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Effect of Intrinsic and Extrinsic Factors on the Storage Stability of Sardine Oil

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Abstract

Oil extracted from pelagic fishes, rich in n-3 polyunsaturated fatty acids (PUFA) like Eicosapentaenoic acid and Docosahexaenoic acid, have numerous health benefits. The oil also contains impurities like di- and mono glycerides, free fatty acids, phospholipids, unsaponifiable matter, metal ions and volatile compounds. Most of these impurities are removed by refining process without affecting valuable n-3 PUFA. However, due to the presence of residual impurities, environmental factors and higher degree of unsaturation, the oil exhibit hydrolytic and oxidative instability during storage. This study was aimed to identify the most detrimental factors causing hydrolytic and oxidative instability and deterioration of n-3 PUFA content in sardine oil during five-week storage. The effect of various extrinsic and intrinsic factors on the storage stability was investigated. The hydrolytic and oxidative instability was estimated by free fatty acid (FFA) content and totox value (TV) respectively. Moisture, sunlight, ferric ions and FFA were found to be most detrimental to oil quality and n-3 PUFA content. Although, addition of phosphotidylcholine and phospholipase-A showed high degree of hydrolytic and oxidative instability, n-3 PUFA destruction was minimal. Interestingly, even in the presence of ferric ions and FFA, phosphotidylcholine and phospholipase-A exhibited n-3 PUFA protection. The exact mechanism by which phosphotidylcholine and phospholipase-A offered protection to n-3 PUFA needs further investigation. From this study, it can be concluded that removing ferric ions, moisture and FFA from crude oil during refining is essential. Further, the refined oil must be stored under dark conditions in airtight containers to retard deterioration of oil quality.

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Keywords

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Totox Value.

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Introduction

The major sources of dietary lipids are edible oils, which include both vegetable and fish oils. Edible oils supply calories, essential fats and helps in absorbing fat-soluble vitamins. Type of lipid consumed plays a major role in the health of an individual. Oil sardines are one of the richest and cheapest sources of n-3 PUFA such as Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA). The beneficial effects of n-3 PUFA in the prevention and treatment of coronary, neuromuscular, immunological disorders and allergic conditions are well documented (Vaisali *et al.,* 2015). Due to this, sardine oil has important industrial applications in food, pharmacy, animal feeds, cosmetics, paint and marine feeds and hence, its quality and stability during storage and transhipment have attracted a considerable attention.

The crude oil extracted from sardines by physical methods consists primarily of triglycerides (TAGs), which contain fatty acids of various chain lengths (including EPA and DHA) in addition to Free Fatty Acids (FFA), primary oxidation products, minerals, pigments, moisture, phospholipids, phospholipases and insoluble impurities (Morais *et al.,* 2001). Spoilage of fish oils occurs in two different ways i.e, oxidative and hydrolytic spoilage. Lipids are susceptible to oxidation in the presence of catalytic systems such as light, heat, enzymes, metals and metalloproteins, leading to complex processes of autoxidation, photooxidation, thermal or enzymatic oxidation, most of which involve free radicals and/or other reactive species as the intermediate. Unsaturated fatty acids are the major reactants affected by such reactions, whether they are present as FFA, acylglycerols or phospholipids (Shahidi & Zhong, 2010). Due to the presence of many double bonds, EPA and DHA are highly susceptible to oxidation. Oxidation generates free radicles like the lipid hydroperoxides, leading to adverse impacts on flavour, colour and the shelf life of the oil. (Choe & Min, 2006; Chen *et al.,* 2011). Early oxidation products such as hydroperoxides further undergo breakdown to generate secondary products like the aldehydes, ketones and alcohols which are responsible for the off flavours and odours from rancid oils. Environmental conditions like air, heat, light, moisture and other intrinsic factors like phospholipids (PL), phospholipases (PLS), free fatty acids (FFA) and metal ions have found to enhances oxidative and hydrolytic spoilage (Vaisali *et al.,* 2015; Chaiyasit *et al.,* 2007; Naz *et al.,* 2004). Reports pertaining to the influence of temperature (Akhtar *et al.,* 2010), light (Choe & Min, 2006; Hemery *et al.,* 2015), moisture (Kim *et al.,* 2014; Kittipongpittaya *et al.,* 2016), metal ions (Chen *et al.,* 2012; Benedet & Shibamoto, 2008; Kapchie *et al.,* 2013), PL and PLS (Kittipongpittaya *et al.,* 2016; Chen *et al.,* 2011; Kittipongpittaya *et al.,* 2014) and FFA (Aubourg, 2001; Kittipongpittaya *et al.,* 2014; Paradiso *et al.,* 2010) on the oxidative stability of various edible oils (vegetable and marine oils) are available. However, no reports are available on the effects of environmental conditions, intrinsic factors on the quality of the n-3 PUFA rich sardine oil.

The rate and mechanism of oxidation in fish oils containing n-3 PUFA is considerably different from the other oils and the oxidative and hydrolytic stability vary greatly, depending on fish species (Boran *et al.,* 2006). Based on the above literature, it is therefore the thrust of this work to study the effects of extrinsic factors and intrinsic factors on the quality of the sardine oil, which is rich in n-3 PUFA. Totox value (TV), a value based on peroxide and anisidine values, gives an idea of the extent of oxidation and FFA, which is an important indicator of hydrolytic degradation of the glycerides were monitored. The extent and the rate of deterioration of n-3 PUFA during the storage period were determined. The ultimate goal was to identify most important factor/s, which affects the storage stability of n-3 PUFA rich sardine oil.

Materials and Methods

Materials

Crude sardine oil was refined by the strategy adopted by Charanyaa *et al.,* (2017). Refining was performed by degumming the oil with 5% orthophosphoric acid (OPA) followed by a two stage solvent extraction with the solvent to oil ratio of 1:1 (w/w) by using methanol as the extracting solvent. Bleaching was performed under vacuum with 3% activated charcoal for 10 minutes at 80°C. Refined oil was stored under nitrogen at -21°C in dark container until use. OPA, methanol, activated charcoal, isooctane, glacial acetic acid, p-Anisidine (0.25%), potassium iodide, starch, sodium thiosulphate, isopropanol, potassium hydroxide, phenolphthalein indicator, wijis solution were purchased from Merck, India. Phospholipase-A (Sigma Aldrich, India),

Phosphotidylcholine (HiMedia, India), Oleic acid (Merck, India) were procured and stored under refrigeration. All the reagents of analytical grade and the solvents of chromatographic grade were used without any purification.

In this study, the oil samples were divided into two groups i.e, one set of oil samples were kept under the influence of environmental conditions (extrinsic factors) namely light intensity, temperature, moisture and combination of all extrinsic factors (C1) (a total of 16 flasks). In addition, another set of oil samples (14 flasks) were maintained in accordance with the experimental conditions of the various intrinsic parameters and a combination of the intrinsic factors (C2) as mentioned further in this section. All the experiments were conducted in duplicates along with control flasks (in duplicate) to eliminate experimental error.

Effect of Environmental (Extrinsic) Factors on the Stability of the Oil.

The effect of light exposure on the storage stability of the oil was studied by exposing 10 g of refined sardine oil samples to three different light conditions (dark, white light, sunlight). First set of oil sample was placed in amber coloured conical flask, second set of oil sample contained in a transparent conical flask, was under the influence of an 8-W white light and the third set of oil in a transparent conical flask was left by the window under the influence of sunlight. All the samples were purged with nitrogen at room temperature (≈30°C) before wrapping the flasks with parafilm and aluminium foils to protect them from insects and air. These oil samples were withdrawn every week for a period of five weeks and analysed for TV and FFA values.

Effect of temperature on oil was studied by storing 10 g of refined oil samples at two different temperatures namely 30°C, and -21°C. The oil samples were placed in the closed amber coloured flasks under nitrogen atmosphere. These samples were periodically withdrawn and were subjected to further analysis.

Effect of moisture content on the storage stability of oil was studied by adding calculated quantity of water to the refined oil samples. The change in TV and FFA values were checked every week for a period of five weeks in 10g of oil taken in duplicates, containing 1000 ppm of moisture placed in amber coloured conical flask under nitrogen atmosphere at 30°C. Refined oil sample stored under darkness in the closed amber coloured flasks under nitrogen atmosphere at 30°C, without any added impurities was considered as control.

Effect of Intrinsic Factors on the Quality of the Oil

Effect of metal ions, phospholipase-A, phospholipid and FFA was studied by adding calculated quantities of each one to the refined oil samples. 1000 ppm each of chloride salts of metal ions namely ferric chloride, mercuric chloride and cupric chloride, PLS (Phospholipase-A), PL (Phosphotidylcholine), FFA (Oleic acid) was dissolved individually in 10 g of oil to study its effects on the TV and FFA values in the oil. These six sets of oil samples in duplicates were maintained at 30°C, purged with nitrogen, and sealed. The samples were collected for chemical analyses every week until the end of five weeks.

Combined Effects of Prominent Extrinsic Factors and Intrinsic Factors

Sunlight, moisture and temperature (30°C), among the environmental conditions were found to degrade the quality of the oil the most, while among the intrinsic parameters, ferric chloride, PL, PLS and FFA degraded the quality of the oil rapidly. Hence, these factors were chosen for the combinational studies (C1 and C2).

Combined effect of prominent extrinsic factors (sunlight, moisture and temperature (30°C)) were studied first (C1 trials). The TV and FFA values were noted every week for a period of five weeks in 10g of oil (duplicates) in transparent flasks under the combined influence of sunlight (light) and 1000 ppm of moisture at ambient temperature (≈30°C).

Next, combined effect of intrinsic factors (phospholipase-A, phospholipid and free fatty acid) were studied (C2 trials). 1000 ppm of ferric chloride, PLS, PL, FFA was together added to 10 g of oil to study its effects on the TV and FFA values in the oil samples. The sample was maintained at 30°C and purged with nitrogen for five consecutive weeks.

Analytical Methodology

The oxidative and hydrolytic spoilage occurring due to the influence of environmental and intrinsic factor/s were assessed by determining TV and FFA content respectively, during the storage period. The n-3 PUFA content of the oil was checked at the end of fifth week for those parameters, which influenced the maximum instability in the oil in order to ascertain the extent of decomposition of valuable n-3 PUFA present in the oil.

Protocols of standard official AOCS methods (Cd 3d-63) were followed to determine the acid value or FFA, which is obtained by the titration of FFA, released from the glycerides present in the bulk oils. Atomic absorption spectrometer (GBC scientific equipment, 932 plus) was used for the determination of metal ion concentration in the refined oil in agreement with the method as given by Aluyor *et al.,* (2009). Moisture content of the oil samples were calculated by drying the samples in the oven and recording the weight of the samples until it reached a constant value (Aidos *et al.,* 2002).

The peroxide value (PV) (Cd 8b-90) and p-Anisidine value (p-AV) (Cd 18-90) of the oil was determined according to AOCS methods. TOTOX value was calculated as;

$$TOTOX = (2 \times PV) + p\text{-}AV \quad ...(1)$$

All the experiments were performed in duplicates and the mean values of the results were presented.

Chromatographic Analysis of Fatty Acid Esters

Fatty acid methyl esters (FAME) were prepared by trans-esterification process (Ichihara & Fukubayashi., 2010) in a GC (Trace 3330 GC Ultra, Thermoelectron Corporation) equipped with a flame ionization detector (FID) , split/splitless injector and DB-5 column (30m x 0.25 mm x 0.2µm). The right inlet and the detector temperatures were set at 280°C and 300°C, respectively. The oven temperature was maintained at 160°C for 1 min and further programmed to185°C at the rate of 5°C/min. This temperature was held for 10 min with a further increase of temperature to 240°C at 8°C/ min which was held for 10 min. The samples were prepared and analysed in duplicates. The conditions adopted here were as per the methodology adopted by Charanyaa *et al.,* (2017). FAMEs were identified and quantified by comparing the retention time of the samples with FAME standards from Sigma Aldrich expressed as per cent of total fatty acids (%). Chrom Cad software was used for the analysis of the chromatograms. Total n-3 PUFA values were subjected to analysis of variance (ANOVA) one way ($p<0.05$); comparison of the means after ANOVA test was performed using Tukey's test. All the samples were analyzed in duplicates and their means were reported by SPSS (16.0) computer program.

Results And Discussion

Fish oil, containing elevated levels of n-3 PUFA, is highly susceptible to hydrolytic spoilage and oxidative deterioration. The extent and the rate of spoilage strongly depend upon storage conditions (extrinsic factors), presence of several impurities and fatty acid profile. In the present study, deterioration rates of refined sardine oil were determined under different storage conditions in the presence of various impurities commonly found in crude sardine oil. The aim of the current investigation was to identify prominent extrinsic and intrinsic factors, which cause oxidative and hydrolytic degradation of oil during storage under controlled conditions. Further, an effort was also made to understand the mechanism of oxidative and hydrolytic spoilage due to each individual extrinsic and intrinsic factors. The FFA content and TV were estimated periodically (once in a week) to determine the hydrolytic spoilage and oxidative deterioration for five weeks.

Table 1: L Composition of refined Indian Sardine oil used in this study

Parameters	Values
Free fatty acid (% oleic acid)	BDL*
Phospholipid (ppm)	BDL*
Iron (ppm)	BDL*
Copper (ppm)	BDL*
Mercury (ppm)	BDL*
Fatty acids (% w/w)	
$C_{14:0}$	26.18
$C_{16:0}$	46.87
$C_{18:1}$	5.06
$C_{18:2}$	5.76
$C_{20:5}$ (EPA)	11.81
$C_{22:6}$ (DHA)	6.1

*BDL-Below detection limit

Refined sardine oil with the initial characteristics as shown in Table 1 was taken for the study. The oil had high EPA and DHA concentration (1.65 times) as compared to other unsaturated fatty acids. At the same time, saturated fatty acids were the predominant ones, present in high concentration (2.54 times) compared to unsaturated fatty acids.

Effect of extrinsic parameters on the storage stability

Light exposure (Dark, white light, sunlight), Temperature (30°C, -21°C) and moisture content (1000ppm) were the extrinsic parameters studied for five weeks storage period. The effect of each independent factor was studied first.

Light Exposure

Sunlight was found to show the highest effect on storage instability compared to white light and darkness. Sardine oil exposed to sunlight was found to attain highest FFA and TV indicating higher hydrolytic and oxidative instability compared to the oil samples maintained under white light and dark conditions during five-week storage (Fig. 1(a) and 1(b)). It was found that PV change was minimal in all the oil samples, whereas p-Anisidine value (not shown) changed drastically. Increase in p-AV led to a high TV indicating that secondary oxidation products were produced by FFA, which is evident from the concurrent increase in FFA and TV. The highest FFA values of 3.34, 3.74, 6.78 in the fifth week for samples placed in dark, white light and sunlight respectively were recorded. These results indicate a positive correlation between light intensity and hydrolytic instability. However, TVs of samples kept in white light for 5 weeks recorded slightly lesser TVs compared to dark conditions. However, TV of sample kept in sunlight and dark condition were 15.76 and 310.06 respectively, showing the detrimental effect of sunlight. As the oil samples were kept under sunlight, there was possibility of few degree increase in temperature. This could have accelerated the conversion of FFA into secondary oxidation products like hydroxyl dimers and trimers which accelerate the oil deterioration. Simultaneously, primary oxidation products (hydroperoxide) might have undergone decomposition introducing oxygen into the oil to increase further oxidation. (Choe & Min, 2006; Hemery *et al.,* 2015). The results obtained here are in accordance with several reports (Naz *et al.,* 2004; Boran *et al.,* 2006). Based on the above results, the oils were stored in dark environment for the rest of the experiments. As the purpose of these experiments was to identify prominent extrinsic and intrinsic parameters affecting hydrolytic and oxidative stability under ambient conditions, rest of the experiments were conducted at 30°C.

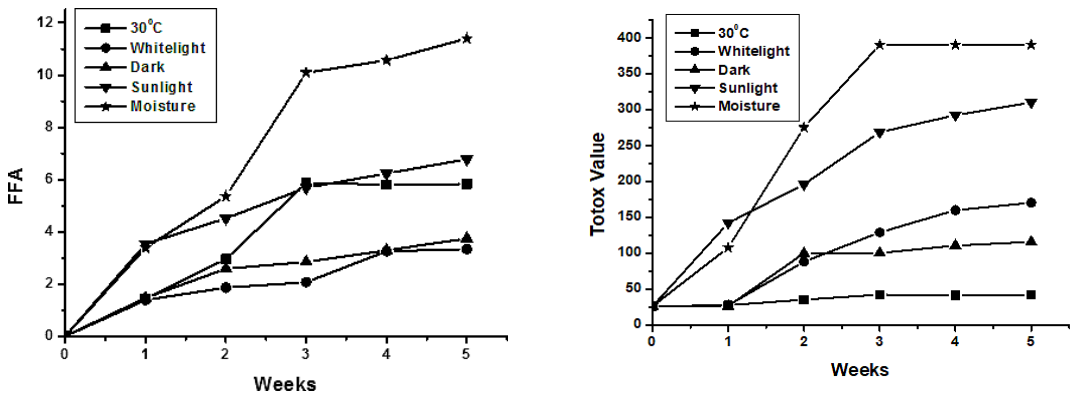

Fig.1: (a) Experimental data on the influence of extrinsic factors (30°C, light and moisture) on FFA release ; Fig. 1(b) Experimental data on the influence of extrinsic factors (30°C, light and moisture) on the TV in the oil for five weeks

Temperature

Oil samples stored at 30 °C in dark conditions recorded the highest FFA and TV (Fig. 1(a) and 1(b)). There was no significant change in FFA and TV in the oil stored at -21°C. The increase in TV at higher temperature was mainly due to the

increase in p-AV (data not shown), which denotes the production of secondary oxidation products. Secondary oxidation products are produced mainly due to the decomposition of hydroperoxides (primary oxidation products). It was also observed that there was a simultaneous increase in FFA and TV in the oil samples which is due to the ability of FFA to quicken the hydroperoxide breakdown (Miyashita & Takagi, 1986). Guillen *et al.,* (2008) in their study on sunflower oil, stored in closed containers at room temperature, reported the accumulation of large quantities of monocyclic and polycyclic aromatic hydrocarbons. Aidos *et al.,* (2002) and Lu *et al.,* (2014) proposed that the stability of crude herring oil and krill oil respectively, was drastically reduced with the increase in temperatures. Furthermore, Boran *et al.,* (2006) reported that oxidative and hydrolytic stability of various fish oils decreased with increase in storage temperature from -18°C to 4°C.

Moisture

Presence of moisture caused high hydrolytic and oxidative instability in oil as indicated by FFA and TV respectively during five weeks storage (Fig.1 (a) and 1(b)). Among all the three extrinsic parameters studied, moisture was found to be the most important factor showing highest hydrolytic and oxidative instability. Interestingly, the FFA and TV reached a value of 10.09 and 390.1 respectively by the end of 3rd week and did not show significant increase in subsequent weeks. This could be due to the tendency of the moisture to hydrolyze triglycerides into di- and mono-glycerides, glycerol and fatty acids and exist in the form of "association colloids" (McClements & Decker, 2000). These association colloids act as reaction site for lipid oxidation and forms volatiles (Kim *et al.,* 2014). The near stagnation in FFA and TV could be due to the formation of volatiles at a rate equal to the formation of secondary oxidation products and glyceride hydrolysis.

Effect of Intrinsic Parameters on the Storage Stability

Metal ions (Fe^{3+}, Cu^{3+}, and Hg^{3+}), PL, PLS and FFA were the intrinsic parameters studied for five weeks storage period.

Metal Ions

Sardine oil is found to contain varying amount of Fe^{3+}, Cu^{3+}, and Hg^{3+} and hence these three were considered for the study. Comparative analyses of FFA and TV of the oil samples containing them show that iron displayed the strongest prooxidant activity in the oil. The distinct dissimilarities in FFA and TV between the metal ions may be because of the type and the chemical state of the metals (Chen *et al.,* 2012, Benedet & Shibamoto, 2008). Change in TV in oil samples containing Cu^{3+} and Hg^{3+} were same as control (Fig. 2(b)), whereas FFA decreased by 39.4 % and 46.6 % respectively (Fig. 2(a)). Significant increase in TV was recorded in oil samples containing Fe^{3+} (4.83 times) (Fig. 2(b)) whereas the FFA decreased by 21.7 % similar to other metal ions studied. In case of Fe^{3+}, TV increased rapidly during first two weeks stabilizing in the third week with a slight decrease in the remaining weeks. This can be attributed to the reduction in the activation energy of the initiation step of lipid oxidation and acceleration of decomposition of the lipid peroxides into peroxyl radicals and superoxide anions (Kapchie *et al.,* 2013). Moreover, the FFA produced creates an anionic milieu which attracts and forms complexes with Fe3+ increasing its solubility. As transition metal ions are known to catalyse both the decomposition of lipid hydroperoxides and accelerate the free radical catalysed oxidation (Fomuso *et al.,* 2002), it can be concluded that FFA and lipid hydroperoxides produced are quickly converted into small volatile molecules. Similar results were reported by Kapchie *et al.,* (2013) in Soybean oil system by ferric ions.

Experimental data on the influence of metal ions on the TV in the oil for five weeks. The refined oil sample stored under dark conditions at 30 °C under nitrogen environment was considered as control.

Phospholipid (Phosphotidylcholine)

Phospholipids (PL) caused a steady increase in FFA and TV over five weeks period and FFA, TV values reached 2 times and 10.29 times respectively as against control experiments (Fig. 3(a) and 3(b)). The role of PL in the hydrolytic and oxidative stability is not very well understood. This is because of the ability of PL to either form lamellar structures or reverse micelles in combination with other minor ingredients such as trace metals and water, due to its intermediate hydrophilic-lipophilic balances (~8). PL such as Phosphotidylcholine used here tends to form reverse micelles and exhibit the prooxidant activity.

Chen *et al.,* (2011) concluded that the reverse micelle formed by the PL facilitate lipid oxidation. Contrary to these reports, Nwosu *et al.,* (1997) have reported that PL (Phosphotidylcholine) exhibited antioxidant properties in salmon oil, whereas they fail to show any antioxidant properties in menhaden oil. They opined that the ability of PL to form reverse micelles in the particular bulk oil could influence antioxidant property of that PL. Lee & Choe (2009) have reported that PL exhibited antioxidant properties in the presence of light in canola oil. Reische *et al.,* (2008) have concluded that antioxidative action of phospholipids is not well understood. King *et al.,* (1992) found that the presence of nitrogen in the PL like phosphatidylcholine displayed efficient antioxidant properties under most conditions which is possibly related to its chelating ability. Similarly, Jiang *et al.,* (2016) in his study found that though PL like phosphatidylcholine inhibited soya bean oil oxidation was responsible for the production of trimethylamine, major source of fishy off odours in the oil. It is likely that antioxidant activity differs among the various phospholipids as a result of the regeneration of primary antioxidants, metal chelation, and decomposition of hydroperoxides. Interestingly, the results obtained by us were contradicting these reports, showing strong prooxidant properties.

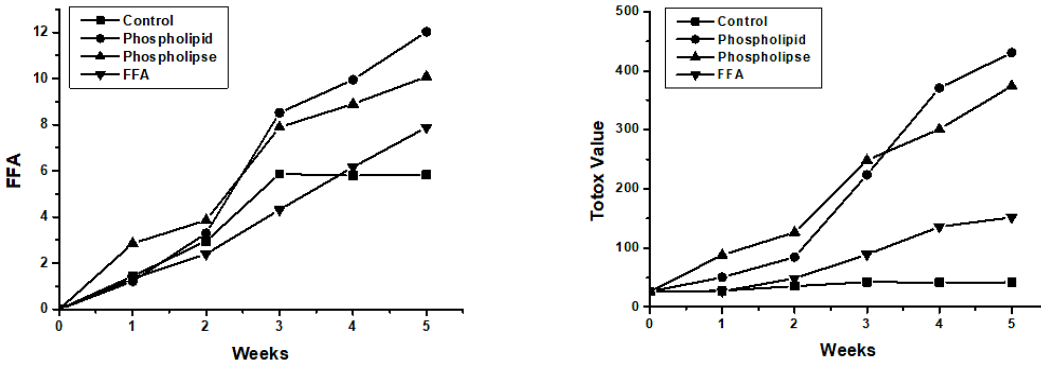

Fig: 3(a) Experimental data on the influence of PL, PLS and FFA on FFA release ; Fig: 3(b)

Experimental data on the influence of PL, PLS and FFA on the TV in the oil for five weeks. The refined oil sample stored under dark conditions at 30 °C under nitrogen environment was considered as control.

Phospholipase-A

It is clear from Figures 3(a) and 3(b), that the addition of PLS also resulted in the steady increase in both FFA (1.7 times) and TV (8.94 times) as compared to control over five weeks of storage. As PLS have the ability to hydrolyze glycerides, inclusion of PLS is expected to increase FFA in the bulk oil steadily. Perhaps the release of FFA could have caused the rapid breakdown of hydroperoxides leading to the high totox values (Kittipongpittaya *et al.,* 2014). Moreover, PLS also hydrolyze sterols and PL, releasing varieties of FFA, which in turn contribute to oxidative instability of bulk oil.

Oleic Acid (FFA)

On addition of oleic acid (FFA) to the bulk oil, it was seen that the FFA and TV increased progressively (1.35 and 3.63 times respectively) over a span of five weeks, though much less in magnitude compared to other factors such as PLS and PL (Fig. 3(a) and 3(b)). The FFA has exhibited prooxidant properties in bulk oil time and again. The prooxidant property is attributed to the carboxyl group, which form free radicals by the decomposition of hydroperoxides (Miyashita & Takagi, 1986). The oxidative instability of bulk oil also depends upon the chain length and degree of unsaturation of fatty acids present in glycerides of bulk oil. An increasing degree of unsaturation of the FFA added led to increasing oxidation development in the reaction systems when the reaction temperature was 30 °C (Aubourg, 2001). The added FFA increases both the primary

and secondary oxidation products in bulk oil (Paradiso *et al.,* 2010). The results obtained here is in consonance with the above reports.

Combined Effect of Prominent Parameters on the Storage Stability

To understand the combined effect of prominent extrinsic parameters (C1), the oil samples were stored under sunlight in the presence of moisture (1000 ppm) at 30°C for 5 weeks and the FFA and TV were estimated periodically(Fig. 4 (b). TV reached the peak in the 4th week and started declining in the fifth week (from 400.6 to 303.3) perhaps due to the liberation of tertiary oxidation products (volatiles). It can be argued that the presence of sunlight increased the rate of liberation of tertiary oxidation products (off flavours) exceeding the rate of formation of secondary oxidation products (Sun *et al.,* 2014). This experiment clearly shows that moisture in combination with sunlight and temperature contributes immensely for both hydrolytic and oxidative instability of fish oil. Kittipongpittaya *et al.,* (2016) investigated the influence of moisture content in combination with minor components, and highlighted its importance in impacting the oxidative instability in the bulk oil.

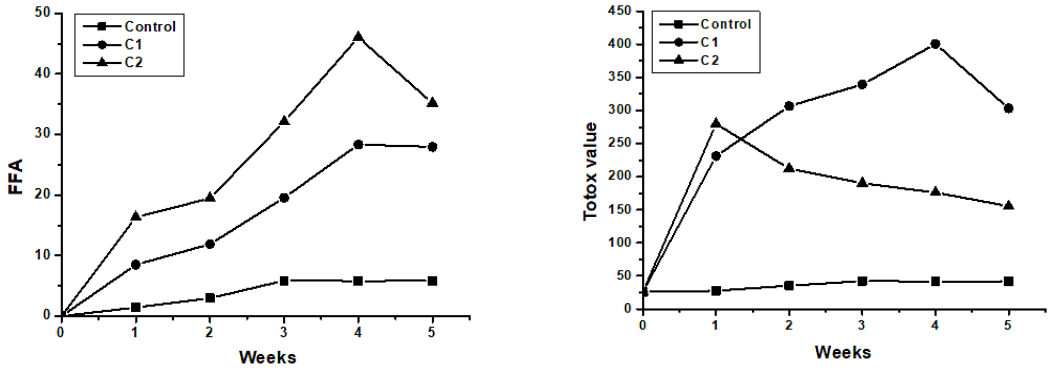

Fig: 4(a) Experimental data on the influence of C1 and C2 on FFA release ; Fig: 4(b)

Experimental data on the influence of C1 and C2 on the TV in the oil for five weeks. The refined oil sample stored under dark conditions at 30 °C under nitrogen environment was considered as control.

Intrinsic parameters showing prominent effect on storage instability of oil were chosen and experiments were conducted to ascertain their combined effect and named as C2. Ferric chloride, Phospholipid (PL), Phospholipase-A (PLS) and free fatty acid (FFA) were added to oil samples (1000 ppm), stored under darkness at 30°C for 5 weeks. FFA of bulk C2 oil showed a marked and progressive increase all throughout the storage period (Fig. 4(a)). The FFA content increased by 6 times (compared to control) at the end of five weeks, showing the highest hydrolytic instability among all the factor/s studied so far. Interestingly, the TV showed a rapid rise in the first week (10.16 times the control), which started to steadily decline over rest of the storage period (Fig. 4(b)). In spite of steady increase in FFA, the fall in TV from the second week indicates the rapid synthesis of volatile matters. Perhaps the rate of formation of volatile matters might have exceeded the synthesis of secondary oxidation products. TV was calculated based on the PV and p-AV and in both the cases PV values recorded throughout were negligible compared to p-AV. This shows that induction period of lipid oxidation was short and large amount of aldehyde and ketonic breakdown products of peroxides (primary oxidation products) were present. At high temperature or in the presence of metals, hydroperoxides are readily decomposed to alkoxy radicals and then form mostly low-molecular weight aldehydes, ketones, acids, esters, alcohols, and short-chain hydrocarbons. Among them, aliphatic carbonyl compounds such as alkanals, trans-2,4-alkadienals, isolated alkadienals, isolated cis-alkenals, trans and cis-2,4-alkadienals, and vinyl ketones are volatile molecules, responsible for the off-flavour. Propanal is the major secondary product formed during the oxidation of n-3 fatty acids which

is highly volatile. Further epoxide groups are also formed during autoauxidation of lipids (Akoh *et al.,* 2008). Escape of volatile molecules and formation of epoxide groups might have resulted in a downward trend in TV.

Effect of Prominent Factors on N-3 Pufa Content of Fish Oil during Five Weeks Storage

The effect of individual parameters such as sunlight, moisture, Iron (Fe^{3+}), Phospholipid (Phosphotidylcholine), Phospholipase (Phospholipase-A), FFA (Oleic acid) and two combinations of factors (C1 & C2) were studied to ascertain their effect on n-3 PUFA content. The oil sample stored at 30°C under darkness in nitrogen environment was considered as control. The FFA values and TV were compared with the control values attained by the end of fifth week and reported (Fig. 5). The DHA and EPA contents of oil samples were estimated after five weeks storage and are compared with that of fresh refined oil. Highest n-3 PUFA deterioration was observed pertaining to moisture and lowest was observed in oil samples having phospholipases, phospholipids and C2 (Table 2). The oil stored at 30°C in darkness under nitrogenous environment also showed minimal n-3 PUFA destruction. Also, in most of the oil samples, DHA deterioration was more than EPA deterioration, which is contrary to the popular perception that DHA in the oil is more stable due to its positioning in the glyceride backbone (Tengku-Rozaina & Birch, 2013). However, couple of reports published in recent times have reported that the DHA deteriorated more than EPA in the fish oil. (Frankel *et al.,* 2002; Akthar *et al.,* 2010), which are in agreement with our results.

Table 2: Effect of various factors on the EPA and DHA proportions (mass per cent of the total fatty acid) after 5 weeks of incubation in the respective conditions under nitrogen atmosphere

Factors affecting the quality of the oil	EPA (%)	DHA (%)	Total n-3 PUFA (%) (EPA+DHA)
Refined oil	11.81±0.01	6.1±0.00	17.91±0.01^{a}
30°C	10.56±0.35	4.11±0.03	14.68±0.38^{b}
Sunlight	6.5±0.16	3.35±0.03	9.85±0.19^{c}
moisture	6.59±0.74	1.64±0.00	8.23±0.74d^{c}
Iron	5.37±0.07	4.06±0.02	9.43±0.05^{cd}
Phospholipases	11.64±0.42	3.52±0.06	15.16±0.48^{b}
Phospholipids	11.04±0.08	3.24±0.08	14.28±0.16^{b}
FFA	4.63±0.24	3.67±0.00	8.3±0.25^{cd}
Combination 1	7.23±0.56	2.49±0.07	9.72±0.63^{cd}
Combination 2	12.06±0.16	3±0.08	15.06±0.25^{b}

Statistical comparison was made among all the factors chosen for the study for individual weeks. The means with the same letters for the different factors are not significantly different ($p<0.05$) by Tukey's test.

The main motivation of investigating the various factors was for a better understanding of the impact of these factors and interactions occurring in the bulk oil. The results obtained above suggest that, oil maintained at -21°C in dark environment resulted in least oxidation (Hemery *et al.,* 2015; Boran *et al.,* 2006). Among the environmental conditions, moisture played the most important role in the oil quality decline. This is supported by the observation that oil under the combined influence of moisture and sunlight at 30°C (C1) showed a drastic increase in FFA and TV and a reduction in n-3 PUFA content. This result confirms the synergistic effect of moisture, sunlight and temperature as the catalyst in the decline of the oil quality, which concur with the results of Park *et al.,* (2014).

FFA, TV attained by oil samples exposed to different conditions was compared with n-3 PUFA deterioration (Fig. 5). Oil samples containing PL

and PLS showed very low n-3 PUFA deterioration (20 and 15 % respectively) by the end of five weeks of storage. Interestingly, the oil samples of trial C2, where a combination of Iron, FFA along with PL and PLS also showed a mere 15% deterioration. On the contrary, oil samples exposed to FFA and iron independently showed maximum n-3 PUFA destruction (57 and 47 % respectively). This shows that PL and PLS retarded n-3 PUFA deterioration. A study by Choe & Min (2006) reported that phosphatidylcholine reduced the oxidation of DHA at 25-30°C in dark. At the same time, increase in TV shows that PL and PLS did not exhibit anti-oxidant properties. Nwosu *et al.,* (1997), had reported that PL exhibited protection to n-3 PUFA in bulk Salmon oil during storage. Lyberg *et al.,* (2005) have reported that DHA containing PL (DHA esterified to PL) showed higher oxidative stability than DHA esterified with glycerol, indicating higher protection offered by PL. The protection offered by PLS for n-3 PUFA needs thorough investigation to understand the mechanism.

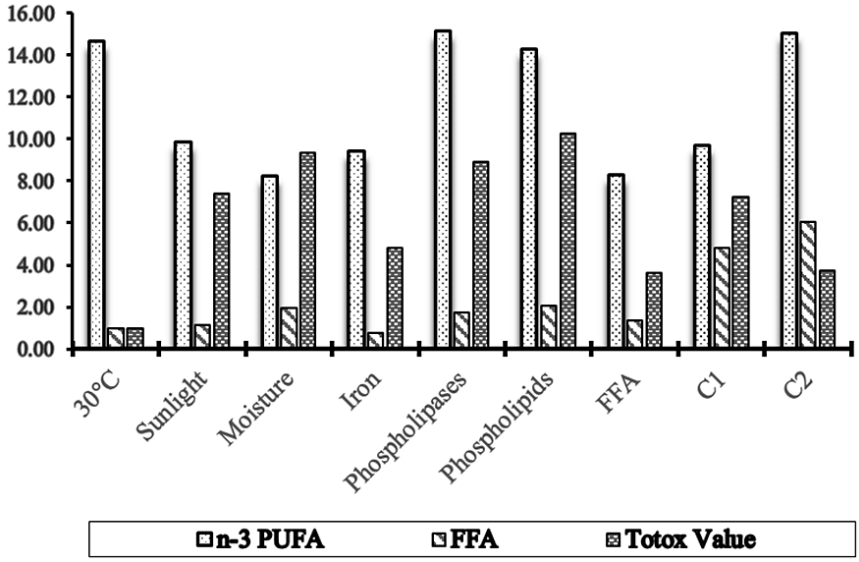

Fig: 5 Effect of selected intrinsic and extrinsic factors which led to the maximum instability in oil, on n-3 PUFA content and storage stability after five weeks of storage at 30°C, in the dark conditions under inert atmosphere. C1- combination of 1000 ppm moisture, sunlight and temperature (30°C). C2- combination of Ferric chloride, PLS, PL, FFA (each 1000 ppm). Residual n-3 PUFA, number of times increase in FFA and TV compared to control experiments are plotted

Conclusions

The aim of the current investigation was to find the most detrimental factor/s for hydrolytic stability, oxidative stability and n-3 PUFA content of sardine oil during five-week storage. Among all the independent factors studied (light, temperature, moisture, metal ions, phospholipids, phospholipase-A, free fatty acid), moisture, sunlight, PL and PLS were found to be most detrimental to the oil quality during 5-weeks storage. Highest n-3 PUFA deterioration was observed in oil sample containing moisture and lowest was observed in oil samples having phospholipases, phospholipids. Interestingly, the oil samples exposed to a combination of Iron, FFA along with PL and PLS also showed a mere 15% deterioration, on the contrary, oil samples exposed to FFA and Iron independently showed maximum n-3 PUFA destruction (57 and 47 % respectively). This shows that PL and PLS retarded n-3 PUFA deterioration. Further investigation is necessary to evaluate the degree of protection offered by various PL and PLS and their mode of action. From this study, it can be concluded that efforts should be

made to remove ferric ions, moisture and FFA as much as possible during refining. The refined oil must be stored in airtight containers protected from sunlight.

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Conflict Of Interest

Authors declare no conflict of interest.

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