of phospholipids was observed during bleaching, which could be attributed to the adsorption by GAC and subsequent flocculation[45]. The refining process reduced the totox value by 75.68% of crude oil. However,

in the second stage of solvent extraction, the totox value increased. This may be due to the increase in iron content (Fe^{++}), contributed by GAC[7]. But, totox value was reduced in bleached oil due to the adsorption of the impurities and metal ions on GAC. The added advantage of GAC as bleaching agent is in the effective removal of the polycyclic aromatic hydrocarbons, which are produced from the oxidation prone fish oils due to oxidation[46]. The moisture content of the crude sardine oil was reduced from 0.22% to 0.12%. The improvement in the oil quality after refining thus reduces the susceptibility of the oil to rancidity and improves its stability.

Predominant fatty acids present in oil were estimated at every stage of refining to understand the change in their concentration (Table 2). GC analysis showed that palmitic acid (C16:0), was the most abundant fatty acid followed by myristic acid (C14:0) ($p < 0.05$). The n-3 PUFA content increased from 16.39% to 17.91% due to refining. This can be explained by the fact that when n-3 PUFA content of crude oil was estimated, both the n-3 PUFA existing in the free form (FFA) and the ester form are converted into FAME and quantified. Thus the estimated value (16.39%) represented both the free form of n-3 PUFA and glyceride ester. However, during refining stage the free form of n-3 PUFA is removed, thus leaving behind only the glycerol ester of n-3 PUFA. Due to the removal of appreciable amounts of phospholipids, FFA and other impurities, concomitant enhancement of n-3 PUFA takes place (From 16.39% and 17.91%). From the Table 2 it can be observed that the fatty acid composition did not change appreciably during every stage of refining except for DHA where the change was significant ($p < 0.05$). The net enhancement of n-3 PUFA content is due to the significant increase in DHA, whereas EPA value did not change much. This could be attributed to positioning EPA (sn-1,3 location) and DHA (sn-2 location)[46]. Due to sn-1,3 location, EPA in the glycerides is prone to hydrolysis and hence some amount of EPA could be present in the form FFA, which is lost during refining. Thus, any concomitant enrichment is nullified by the pro-

Table 2   Fatty acid profile of Indian Sardine oil during various stages of refining.

Fatty acids (% w/w)	Crude (% w/w)	Degummed oil (% w/w)	Solvent (1) extracted oil (% w/w)	Solvent (2) extracted oil (% w/w)	Bleached oil (% w/w)
14:0	33.93 ± 3.10[a]	34.31 ± 4.99[a]	26.77 ± 2.25[a]	25.33 ± 0.65[a]	26.18 ± 5.97[a]
16:0	37.58 ± 4.71[a]	33.56 ± 1.06[a]	44.56 ± 1.78[a,b]	48.9 ± 1.49[b]	46.87 ± 1.84[a,b]
18:1	5.64 ± 2.18[a]	4.21 ± 1.37[a]	5.34 ± 0.82[a]	5.43 ± 0.09[a]	5.06 ± 0.50[a]
18:2	5.64 ± 1.12[a]	4.86 ± 1.28[a]	5.34 ± 0.82[a]	5.41 ± 0.17[a]	5.76 ± 0.65[a]
20:5 (EPA)	11.19 ± 0.18[a]	10.06 ± 0.05[b]	11.28 ± 0.16[a]	11.56 ± 0.49[a]	11.81 ± 0.02[c]
22:6 (DHA)	5.20 ± 0.07[a]	5.46 ± 0.08[b]	5.81 ± 0.03[c]	5.89 ± 0.02[c,d]	6.1 ± 0.18[d]

Mean ± SD, *n*=3.

[a, b, c, d] Means with different superscripts letters within a row are significantly different at $p < 0.05$.

gressive loss of EPA during refining along with the rest of the FFA. Where as in case of DHA, due to its sn-2 positioning in the glycerides, it is less prone to oxidation and hydrolysis, and perhaps the DHA estimated in crude oil was mostly from Glycerol esters. Due to this, loss of DHA during refining did not take place, and thus a concomitant enrichment was witnessed. This result concurs with the findings of Noriega-Rodriguez *et al.*[26] on sardine (*Sardinops sagax* caerulea) oil refining. In this discussion, more emphasis is laid on the amount of nutritionally important components, i.e. the n-3 PUFA and its preservation over the course of the tailor made refining process which is the main aim of the work.

In the current refining strategy, phospholipids were removed through the degumming process with OPA. The alkali neutralization process was replaced with solvent extraction to remove FFA. Further improvement in the FFA removal by solvent extraction was achieved by employing membrane assisted solvent extraction. The conditions adopted in the bleaching process were very mild. GAC performs both bleaching and deodorization simultaneously and excludes the high temperature (180–220℃) deodorization step using steam[47]. It also effectively removes undesirable metal ions, FFA and phospholipids without causing destruction to n-3 PUFA.

4 Conclusions

Complexity of the Indian sardine oil and the need for removal of undesirable impurities while retaining the valuable ingredients in the oil has made refining even more important. The tailor made strategy includes degumming, deacidification by solvent extraction, which replaces the conventional alkali neutralization, bleaching with GAC was found to be an effective refining process. Further improvement in deacidification was achieved by employing PTFE membrane assisted solvent extraction. This strategy was able to produce sardine oil of superior quality (≈ 76% by weight), by removing almost all the impurities with minimal oil loss, processed at ambient temperature without any loss of n-3 PUFA content.

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References

1) Chakraborty, K.; Joseph, D. Production and characterization of refined oils obtained from Indian oil sardine (*Sardinella longiceps*). *J. Agr Food Chem.* **63**, 998-1009 (2015).

2) Nettleton, J.A.; Katz, R. n - 3 Long-chain polyunsaturated fatty acids in type 2 diabetes: a review. *J. Am. Diet. Assoc.* **105**, 428-440 (2005).

3) Morais, M.M.; Pinto, L.A.A.; Ortiz, S.C.A.; Crexi,V.T.; Silva, R.L.; Silva, J.D. Study of fish oil refining process. *Rev. Inst. Adolfo Lutz.* **60**, 23-33 (2001).

4) Bimbo, A.P. Processing of marine oils. in *Long-chain omega-3 specialty oils* (Breivik, H. ed.), Oily Press Lipid Library, P.J. Barnes & Associates, Bridgwater, UK, pp. 77-109 (2007).

5) Akoh, C.C.; Min, D.B. *Food Lipids: Chemistry, Nutrition and Biotechnology*. 2nd ed. Marcel Dekker, USA (2002).

6) Koning, A.J.D.; Mol, T.H. Rates of free fatty acid formation from phospholipids and neutral lipids in frozen cape hake (*Merluccius* spp) mince at various temperatures. *J. Sci. Food Agr.* **50**, 391-398 (1990).

7) Thanonkaew,A.; Benjakul, S.; Visessanguan, W.; Decker, E.A. The effect of metal ions on lipid oxidation,colour and physicochemical properties of cuttlefish (*Sepia pharaonis*) subjected to multiple freeze–thaw cycles. *Food Chem.* **95**, 591-599 (2006).

8) Berdeaux, O.; Fournier, V.; Lambelet, P.; Dionisi, F.; Sebedio, J.L.; Destaillats, F. Isolation and structural analysis of the cyclic fatty acids monomers formed from eicosapentaenoic and docosahexaenoic acids during fish oil deodorization. *J. Chromatogr. A.* **1138**, 216-224 (2007).

9) Vaisali, C.; Charanyaa, S.; Belur, P.D.; Regupathi, I. Refining of edible oils: a critical appraisal of current and potential technologies. *Int. J. Food Sci Tech.* **50**, 13-23 (2015).

10) Rodrigues, C.E.C.; Gonçalves, C.B.; Batista, E.; Meirelles, A.J.A. Deacidification of vegetable oils by solvent extraction. *Recent Pat. Eng.* **1**, 95-102 (2007).

11) Sreenivasan, K.; Viswanath, D.S. Refining of cottonseed oil using solvents. *Indian J. Technol.* **11**, 83-90 (1973).

12) Bhattacharyya, A.C.; Bhattacharyya, D.K. Refining of high FFA rice bran oil by alcohol extraction and alkali neutralisation. *J. Oil Technol. Assoc. India* **15**, 36-38 (1983).

13) Pina, C.G.; Meirelles, A.J.A. Deacidification of corn oil by solvent extraction in a perforated rotating disc column. *J. Am. Oil Chem. Soc.* **77**, 553-559 (2000).

14) Krishna Kumar, N.S.; Bhowmick, D.N. Separation of fatty acids/triacylglycerol by membranes. *J. Am. Oil Chem. Soc.* **73**, 399-401 (1996).

15) Bhosle, B.M.; Subramanian, R. New approaches in de-