STUDIES ON THE PRODUCTION OF n-3 POLYUNSATURATED FATTY ACID GLYCERIDE CONCENTRATE FROM INDIAN SARDINE OIL

Thesis

Submitted in the partial fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

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DECLARATION

I hereby *declare* that the Research Thesis entitled "**Studies on the production of n-3 polyunsaturated fatty acid glyceride concentrate from Indian sardine oil"** which is being submitted to the **National Institute of Technology Karnataka, Surathkal**, in partial fulfilment of the requirements for the award of the Degree of **Doctor of Philosophy** in the Department of Chemical Engineering, *is a bonafide report of the research work carried out by me*. The material contained in this Research Thesis has not been submitted to any University or Institution for the award of any degree.

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CERTIFICATE

This is to *certify* that the Research Thesis entitled "**Studies on the production of n-3 polyunsaturated fatty acid glyceride concentrate from Indian sardine oil"** submitted by **Ms. Charanyaa Sampath (Register Number: 123015CH12F04)** as the record of the research work carried out by her, *is accepted as the Research Thesis submission* in partial fulfilment of the requirements for the award of degree of **Doctor of Philosophy**.

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ABSTRACT

Indian oil sardine (*Sardinella longiceps*) is a chief pelagic fishery resource of India and 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). The beneficial effects of n-3 PUFA in the prevention and treatment of coronary, neuromuscular, immunological disorders and allergic conditions are well documented. The crude oil extracted from Sardines contains mainly glycerides in addition to several undesirable components viz., free fatty acids, primary oxidation products, metal ions, pigments, moisture, phospholipids, phospholipases and insoluble impurities. Due to this, oil is highly susceptible to spoilage during storage and transhipment. Through a comprehensive study, a tailor made strategy was developed consisting of degumming, deacidification by solvent extraction, bleaching with granular activated carbon to eliminate all the aforesaid undesirable components, while retaining n-3 PUFA glycerides. This strategy was able to produce sardine oil of superior quality with minimal oil loss, without any loss of n-3 PUFA content. The effect of various extrinsic factors (light, temperature and moisture content) and intrinsic factors (metal ions, phosphotidylcholine, phospholipase-A and oleic acid) on storage stability of refined oil was undertaken to identifying the most detrimental factor during five-week storage. Moisture, ferric ions, oleic acid and sunlight were found to cause highest oxidative and hydrolytic instability and highest reduction in n-3 PUFA content. Interestingly, even in the presence of ferric ions and oleic acid, phosphotidylcholine and phospholipase-A exhibited n-3 PUFA protection in spite of high oxidative and hydrolytic instability. In order to enhance n-3 PUFA content in the refined sardine oil, lipase mediated hydrolytic removal of unwanted fatty acids was attempted using *Candida rugosa* lipase (CRL) and *Pseudomonas cepacia* lipase. In order to facilitate reuse of CRL and to achieve higher efficiency and thermal stability, CRL was bioimprinted and immobilized. The preparation of immobilized enzyme and the hydrolysis of oil were optimized. The n-3 PUFA content in the deacidified oil was enriched up to 2.83-fold using bioimprinted immobilized lipase. The resultant oil had negligible di- and triglycerides content with the increase in the monoglyceride content. This proves higher efficiency in the hydrolysis of ester bonds of fatty acids,

other than n-3 PUFA. The LC-MS data analysis of oil hydrolyzed by CRL-CLEA revealed the presence of increased quantities of monoglycerides of EPA and Palmitic acid (PA). Reusability studies showed the bioimprinted – immobilized lipase could be reused up to 5 runs without a substantial reduction in its performance.

Keywords: Bioimprinting, *Candida rugosa* lipase, cross-linked enzyme aggregates, Docosahexaenoic acid, Eicosapentaenoic acid, *Pseudomonas cepacia* lipase, PTFE, Refining.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABRREVIATIONS

AAS	Atomic Absorption Spectrometry
ALA	Alpha linolenic acid
ANOVA	Analysis of variance
BSA	Bovine Serum Albumin
CLEA	Crosslinked Enzyme Aggregates
CRL	*Candida Rugosa* lipase
DHA	Docosahexaenoic acid
DOH	Degree of Hydrolysis
DPA	Docosapentaenoic Acid
EFA	Essential Fatty Acids
EPA	Eicosapentaenoic acid
FFA	Free Fatty Acids
GAC	Granulated Activated Charcoal
GC	Gas Chromatography
HP	Hydratable Phospholipid
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography- Mass Spectrometry
MA	Myristic Acid
MF	Microfiltration
n-3 PUFA	n-3 Polyunsaturated Fatty Acids
NHP	Non Hydratable Phospholipid
OA	Oleic Acid
OPA	Orthophosphoric Acid
PA	Palmitic Acid

p-AV	p- Anisidine Value
PCL	*Pseudomonas cepacia* lipase
PEI	Polyethyleneimine
PL	Phospholipid
PLS	Phospholipase
PPG	Polypropylene Glycol
PTFE	Polytetrafluoroethylene
PV	Peroxide Value
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscopy
TAG	Triacylglycerols
UF	Ultrafiltration

NOMENCLATURE

Symbol	Description
°C	Degree centigrade
g	Acceleration due to gravity
g	grams
L	litre
M	Molar
mol	Moles
rpm	revolutions per minute
U	Enzyme Unit
% (w/w)	Percentage weight by weight
% (v/v)	Percentage volume by volume

CHAPTER 1

INTRODUCTION

Chapter 1

Since 1980's, lot of epidemiological studies began to bridge the relationship between fish/ fish oil consumption due to the low incidence of cardiovascular diseases in the Eskimo population (Dyerberg 1986). From then on, the amount of clinical and biological studies about n-3 PUFA has increased, being voluminous in the current available information related to their mechanism of action, its effects and health repercussions. Omega-3 fatty acids, especially EPA and DHA, have amassed a lot of attention among the scientific community and the current society since several scientific studies have proved the positive effect of these compounds on human health. Vegetable and fish oils are the most important natural sources of n-3 PUFA. It's been estimated by the United States Department of Agriculture (USDA) that the oil production has increased from 140 million metric tonnes in 2010 to 161 million metric tonnes in 2013 to meet the increasing world market demands for refined oil (Vaisali et al. 2015). Plant derived PUFA are obtained from the sources such as palm, olive, soybean, sunflower, coconut, corn, cottonseed and peanut which are widely used, while marine derived PUFA are obtained from the various oils especially mackerel, sardines, salmon, anchovy, cod liver, sword fish, hake, etc., (Vaisali et al. 2015). There exists a compositional difference between marine and plant derived PUFA (Moffat et al. 1993, Aursand et al. 1994, Ghazani and Marangoni 2013 and Ramos et al. 2009). Marine oils are known to contain fatty acids of nutritional and medical significance (EPA 20:5 n-3 and DHA 22:6 n-3), whereas, vegetable oils are known to be natural sources of n-6 PUFA. Vegetable oils are domestically utilised in most countries. Oleic acid (18:1), LA (18:2) and ALA (18:3) contributes to the major part of fatty acid content in vegetable oil. ALA is an essential fatty acid as it acts as a precursor for the synthesis of EPA. Vegetable oils like soybean and rapeseed oil are known to contain more n-6 PUFA due to which high concentrations of 4-hydroxy-2-trans-nonenals are found in vegetable oils upon oxidative deterioration, as they are derived from n-6 PUFAs (Hua et al. 2016). Whereas, fish oils have high concentration of both n-3 and n-6 PUFAs due to which wide varieties of harmful alkenals are found in fish oil than vegetable oil upon oxidative deterioration.It has been reported by Davis and Etherton (2003), that human body could not synthesize EPA very

efficiently except at high doses of LA. Thus, it becomes necessary to provide dietary supplements of EPA as it act as precursors for DHA synthesis. This is overcome by the consumption of fish oils which has both EPA and DHA content. Though fish oils have overtaken vegetable oils in their commercial importance, vegetable oils are still widely used due to their various attributes viz., appearance, taste, smell, cultural preference and ease of production.

The n-3 PUFA from fish oil is obtained commonly by physical processes like cooking and pressing followed by centrifugation from fish or fish by products. Pelagic fisheries resources of India are essentially of multispecies and multisector fisheries. 240 species in general contribute to this sector. The total world fish capture from marine waters and aquaculture was of the order 167.2 million tonnes in the year 2014, out of which 21 million tonnes was processed to produce fish meal and fish oil (FAO 2016). Indian fish catch during 2007 was 4.0 million tons, out of which 4.7% was used for fish meal and fish oil production. The fish oil production in South India is estimated to be 34 thousand tonnes, out of which 30.5 thousand tonnes is produced in Karnataka alone (Ponnusamy et al. 2012). However, among the various fish oils, oil from anchovies and sardines are the most significant and are found to have very high quantities of EPA and DHA (Mohanty et al. 2004). The Indian oil sardine is distributed along the coast of Somalia, Gulf of Oman, west of Pakistan, West coast of India around Srilanka to East coast of India. Very large shoals of *Sardinella longiceps* occur in Somalia coast. It is distributed along entire Indian peninsula, but highest shoaling is observed along the coast of Kerala and Karnataka (Raja 1969). The bulk sardine fish catch spreads over October to March along west coast of India and is used for the production of fish oil and fish meal. Fourteen species of Sardines are found in the Indian waters, of which, *Sardinella longiceps* (Oil Sardines) is the one which contributes to 15% of the total fish oil production in the country. Kerala, Karnataka, Goa, Maharashtra and Gujarat are the states which contribute to the marine fish production with Kerala and Karnataka being the most productive zones in the west coast. About 20,000 tons of unrefined fish oil (chiefly sardine oil) is being exported per annum from undivided South Canara district of Karnataka alone. The unrefined fish oil generally contains about 20% (by weight) of n-3 PUFA. The medical benefit

of n-3 PUFA and the abundance of sardines in these regions provided motivation to focus on the refining of sardine fish oil and enrichment of PUFA in it. The oil from sardines obtained from Mukka fish oil Industries, Mangaluru was hence chosen for the studies. Crude fish oil is not suitable for human consumption due to the presence of various impurities like free fatty acids (FFA), phospholipids, metal ions, etc. The presence of low amounts of n-3 PUFA and the need to produce flavour-neutral, light-coloured and stable oil (physically and oxidatively) in order to meet the demands of industries and consumers, makes refining of oil and subsequent concentration of n-3 PUFA in the oil indispensable. Oils exhibit oxidative and hydrolytic instability during storage and transhipment and the degree of spoilage depends on the lipid profile, presence/quantity (high or low) of impurities and prevailing environmental conditions. Oxidation takes place either in the presence of light (photooxidation), dark (autooxidation) or due to enzymes (enzymatic oxidation). The accelerated oxidation rate due to the higher degree of unsaturation makes it necessary to stabilize the oil immediately after extracting the oil. Hence, several refining techniques have been proposed and applied at large scale, to improve the quality of oil. The processing stages are to be designed to target the major class of impurities one step at a time, thereby resulting in a high quality product. Since consumers expect bland and odourless oil with high oxidative stability and good amount of n-3 PUFA content in the oil, effort is made to remove odoriferous compounds along with pigments, gums and metal traces efficiently (Farhoosh et al. 2009, Lamas et al. 2016) with the subsequent storage of the refined oil under conditions which limit the deterioration of oil. Various factors that affect the composition of oils are fish species, geographical location of the source, sex, age, size, temperature of the environment and method of oil extraction (Choe and Min 2006). To develop these production technologies from natural sources has become a major challenge since the quality of the PUFA is of utmost concern. This necessitates the requirement of methods that convert the poor quality oil into oil rich in n-3 PUFA. Refining in the industries generally is carried out by two different strategies namely physical and chemical refining. The steps involved in physical refining include degumming, bleaching, and deodorization and chemical refining is inclusive of an additional neutralization step specifically focused on neutralizing the excess FFA in the oil. In the case of chemical (wet) refining,

chemicals are made use of for the removal of impurities while in physical refining, steam is used to strip the FFA along with impurities and other unsaponifiable matters under vacuum. The main objective of refining is to remove the contaminants that adversely affect the quality of oil, thereby increasing the shelf life and consumer acceptance. During the past few years, extensive efforts have been made to develop refining technology, using either conventional physical/chemical processes or several unconventional processes including biological and membrane processes. The domestic use of fish oils are yet to be practised worldwide as there is no commercially viable production technology available to produce colour less, odour free, neutral, bland tasting fish oil without losing n-3 PUFA and other valuable components. Hence there is an on-going effort to develop refining technology for every variety of edible oils (vegetable/fish) so as to produce nutritionally rich, colour less, odour less, neutral and bland tasting oil. Though Dijkistra (2009) has critically analysed the edible oil processing at industrial level, there are limited reviews on all the available technologies for wide range of edible oil varieties. Recent demand for enriched fish oils with high nutritional benefits requires a more crucial analysis of all processes along with an insight on the compositional variation that affect each process. In a review by Wijesundera et.al (2011), it has been reported that aquatic life has been widely exploited due to the benefits of essential fats from marine sources, thus reducing the fish population by 90%. This imposes a need to develop highly efficient refining process to protect the significant components rather than continuous exploitation.

Nowadays, the diet involves an increased consumption of food that contains increased quantities of saturated fatty acids and lesser amounts of n-3 PUFA. Nutritionists suggest the consumption of fish and green vegetables as a source of n-3 PUFA in order to prevent diseases and hence n-3 PUFA is being sold in the form of nutritional supplements and functional food products. Many chemical methods are being explored for the production of concentrates of n-3 PUFA in the fish oil. Since these techniques delivers disadvantages, the effectiveness of the lipases for enhancement of n-3 PUFA like EPA and DHA from fish oil has been exploited (Wanasundra and Shahidi 1998). They have been used for the production of value added food products

with medicinal properties because of their ability to catalyze reactions such as hydrolysis and trans-esterification (Sharma et al. 2013). These lipases remain active at the oil-water interface causing the release of FFA by the attachment of acyl group on the positive side chain and hydrogen ion on the negative side chain in the active site (Sharma et al. 2013). The presence of carbon-carbon cis-double bonds in EPA and DHA results in the bending of the chains enhancing the steric hindrance for the approaching lipase (Casas-Godoy et al. 2014). This result in the selective hydrolysis of ester linkages of non-PUFA glycerides like saturated and monounsaturated fatty acids more efficiently , in turn concentrating EPA and DHA due to the failure of lipases to cleave their ester linkages (Wanasundara and Shahidi 1998).

It is well known that the use of commercial lipases for the very purpose of concentration of EPA and DHA in the oil leads to the wastage and limited reuse of these enzymes. For this reason, enzyme preparations designed with a high activity and stability has attracted considerable attention over the native enzymes. Enzyme immobilization, lipid coating and bioimprinting are the recently employed techniques to modify lipase (Yan et al. 2010). Identifying more specific enzymes and the techniques most suitable for the enrichment of n-3 PUFA is still a major challenge in the edible oil industry.

1.1. SCOPE OF RESEARCH WORK

Currently, majority of n-3 PUFA concentrates are commercially derived from marine oils such as menhaden, salmon, mackerel, sardines, krill oils etc. The production involves steps like fish oil extraction, refining, concentration and stabilization. Conventional processes comprising of physical and chemical methods, are the most common steps employed in the current industrial production of n-3 PUFA. However, these techniques involve the destruction of the desirable components due to harsh chemicals and high temperatures. Although several reports are available on the refining of edible oils (Vaisali et al. 2015), the methodologies adopted for other edible oils cannot be extended to the fish oils due to the diverse nature of oils with respect to its composition. This enunciate the need for optimization of various stages of refining process that removes the impurities, minimising the oil loss while retaining the beneficial constituents like the n-3 PUFA in particular to fish (sardines) oils. There

are few reports on the refining of fish oils but reports pertaining to the refining of Indian Sardine oil and optimization of various stages of refining are rather exiguous. Therefore, there is a pressing need for designing a sustainable integrated refining process for Sardine oil keeping in mind the complexity of the oil composition.

Sardine oils contain high levels of EPA and DHA which in turn are composed of high number of double bonds making them highly susceptible to oxidation apart from the impurities that are present in the oil. Prevention of spoilage of fish oils has been a significant challenge for the food industry especially since there is a high demand for bland and odourless oil with a high oxidative stability. Therefore, proper storage is required not only to preserve the delicate taste of the oil, but also to ensure that it does not spoil and become rancid. Ensuring proper storage conditions will lead to a prolonged shelf life to ensure the healthy nutrients remain intact. The untoward side effects witnessed during oxidation geared towards the tailoring of storage conditions of the oils. Several reports are available on the oxidation studies of various edible oils under the effect of varied storage conditions, but to our knowledge, very few studies have been published corresponding to the effects of environmental conditions, intrinsic factors and storage time on the quality of refined Indian Sardine oil which is rich in n-3 PUFA. Attempts to identify most significant factors (environmental and intrinsic) for a particular span of time affecting the storage stability of the Indian Sardine oil are paltry. An understanding on the hydrolytic and oxidative deterioration of the oil presented in this study may deliver a good source of information on the most unfavourable factors affecting the quality of the oil which provides an insight on the storage environment of the fish oil.

The production of n-3 PUFA rich concentrates of good quality, for manufacturing n-3 PUFA rich supplements and foods, annunciate the need to study novel and competitive methods involving the production of these concentrates. Majority of the industrial processes for the production of n-3 PUFA concentrates on a large scale involve the extraction of oil followed by the concentration of n-3 PUFA concentrates in the oil. The methods generally used by many of the industries to produce n-3 PUFA concentrates display a major drawback of loss of inherent quantities of EPA and DHA during the process. This can be avoided by exploiting the usefulness of certain

enzymes namely lipases for the enhancement of n-3 PUFA. Owing to the uniqueness of the various sources of lipases from the microorganisms, these lipases can be expected to possess unique properties which lead to the good hydrolysis efficiency and in turn enrich the EPA and DHA content in the oil. Several researches are already available on the hydrolysis of fish oils using lipases (Chakraborty et al. 2010). Nevertheless, the instances of hydrolysis of refined fish oil using the two enzymes modified using solvents and surfactants and consequent optimization of hydrolysis process under various conditions are scarce, in particular with Indian Sardine oil. Further, the use of commercial soluble enzymes leads to the wastage and limited reuse of the lipases. To avoid this, modification of enzyme preparations by techniques like carrier free immobilization and bioimprinting are attracting considerable attention. Cao et al. (2003) have reviewed numerous carrier free immobilization techniques. Reports on the successful preservation of bioimprinted characteristics of lipase by carrier free immobilization technique in aqueous environment are inadequate especially with respect to Indian Sardine oil. Additionally, the use of these modified enzymes for the production of n-3 PUFA rich Indian Sardine oil is insufficient.

1.2. OBJECTIVES

Considering the above observations and bearing in mind the ambit of the planned work mentioned above, the following objectives were suitably laid

1. Optimization of various stages of refining processes of Indian Sardine oil.
2. Studies on the effects of intrinsic and extrinsic factors on storage stability of sardine oil.
3. Enhancement of n-3 PUFA by hydrolysis using commercial lipases and prepared (bioimprinted and cross linked) *Candida rugosa* lipase.

1.3. ORGANIZATION OF THESIS

Detailed study conducted on “Studies on the production of n-3 polyunsaturated fatty acid glyceride concentrate from Indian Sardine oil” has been presented in this thesis. The outcome of the study has been organized as chapters under following headings.

CHAPTER 1: Introduction

This chapter introduces the said research and discourses the background and the necessity of the research work. The need to produce good quality oil enriched with n-3 PUFA from Indian Sardine oil for the pharmaceuticals and industrial applications has been discussed here. The scope and the objectives of the work have also been stated.

CHAPTER 2: Review of literature

This chapter presents a detailed bibliographical search of the various refining processes, preserving the stability of fish oil and enrichment methods of n-3 PUFA pertaining to the above stated objectives. The processes and methods have been discussed in detail and the most pertinent works on these fields are consolidated.

CHAPTER 3: Refining of Indian Sardine oil

This chapter presents the experimental details and results pertaining to the refining of crude Indian Sardine oil. A tailored made step by step method was devised for the crude fish oil refining and the optimization of each stage of refining and efficiency of each refining steps were reported.

CHAPTER 4: Storage stability of Indian Sardine oil

This chapter gives an account on the effects of the intrinsic and extrinsic parameters on the storage stability of the oil. The study entails a comprehensive study of fish oil hydrolytic and oxidative instability in the presence of various environmental/intrinsic factors. Also, an elucidation of the effect of most marked extrinsic and intrinsic factors on the n-3 PUFA deterioration in the oil was anticipated in order to highlight its shelf life and select the best storage conditions of fish oil.

CHAPTER 5: The lipase mediated enhancement of n-3 PUFA in the oil

This chapter presents the experimental details and results of n-3 PUFA enrichment by using commercially available lipases (*Candida rugosa* and *Pseudomonas cepacia*). Outcome of optimization studies for the production of Sardine oil concentrate were

discussed. Further, preparation and optimization of bioimprinted, cross-linked enzyme aggregates of *Candida rugosa* lipase and its performance were presented in this chapter to establish its implications on the sardine oil quality.

CHAPTER 6: Summary and Conclusions

This section offers a brief summary and conclusions of the findings of this research work on the studies of refining, storage stability and enhancement of n-3 PUFA of Indian Sardine Oil with some recommendations for the future work.

CHAPTER 2

REVIEW OF LITERATURE

Chapter 2

This section provides a detailed literature survey concerned to the objectives laid as mentioned in chapter 1. Firstly, a brief mention about the statistics of the sardine fish oil production in India followed by introduction of essential fatty acids, focusing on the most relevant studies published over the past few years about their role in human health. A thorough review of various methods and recent advances in the field of edible oil processing and refining, including conventional methods (physical and chemical) were provided. Further, it also propounds various factors that affect the quality of edible oils during storage and gives an outline of various studies carried out by researchers elsewhere to ascertain the effect of those factors on storage stability of edible oils. The last part of the chapter commentates on the details of recent studies highlighting the development and the use of lipases to enhance the n-3 PUFA content of edible oils.

2.1. SARDINE OIL

7.8 million tons of fish out of the 75 million tons per year of the total world fish catch is being processed to produce about 2.6 million tons of fish meal and 0.39 million tons of fish oil. In the year 2007, the Indian fish catch was 4 million tons, out of which 4.7% was used for fish meal and fish oil production. More than 16 fish meal plants are operating in India (Aswathy and Narayanakumar, 2013, FAOUN). 51.6% of the 240 species contributing to the marine fishing is shared by pelagic fishes. 15% of the 51.6% of the pelagic species is predominantly *Sardinella longiceps* (Oil Sardine) which is the major species of the fish oil production in the country. Other 13 species of Sardines termed as lesser sardines contribute to 3-7% of the marine fish catch (Mohanty et al. 2008). Fish oil is being used in leather tanning, soap manufacture, biodiesel production and aquaculture feed production. About 20,000 tons of unrefined fish oil, chiefly Sardine oil, is being exported per annum from South Canara district of Karnataka alone. Average unrefined fish oil price in international market was $ 1,330/Ton in May 2011. The unrefined fish oil contains about 20% (by weight) of n-3 Poly Unsaturated Fatty Acids (n-3 PUFA). The international market, bulk price of n-3 PUFA is about $ 235/kg.

In view of the statistics as mentioned above, therefore, from an economic standpoint, the vast availability of sardines along the coastlines of Western Ghats and the presence of large amounts of EPA and DHA in these fishes qualifies them as an important group of pelagic fishes with considerable economic potential as the starting material for the further studies.

2.2. n-3 POLYUNSATURATED FATTY ACIDS (PUFA)

2.2.1. Essential fatty acids (EFA)

Fatty acids are compounds formed by a hydrocarbonated chain and a carboxylic group that is normally attached to a glycerol backbone to form acylglycerides (mono, di- or triglycerides).

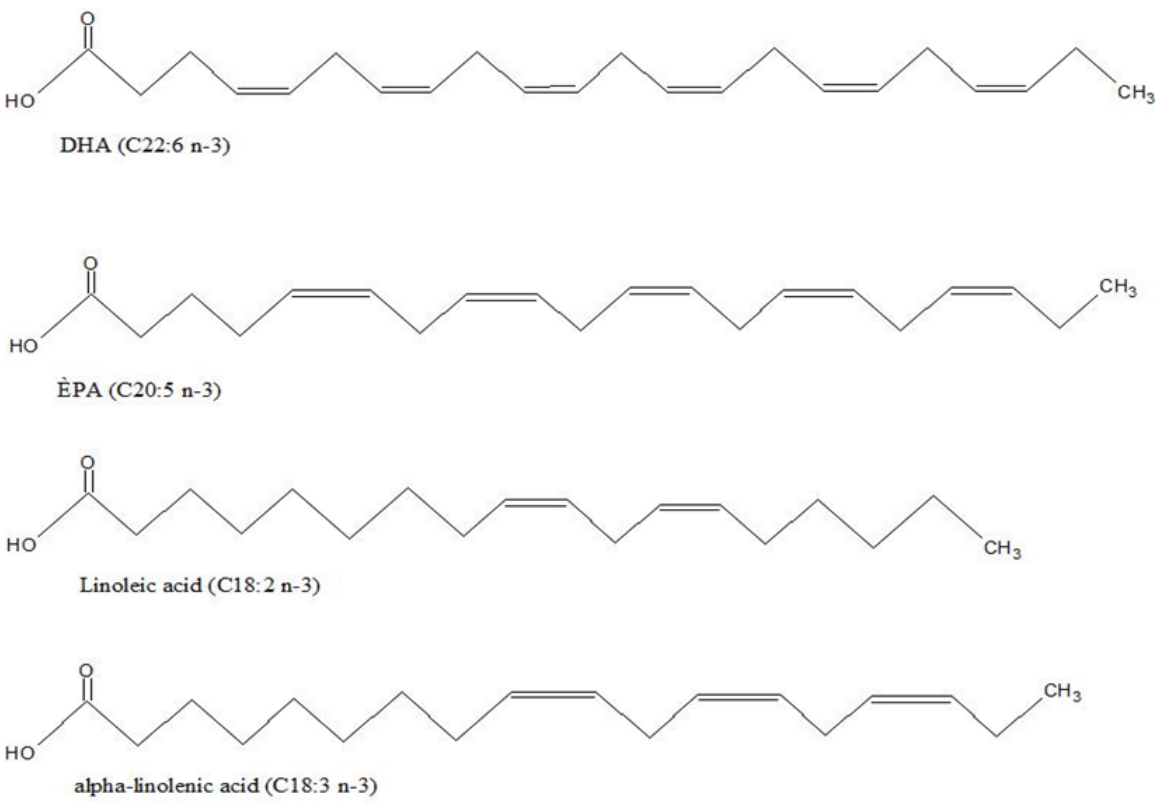


Figure 2.1: Important Essential Fatty Acids (EFA)

Table 2.1: Most common fatty acids added in human diet

Type		Fatty acids	Carbon number	Number of double bonds (C=C)	Formula
Saturated fatty acids (SFA)		Butyric	4	0	C4:0
		Caproic	6	0	C6:0
		Caprilic	8	0	C8:0
		Capric	10	0	C10:0
		Lauric	12	0	C12:0
		Myristic	14	0	C14:0
		Palmitic	16	0	C16:0
		Stearic	18	0	C18:0
		Araquidic	20	0	C20:0
		Behenic	22	0	C22:0
		Lignoceric	24	0	C24:0
Monounsaturated fatty acids (MUFA)		Palmitoleic	16	1	C16:1
	n-9	Oleic	18	1	C18:1
		Gadoleic	20	1	C20:1
	n-11	Gondoic	20	1	C20:1
	n-13	Erucic	22	1	C22:1
Polyunsaturated fatty acids (PUFA)	n-6	Linoleic (LA)	18	2	C18:2
		γ- Linoleic	18	3	C18:3
		Dihomo-γ- Linoleic	20	3	C20:3
		Araquidonic	20	4	C20:4
	n-3	α- Linoleic (ALA)	18	3	C18:3
		Stearidonic	18	4	C18:4
		Eicosapentanoic(EPA)	20	5	C20:5
		Docosapentanoic (DPA)	22	5	C22:5
		Docosahexanoic (DHA)	22	6	C22:6

Table 2.1 registers the most common types of fatty acids and they are categorized in the table based on the number of unsaturation as saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) and

according to the position of first carbon-carbon double bond from the COOH functional group, named as n-3, n-6, n-9, n-11 and n-13. SFA as the name suggests, are saturated with hydrogen and are straight chain hydrocarbons with even number of carbon atoms while MUFA and PUFA have one carbon-carbon double bond and multiple carbon-carbon double bonds, respectively, which may occur in different positions. PUFA with the first double bond between the third and the fourth carbon atom are named as n-3 fatty acids and PUFA of n-6 fatty acids have their first double bond between the sixth and the seventh carbon atoms.

PUFA in general are synthesized only by plants and phytoplanktons which are essential for mammals (Vaisali et al. 2015). The human body can synthesize palmitic acid, palmitoleic acid or oleic acid. However, it is unable to produce fatty acids with the double bonds at position n-6 and n-3 such as linoleic acids, EPA and DHA, consequently these are considered as essential fatty acids (EFA) and must be supplemented externally through diet.

Fatty acids like arachidonic acids (n-6), EPA, DPA and DHA (n-3) are synthesized from parent fatty acids like LA and ALA by a series of enzymatic reactions based on the desaturation and elongation of hydrocarbonated chain (Figure 2.2). Figure 2.2 makes it evident that LA and ALA compete for the same enzyme (desaturase), and a high consumption of vegetable oils leads to the high levels of LA in the organism, which implies a lower conversion of ALA into EPA and DHA.

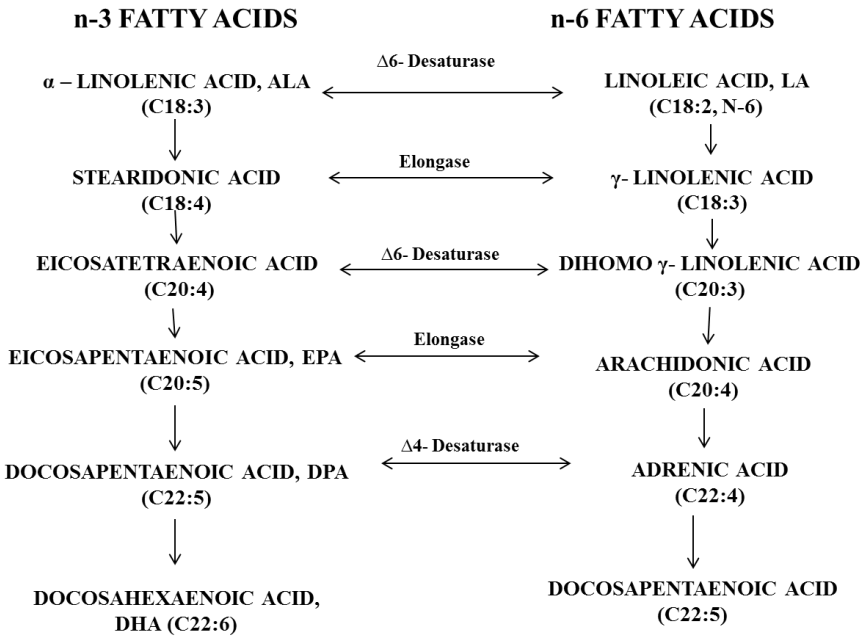


Figure 2.2: Pathways for the synthesis of n-3 and n-6 fatty acids (Adapted from Simopoulos 1991).

2.2.2. n-3 PUFA in human health and diet

The consequences of major fatty acids in human health have been the topic of interest of many scientific researches and reviews in the last few years. A book edited by Chow (Chow 2000) and a review article on dietary fat and cancer risk published by Chen et al. (2007) gives a compilation of the effects of n-3 PUFA in human health. There are also ample research dealing with n-3 PUFA since the time it was realised by Dyerberg and group (1986) about the effects of n-3 PUFA on the cardiac health of the eskimos. Several epidemiological and clinical studies have dealt with the mechanism of the effects of EPA and DHA in human health (Dyerberg 1986 and Kromann et al. 1980). A brief mention of some of the review articles which compile the effects of n-3 PUFA in human health is made in the following Table 2.2.

Table 2.2: Studies on the implications of n-3 PUFA on human health

No	Effects on human health	References
1.	n-3 PUFA resulted in the lowering of the blood pressure in patients with hypertension over the course of one year study	Bagge et al. 2017
2.	Strong evidence of long chain-PUFA on cardiovascular and rheumatoid arthritis	Ruxton et al. 2005
3.	Positive effect of long chain n-3 PUFA in treating asthama	Reisman et al. 2006
4.	Potential use of n-3 PUFA in the treatment of psoriasis	Zulfakar et al. 2007
5.	Reviews on the effects of n-3 PUFA on bowel diseases	Diamond et al. 2008, Razack et al. 2007, Turner et al. 2008
6.	Treatment of mental illness	Clayton et al. 2007, Mazza et al. 2007, Ross et al. 2007
7.	Prevention of various types of cancer	Calviello et al. 2007, Chen et al. 2007, MacLean et al. 2006.

Various researches have proposed the mechanisms by which n-3 PUFA act in the humans (Shaikh et al. 2008, Massaro et al. 2007). Many scientific reports generally advises an intake of n-3 PUFA through the foods, which have them, or as a supplement (Vaisali et al. 2015). Additionally, many experts have suggested the ratio of n-6: n-3 PUFA of 5:1 or less (3:1) to provide health benefits (WHO/FAO 1994).

Considering the importance of edible oil in our daily diet, it is vital to know the source and study the composition of the oils prior to subjecting it to any process. This step becomes critical because the choice of the refining process based on the components in the oil decides the final quality of the oil. In the next section, a brief

account of various methods generally employed in the production of refined edible oils is presented.

2.3. REFINING OF EDIBLE OILS

Crude fish oil is the lowest quality of oil which is not suitable for human consumption. These are used in the industry as raw materials to obtain high quality fish oil with enriched EPA and DHA. Most of the industries extract the oil by pressing and centrifuging the fish oil which was cooked previously. This is the most common type of extraction used in the industries although methods based on protein digestion have been suggested in the literature to avoid the use of high temperatures which improves the oil quality. Wet pressing method is the most traditional process involved in obtaining crude fish oil from the whole fish or its parts as is stated by Food and Agriculture Organization of United Nations (FAO 1986). Several steps involved in production of fish oil includes, cooking of the fish, pressing of the cooked fish and filtration or centrifugation to recover the oil from the micelles that is formed due to the presence of steam while cooking.

The production of oils and fats from vegetable and marine sources with bland and neutral taste even after several months of storage, while retaining their nutritionally important constituents is the challenge, every edible oil industry faces today. To achieve such characteristics, detrimental and flavour producing compounds have to be removed. While retaining some of the most desirable components of edible oils like acylglycerols, n-3 PUFA, the undesirable components like phospholipids, FFA, oxidation products, trace metals and waxes have to be removed by refining steps. Hence the refining process has to be designed in such a way that the undesirable compounds are eliminated, minimising oil loss and maximising the availability of beneficial constituents in order to aim for higher quality standards for EPA and DHA contents in the oil.

Table 2.3: Characteristic differences of major vegetable and fish oils due to the components present in them.

Type of oil	Essential Nutritional Components	Undesirable components	Salient features
Vegetable oils			
Soybean Palm Rice bran Canola	Acylglycerols, Tocopherols Carotenoids n – 6 fatty acids	Phospholipids, Free fatty acids, Pigments, Metal ions Moisture	• More of saturated fatty acids. • Better oxidative stability due to the presence of tocopherols and carotenoid compounds.
Fish oil			
Sardine Menhaden Salmon Krill Salmon Tuna Cod liver Sea blubber	Acylglycerols, n - 3 and n - 6 fatty acids	Phospholipids, Free fatty acids, Oxidation products, Metal ions, Pollutants like PCBs and dioxins	• Increased quantities of unsaturated fatty acids • High rates of oxidation due to unsaturation, presence of pro-oxidants • Extremely short induction time. • Presence of large quantity of impurities due to rapid oxidation and inherent impurities.

(Adapted from Belur et al. 2017)

The main procedure to remove impurities involves several steps such as degumming (to separate phospholipids and waxes), deacidification or neutralization (to strip the FFA and decrease the oil acidity), bleaching (to absorb the pigments and the remains of impurities) and finally deodorization (to remove the smelly components). This sequence provides several drawbacks since it utilizes the chemicals which in turn contaminate the environment. It also leads to the losses of neutral oil containing high FFA contents which results in the significant loss of n-3 PUFA contents (Vaisali et al. 2015).

The conventional refining processes fall under two broad classifications namely, physical and chemical refining. The principle difference between the two methods lies in the removal of FFA either by the addition of chemicals or by the removal through distillation while the following steps remain common for both the processes for the removal of the impurities like FFA, phospholipids, sterols, pigments and toxic compounds (metal ions, dioxins, PCB and smelly compounds) to produce good quality oils (Belur et al. 2017).

In case of chemical or wet refining process, the impurities are removed by contacting oil with certain chemicals. It involves several stages including degumming, deacidification, bleaching and deodorization. The solvent extracted oil usually possesses a number of desirable and undesirable compounds and is further subjected to bleaching and deodorization. While acylglycerols, tocopherols, carotenoids, phytosterols and polyphenols are some of the essential components of oil (Table 2.3), phospholipids, FFA, metal ions, oxidation products and other volatiles, reduce the oil quality (Charanyaa et al. 2017) (Table 2.3). Physical refining makes use of steam to strip the FFA, impurities and unsaponifiable matters from the oil under vacuum. The procedure has shown acceptable results only when the initial quality of the oils is good. The oils are subjected to steam refining only after the initial pre-treatment of the oils. The pre-treatments involved in this process is very stringent and removes certain non-volatile impurities while the volatiles and other trace impurities are removed by steam distillation (Bhosle and Subramanian 2005).

Some of the patents about edible oil extraction in the recent years have been tabulated below:

Table 2.4: Recently published patents on edible oil refining (From 1995 to 2017)

Year	Title	Inventors	Patent number
1996	Enzymatic mucilage removal process	F. Loffler, H. Plainer, H. Ottofrickenstein and B. Sprossler	233528

Year	Title	Inventors	Patent number
2000	An automatic device for detoxification of aflatoxin contaminated edible oils by photolysis	T. Shantha et al	218200
2001	Method and plant for extracting fish oils and the resulting products	P. Barrier and J.Y. Rousseau	US6214396
2001	Process of producing edible quality refined fish oil	J.B. Crowther, B.H. Booth and D.D. Blakewell	WO014158
2001	Process for the preparation of rice bran oil low in phosphorus content	N.B. Kaimal, Thengumpillil, R. Ongole and S.B. Potula	US6706299
2001	Degumming of edible oils by ultrafiltration	S.S.Koseoglu, W.E.Farr, W.F.S. De Greyt and M.Kellens	US 6797172
2004	A process for the production of degummed and dewaxed rice bran oil for physical refining	D.R. Sobankumar, L.Rajam, A.Sundaresan and C. Arumughan	248905
2004	Method and apparatus for processing vegetable oil miscella, method for conditioning a polymeric microfiltration membrane, membrane, and lecithin product	B. Jirjis, H.S. Muralidhara and D.D. Otten	US7923052
2005	A process for the preparation of oryzanol enriched rice bran oil	S.Khatoon and A.G.G. Krishna	270186
2007	A process for the production of oil and protein from fish waste	J.B. Cloughley	BG2428682
2007	Process for the separation of anchovy meat and anchovy oil	L.B. Tae and C.B. Soo	KR100718551
2008	Method from preparing high purity edible fish oil from course fish oil	S. Yong	CN101297708
2008	Edible oil degumming method	Z. Zhenghui	CN101396053

Year	Title	Inventors	Patent number
2010	Method for extracting crude fish oil in leftovers of tilapia	L. Hai et al	CN101768507
2010	Method for dry degumming and refining of edible rice bran oil	F. Chengliang and H. Gensheng	CN101805667
2011	Method for deacidification of alkylation reaction product	F. Yiqun, L. Mei, X. Weihong and X. Nanping	CN102021016
2012	High acid value oil deacidification membrane separation dehydration device	Y. Jielian , W. Zhenfen , Y. Na , P. Qilin , W. Hua , S. Youyong , C. Hengjie	CN202297554
2015	Removal of free fatty acids from glyceride oils	M. Atkins, F. Coleman, P. Goodrich	WO2015079262

In the last few years, the demand for food grade and pharmaceutical grade fish oils ordained for human consumption has increased noticeably. Some of the common refining steps employed in industry have been reviewed below.

2.3.1. Degumming

Oils produced by traditional mechanical pressing and solvent extraction contain gum like substances. These gums otherwise called phospholipids, which tend to absorb some amount of water present or provided during the extraction process are called hydratable phospholipids (HPs). Phosphatidylinositol (PI) and phosphotidylcholine (PC) are highly hydrophilic in nature and are categorized under HPs. The presence of five free hydroxyl groups on inositol moiety makes PI strongly hydrophilic while the presence of positive charge of trimethylamino group in PC makes this phospholipid hydrophilic. Another class of phospholipids comprises mainly non hydratable phospholipids (NHPs). These phospholipids are hard to remove from oil unlike the HPs due to their existence mainly as calcium or magnesium salts of phosphatidic acid

and phosphatidylethanolamine. The principle difference in the polarity of HPs and NHPs is in the size and structure of phosphorus groups of the phospholipids. The larger the phosphorus groups of the phospholipids the more hydratable they are. Since phospholipids are emulsifying in nature, they form clusters with other valuable components in oil. Separation of these clusters by sedimentation / centrifugation or during subsequent refining stages will result in loss of quality (Jiang et al. 2015). They tend to decompose easily and cause dark colouration during heating. These molecules tend to cause serious foaming during frying due to the surfactant like properties and hence must be removed from oils. The ideal phospholipid content in oil should be less than 5mg/kg for any further refining stages (Yang et al. 2008).

2.3.1.1.Water degumming

Degumming was initially designed as a pre-treatment stage for physical refining of oils. High phospholipid content in soybean and canola oils render the process of degumming as an important step. The most conventional method of removal of phospholipids is water degumming, where the oil is treated with the addition of 1 to 3% of water at elevated temperatures after which it is centrifuged (Lamas et al. 2016). Reverse micelles are formed on the addition of water molecules to the oil due to the presence of hydrophilic head of phosphate group present in the phospholipids. The fatty acid forms the monolayers and bilayers over the reverse micelles due to the interaction between the fatty acids to the hydrophobic tails of the phospholipids (Li et al. 2016). The differences in the density of phospholipid cluster and the bulk oil makes it easy to centrifuge and separate the phospholipids as a pellet from the bulk oil. However, the percentage of water addition to the oil differ with the phospholipid content in the oil, as it is stated that the amount of water added should be equivalent to the amount of phospholipids initially present.

In a report involving degumming of rice bran oil, Central Composite Rotational Design (CCRD) was used to study the effect of water concentration and temperature (Engelmann et al. 2016). It was identified that higher temperatures of range 56 to 84°C showed a negative effect on the reduction of phospholipid content due to the solubilization of hydrated gums at higher temperature. It was registered that there was

a drastic reduction (99%) in phosphatidylcholine content within 5 minutes of contact time with water, whereas phosphatidic acid, phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine showed moderate reduction (Pan et al. 2000). This indicates the hydratable nature of phosphatidylcholine unlike other phospholipids. However, the kinetic extraction of phospholipids by water degumming indicated a contact time of 35 minutes for effective removal of HPs in sunflower oil. For oils with high phospholipid content like soybean oil, water degumming has the ability to produce an average quality of oil with a phosphorous content of 60-200 mg/kg (Saravanan et al. 2006). Such high phosphorous content even after degumming showed that water degumming did not reduce the phospholipid content which was due to the presence of NHPs which exist as salts of phosphatidic acid and phosphatidylethanolamine (Ghazani and Marangoni 2013).

2.3.1.2. Acid degumming

The phospholipase D influences the increase in the amount of NHPs in crude vegetable oil which in turn depends on the temperature, moisture levels in seeds and the cellular damage (Pan et al. 2000). These phosphatides form emulsions during further refining stages, leading to loss of oil. Since these compounds hinder the subsequent processing steps, it becomes essential to remove them. Acid degumming was resorted to for the successful removal of NHPs to avoid the disadvantages that the water degumming process posed. Acids that are commonly used for this purpose are phosphoric acid, citric acid, oxalic acid and tartaric acid, depending on the type and amount of NHPs present. They act by decomposing the salt/metal complexes of phospholipids, further allowing hydration by water (Zufarov et al. 2008). These NHPs become hydratable during association with monovalent ions from the acids and results in reverse micelle formation, which can be easily separated by centrifugation on addition of small quatities of water. In a study involving the comparison of different acids to identify the best acid for degumming of *Silybum marianum* seed oil, citric acid showed maximum efficiency (60%) in phospholipid reduction (Mei et al. 2013). It was also noted that 25 min and 35 min of optimal contact time is required to eliminate the NHPs by citric acid and phosphoric degumming of sunflower oil respectively (Pan et al. 2000). It was identified that the increasing concentrations of

acid increased the decomposition of NHPs based on the comparative acid strengths (Mei et al. 2013). Although several literatures state considerable oil loss in acid degumming, this method is used widely in the industries since it is cost-effective with successful utilisation of the waste (Greyt 2012). Acid degumming has become the usual practice in the U.S. soybean industry. A small amount (0.05%–0.2%) of concentrated phosphoric acid (75%) is added to warm oil (70°C) followed by stirring for 5–30 min and gums are separated either by centrifugation or filtration. Phosphoric acid is added to make the NHPs more hydratable by binding calcium and magnesium ions before adding water. Phosphoric acid pretreatment also partially removes chlorophyll from the oil (Akoh and Min 2008). Recently Gordon et al. (2015) have claimed efficient acid/water degumming by employing hydrodynamic cavitation processing (Patent: US 2015/0057460 A1)

2.3.1.3. Other conventional degumming technologies

A variation to this conventional process is the dry degumming method which is used for low phospholipid content oils like palm and coconut oils (Akoh and Min 2008). In this case the oil is treated with acid so that phospholipids agglomerate, after which they are removed during bleaching. In superdegumming process a strong solution of citric acid is added to oil at 70°C, and the mixture is stirred and cooled to 25°C to precondition the gums. Water is then added and stirred for an additional 3 h to hydrate the gums. This process causes the phosphatides to form liquid phospholipid crystals, which are easily removed during centrifugation (Akoh and Min 2008).

The efficiency of these conventional processes reduces with the increase in amount of NHPs (Jiang et al. 2015). While the standard phosphorous content that is allowed in an oil is <10ppm, the amount that is usually found after such conventional degumming is 15-80 mg/kg depending on the type and quality of the oil used (Yang et al. 2008). Several newer techniques are being employed for the degumming of crude oils due to the loss of oil during the further refining steps which is due to the presence of such high concentration of phospholipids.

2.3.1.4. Emerging technologies in edible oil degumming

Two different methods are pursued to eliminate phospholipids. One technique involves the use of semipermeable membranes to separate the phospholipids from bulk oil. This technique works on the property differences between the target molecules (phospholipids) and glycerides. The second method involves the decomposition of phospholipids using phospholipid specific hydrolases with the simultaneous removal of the undesired hydrolysis products.

2.3.1.4.1. Membrane degumming

Oil degumming is exposed to different varieties of microfiltration (MF) and ultrafiltration (UF) membranes. The separation of the oil is affected by the type of membrane material, pore size of the membrane, the components present in oil and the interaction of oil mixture with the membrane surface (Manjula and Subramanian 2006). The mechanism of separation using UF membrane is by molecular size exclusion whereas the non-porous membranes functions by reducing the phospholipids in the oil by the interaction between the solute and the membrane material (Coutinho et al. 2009). The available reports on the membrane degumming can be performed by passing crude solvent free oil oils or passing oil solvent mixture through the membranes for the purpose of degumming. In the industry, refining and handling of crude oil is considered easier than handling oil solvent mixture. Hence, studies using crude oils were conducted to evaluate the efficiency of membranes for degumming. Subramanian et al. (2004) in their studies claimed that there is no significant difference between the molecular mass of phospholipids and glycerides for efficient membrane application in degumming process. However, as phospholipids tend to possess emulsifying properties, they exist in the form of micelles without any need for external addition of surfactant molecules.

With this consideration, several reports involving direct membrane degumming of crude vegetable oil was performed (Subramanian and Nakajima 1997, Subramanian et al. 2001, Koris and Vatai 2002). The microfiltration of crude soyabean oil using polyethylene membranes could result in 12% reduction of phospholipid content indicating that the phospholipid micelle formed in the oil is smaller than the pore size of 0.01-0.03 μm (Subramanian and Nakajima 1997). Due to the inefficiency of

polyethylene membrane for the rejection of phospholipids, polymeric composite membrane was further tested for degumming of crude soybean and rapeseed oil to find 99.5% reduction in phospholipid levels (Subramanian and Nakajima 1997).

The drawback of performing degumming of crude oil using membranes was the drastic flux drop at the initial experimental stage, which was attributed to the pore blocking, concentration polarisation due to the deposition of the phospholipids on the membrane surface and cake formation by the rejected solids (Pagliero et al. 2005, DeSouza et al. 2008). Considering these limitations of the membrane efficiency, Ulusoy and team aimed at overcoming these challenges by altering the membrane surface properties of polyvinylidenefluoride (PVDF) membrane to degum crude soybean oil (Ulusoy et al. 2014). The altered membrane with improved surface properties showed a better phospholipid rejection from 58.9% to 75.5% (crude degumming) with the consistent maintenance of flux similar to the unmodified membrane. Although the efficiency of phospholipid reduction in membrane degumming of crude oil is industrially satisfactory, the low flux of the process made this technology energy intense. The degree of phospholipid removal in dry degumming ranges between 60-99%.

Since crude oil membrane degumming posed challenges with respect to flux and fouling, Wibisono and Widodo (2015) wanted to try the possibility of dry degumming of corn oil by developing a computational fluid dynamic (CFD) model to predict the thickness of concentration polarisation in tubular ceramic membrane. It was concluded from their model that increasing the Schmidt (Sc) number significantly reduces the retention layer thickness. Since Sc number is a dimensionless number based on the ratio of momentum viscosity and mass diffusivity, use of solvent to reduce the viscosity of the oil is considered the best approach. Hence, further approaches involving the use of oil solvent mixtures was carried out. The micelles formed on contact of oil with the solvent are known to be effectively separated by membrane process because of its molecular weight (20000 Da) (Manjula and Subramanian 2006, Ribeiro et al. 2008). Although the process is advantageous, the dependency of the efficiency of separation on critical micellar concentration (CMC) i.e. amount of solvent required for CMC becomes crucial. Hence, hexane is added to

crude oil to form micelles generally with 30-40% of oil in hexane (Coutinho et al. 2009).

Several polymeric membranes including polyethersulfone (PES), polyamide, (PVDF), polyimide membranes, ceramic membranes have been used for vegetable oil degumming and inorganic membranes for degumming hexane oil micelle (Ochoa et al. 2001, Garcia et al. 2006, Saravanan et al. 2006, Manjula and Subramanian 2009, Tres et al. 2010, Marenchino et al. 2006). A 99.3% of phospholipid reduction in soybean oil micelle was achieved by a laboratory cast PVDF membrane with a higher flux (Ochoa et al. 2001, Pagliero et al. 2004).

An interesting fact on challenges of membrane degumming is that, the behaviour of the membrane tends to vary depending on the type of oil used. For instance, corn oil and sunflower oil are known to contain higher wax content along with the presence tocopherols. Membranes to be used for this purpose should be conditioned to improve the membrane properties for maximum reduction (DeSouza et al. 2008). Similarly, for degumming of soybean oil with high phospholipid content, multiple stages of membrane filtration can be considered to improve the efficiency of the process. In general, polyamide and PVDF and ceramic membranes were found to be more stable than PES membranes. Table 2.5 presents a summary of various membranes used for the reduction in phospholipids.

Table 2.5: Different membranes used for degumming of vegetable oils

Type of oil	Membrane Used	Phospholipid content (mg/kg)		References
		Before	After	
Soybean oil/hexane micelle	Polyvinylidefluoride (PVDF)	1200	8	Ochoa et al. 2001
Neutralised (5%) sunflower oil	Microporous cellulose membrane	326	2	Pioch et al. 1998
Neutralised (20%) Sunflower oil	Microporous cellulose membrane	67	1.2	Hafidi et al. 2005
25% soybean oil / hexane micelle	Polyvinylidefluoride (PVDF)	1297	1.6	Pagliero et al. 2005

Type of oil	Membrane Used	Phospholipid content (mg/kg)		References
		Before	After	
25% Soybean oil/hexane micelle	Polyethersulfone	178	18	Moura et al. 2005
Crude rice bran oil/hexane micelle	Polyimide	499	18	Saravanan et al. 2006
Rice bran oil/ hexane micelle	Ceramic membrane	322	16.1	Subrahmanyam et al. 2006
25% soybean oil/hexane micelle	Inorganic membrane (15Kda)	-	(95% reduction)	Marenchino et al. 2006
30% Sunflower oil/hexane micelle	Polyethersulfone	120	6	Garcia et al. 2006
35% Corn Oil/hexane micelle	Ceramic membrane (0.05 µm)	350	23	DeSouza et al. 2008
32% Soybean oil/hexane micelle	Ceramic membrane	803	2.2	Ribeiro et al. 2008

(Adapted from Belur et al. 2017)

Several reports on the reduction of FFA content and some amount of pigment removal along with phospholipid reduction in several vegetable oils (Subramanian et al. 2004, Garcia et al. 2006) have been documented. Although the flux oil –hexane mixture (micelle degumming) through the membrane process is high, membranes that shows good stability in hexane is limited and hence the literature on degumming oil-hexane micelle is rather inadequate.

The main disadvantage of membrane degumming of crude oil is the low permeate flux, and the poor stability of membranes in the presence of solvent. However, several studies are continuing to evolve (Ulusoy et al. 2014), which aims at addressing these challenges. With this accumulating knowledge on the membrane based degumming of oil, few industries like ADM and Cargill have commercialised this particular operation (Ulusoy et al. 2014). However, in order to employ membrane application to

a broad variety of edible oil degumming at industrial level, development of newer membranes is needed to address the shortcomings of these processes.

2.3.1.4.2. Enzyme degumming

Phospholipase is generally the enzyme that is used for the enzymatic degumming of edible oils. This was first reported by Roehm and Lurgi regarding the Lurgi's EnzyMax process (Mei et al. 2013). In this study, degumming was carried out in three steps namely, pH adjustment of the oil followed by the addition of appropriate enzyme concentration and gum separation from oil in the same sequential manner. The advantage of employing phospholipase mediated degumming is that it removes both HPs and NHPs in a single step. Phospholipases A_1, A_2, B, C and D are the different enzymes used for degumming, with phospholipase A_1 being the most common and widely studied enzyme for this application. All these enzymes change the nature of phospholipids and results in the reduction of the emulsifying properties.

The mechanism of degumming exhibited by each of the enzymes is different. Phospholipase A_1 (PLA_1) act by hydrolyzing the *Sn*-1 ester bond in phospholipids with the release of lysophosphatidylcholine acid and fatty acid. The hydrophilic nature of the released choline acid makes it easy for its removal. Whereas PLA_2 enzyme catalyzes the hydrolysis of *Sn*-2 ester bond, producing *Sn*-1-lysophosphatidylcholine acid and fatty acid. While other phospholipases catalyze the hydrolysis and lead to release of fatty acid, phospholipase C (PLC) hydrolyze the bond between acylglycerols and phosphate group, resulting in the liberation of diacylglycerol (Qu et al. 2016). Likewise, phospholipase B (PLB) catalyze the hydrolytic cleavage of two acyl groups resulting in phosphoglycerate, which is considered more hydrophilic than the lysophospholipids produced by other enzymes (Jiang et al. 2011). A pictorial representation of the mechanism by which different phospholipases act is shown in Figure 2.3.

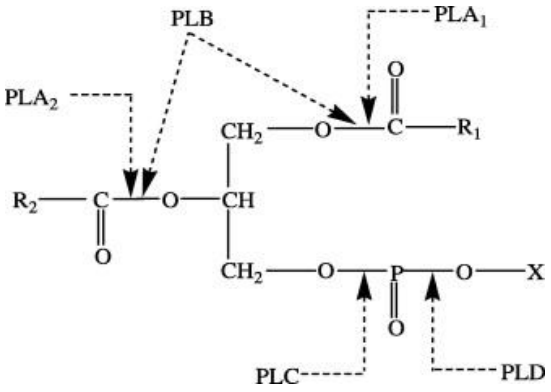


Figure 2.3: Mechanism of action of different types of phospholipases (Adapted from Jiang et al. 2011).

PLA_1 and PLA_2 are also shown to increase the FFA content in oil during hydrolysis by cleaving acyl bonds. Theoretically an increase in 0.036% of FFA concentration can be expected from the hydrolysis of 0.1% phospholipids (Greyt 2012). But the same phenomena cannot be seen during the hydrolysis using PLC. PLC is known to cleave the phosphorous and oxygen bond releasing diglyceride and phosphate ester and hence does not result in the release of FFA (Jiang et al. 2014b). Hence, PLC can be considered for degumming sterol rich oils. Also, degumming sunflower oil with PLA_1 showed a significant reduction in iron and copper content (Lamas et al. 2016). This could extend its use for degumming oil with high metal content. PLD leads to the formation of phosphatidic acid which inturn results in the increase in the NHP in the oil. This increase in the NHP is not desirable since it increases the degumming steps further and also leads to the oxidative deterioration of the oil in a much fater rate (Kock 1981). This is the reason for the reduced usage of PLD in the degumming of oils (Gunstone et al. 1994).

The problem with all these enzymes is their inherent activity towards both phospholipids and triglycerides. Jiang et al. (2014b) recommended the use of higher temperatures above 40°C in order to partially suppress the lipase activity so that the phospholipase activity predominates. The use of high reaction temperatures not only increased the phospholipase activity but also reduced the viscosity of the reaction components thus enabling the contact of enzyme at the interface to a large extent. However, with the further increase of the reaction temperature, low activity due to the poor stability of enzymes was witnessed.

It is evident that all the enzymes used for degumming act by hydrolyzing the acyl bonds of phospholipids, converting them to more hydratable forms. After enzymatic treatment these phospholipids are easily removed by centrifugation. The phospholipase catalyzed degumming occurs at the interface between the aqueous phase with enzyme and the oil phase. Thus increasing the interfacial area might result in increase in rate of reaction, thereby reducing the time taken for degumming. For attaining better degumming results, use of ultrasound technology can be considered for improving the interfacial area and reaction time. The bubbles that are generated due to the effect of ultrasound, collapse near the boundary of two immiscible liquids and the resultant shock wave results in efficient mixing and enhancement of heterogenous reactions (Jiang et al. 2014a). It was also identified that the reaction rate reached its maximum, within 2 hours of reaction with reduction in phospholipids to less than 10mg/kg in rapeseed oil. The kinetics of the phospholipid decomposition during the course of enzymatic degumming revealed that PLA_1 significantly reduced the phosphatidylcholine content to glycerophosphophorylcholine and phosphatidylethanolamine to glycerophosphoethanolamine indicating a possibility of acyl migration which further substantiates the increase in FFA (Jiang et al. 2014b).

In spite of the advantages posed by the enzymes for degumming, industrial use of phospholipase is still low due to high enzyme cost and the inability to efficiently separate and reuse the enzyme. Also, the long reaction time of 4.5 to 6 hours is a major drawback at large scale degumming. To avoid this problem, recombinant enzymes are being studied for decreasing the reaction time and increasing efficiency (Huang et al. 2014). Another possible solution to this problem is the use of immobilized phospholipase, as they are known to show excellent stability and reusability, ultimately reducing the operating costs. Further, immobilized enzymes show more stability during storage and reaction, due to which they are known to exhibit better catalysis. However, industrial separation of immobilized enzyme from the reaction medium by centrifugation and filtration is considered energy intensive and difficult. Most recent developments taken place in enzyme degumming is compiled in Table 2.6.

Table 2.6: Recent studies on enzymatic degumming using different free and immobilized phospholipases

Enzymes used	Oil used for degumming	Immobilization material	Phospholipid content (mg/Kg)			References
			Before degumming	Degumming with free enzyme	Degumming with immobilized enzyme	
Phospholipase A_2	Water degummed soybean oil	Magnetic particles cross-linked with sodium alginate Magnetic chitosan microparticles	142.2	-	14.1 15.6	Qu et al. 2016
Phospholipase A_1	Water degummed soybean oil	Magnetic Fe3O4/SiOx-g-P(GMA) nanoparticles	138	7.0	9.0	Yu et al. 2013
Phospholipase A_1	Crude soybean oil	Lecthin bioimprinted PLA_1 immobilized on diatomite	406.9	62.9	7.3	Li et al. 2016
Phospholipase A1	Crude rice bran oil		390		10	Manjula et al. 2010
Phospholipase A_1	Sunflower oil	Gelatine hydrogel	400	-	50	Sheelu et al. 2008
Phospholipase B	Water and acid	-			-	Jiang et al. 2011

Enzymes used	Oil used for degumming	Immobilization material	Phospholipid content (mg/Kg)			References
			Before degumming	Degumming with free enzyme	Degumming with immobilized enzyme	
	degummed Soybean oil		90.1	7.3		
	Rapeseed oil		83.6	5.7		
	Peanut oil		63.4	3.0		
Phospholipase A_1 Phospholipase C	Water and acid degummed soybean oil	-	28.7	<10 18.2	-	Jiang et al. 2014b
Phospholipase A_1	Crude sunflower oil		474.7	2.61	-	Lamas et al. 2016

(Adapted from Belur et al. 2017)

Thus magnetic nanoparticles are being used as alternative, because of their ease of separation from reaction medium under magnetic field (Yu et al. 2013). Nano polymers are being widely used for immobilization of enzymes. In a study, the immobilization of PLA_2 enzyme registered a considerable broadening of the pH and temperature stability of an immobilized enzyme than its free form. It was also noted that the FFA content was reduced after degumming with immobilized PLA_2 with a 0.35% increase in refining efficiency (Qu et al. 2016). All the available literature on these new approaches is employed specifically for vegetable oil. However, no literature is available on enzymatic degumming of fish oil. Due to mild processing conditions, enzymatic degumming looks highly promising for fish oil as well.

Since degumming stage do not involve very high temperatures and harsh chemicals, the inherent essential components in oils are not affected extensively in comparison

with other processing steps. Also, Ortego-Garcia and team found that degumming of vegetable oil by conventional acid degumming retained all the essential fatty acids along with negligible loss of tocopherols (Ortego-Garcia et al. 2006). However, no reports are available on the effect of degumming on marine oils and the n-3 PUFA content.

2.3.2. Deacidification of oil

Deacidification, as the name suggests, involves the removal of the acidic component in the oil like the FFA along with the phospholipids components which were not separated during degumming together with dirt and other impurities that are saponifiable. This step is thought to be the most difficult and delicate in the refining of oil due to its economic impact on refining. The need for this process is due to the susceptibility of FFA to oxidation leading to rancidity. FFA is found to be one of the major factors triggering hydrolytic and oxidative deterioration (Paradiso et al. 2010). Thus, the presence of FFA in the oil must be avoided as much as possible. Several methods like chemical, physical, micella refining have been employed in industy. Also, new approaches like biological deacidification, chemical reesterfication, and supercritical fluid extraction, membrane processing and liquid-liquid extraction (solvent extraction) are also being explored for deacidification of oils (Belur et al. 2017).

2.3.2.1. Alkali neutralization of oils

Alkaline neutralization involves the reaction between FFA and an alkaline solution like sodium hydroxide/ calcium hydroxide/ potassium hydroxides. Sodium hydroxides tend to saponify the triglycerides as they are highly concentrated in turn leading to the loss of oil. Hence, it is used in conjugation with sodium carbonate or is diluted using hot water (Gunstone et al. 1994). Caustic soda and oil are agitated intensively and passed into a disc separator which removes the soap from the oil. Following this process, washing of the mixture is performed at high temperatures, high mixing intensities for a long duration of contact to attain low residual soap content. Hot water, generally hotter than oil is used for this purpose. Finally, the washed oils are

sent for vacuum drying to remove the moisture from the oil. Use of sodium bicarbonate, sodium carbonate and sodium hydroxide for rice bran oil neutralization was explored at 150°C-210°C at 2-4 mm Hg pressure. The results showed that all the alkali gave almost comparable results though the oil recovery was highest in case of calcium hydroxide (De and Patel 2011). Use of perforated plates to increase the contact between alkali and FFA reduced the oil loss (Tang et al. 2013). Use of nano reactors has been a recent advancement in the alkali treatment wherein the oil is mixed with alkali under a high pressure of 40-80 bars which provides efficient neutralization (Vaisali et al. 2015). It is observed that the FFA content in the oil has a direct bearing on the oil loss which in turn affects the yield during the refining process such as in the case of marine oils and rice bran oil (Vaisali et al. 2015, Bhosle and Subramanian 2005). One of the major disadvantages of alkali refining is the loss of glycerides due to saponification. The loss of valuable nutrients such tocopherols and carotenes is another concern. In spite of these disadvantages, this technique is used by many industries due to the successful reduction in FFA to acceptable levels. To combat the disadvantages faced by alkali refining, physical and micella refining are adopted by the industries.

2.3.2.2. Physical refining

Physical refining makes use of steam to strip the FFA, impurities and unsaponifiable matters from the oil under vacuum. Here the removal of FFA proceeds with the attack of water at the carbonyl centre of the FFA. The procedure has shown acceptable results only when the initial quality of the oils was good. Pre-treated oils are further subjected to steam refining. The pre-treatments involved in this process is very stringent and removes certain non-volatile impurities while the volatiles and other trace impurities are removed by steam distillation. This process is very similar to deodorization and hence both neutralization and deodorization can be performed in a single step as the required equipment for both the processes is the steam distillator. The operating conditions are designed in a way that FFA and other odorous substances are simultaneously removed. Though this process results in very good quality oil with low FFA contents, less oil losses without the release of soapstocks, it also possesses several disadvantages like high operating temperatures ranging

between 240°C to 270°C, large operating times and high operating costs due to the energy for steam production and distillation. This technique cannot be used for those oils which are heat sensitive such as the cotton seed oil (Bhosle and Subramanian 2005). The use of high temperatures results in the formation of side reaction products and trans isomers (Fournier et al. 2007). One of the reports on physical deacidification, states that there was a reduction in the tocopherol content and carotenes in the palm oil (Ooi et al. 1996). Another study by Ghazani and Marangoni (2013) found that physical refining cannot be used for canola oil since the linolenic acid was converted into trans isomers at 240°C and nearly 27.5% of tocopherol in the oil was lost. Cvengros (1995) performed physical refining of edible oils such as rapeseed oil and sunflower oils where the acid number was reduced to 0.09 mg KOH/g from 1.98 mg KOH/g and 0.08 mg KOH/g from 0.98 mg KOH/g for the oils respectively. In a study on physical refining of grape seed oil using molecular distillation at 220°C at high feed flowrates of 1.5ml/min resulted in oils having 0.1% of FFA and a high recovery of tocopherol content. (Martinello et al. 2007). Physical refining of Sardine oil is being practiced with an operating temperature of 170°C to 180°C, in several fish meal industries in India (Personal communication). The oil produced is consistent with the market requirement, without appreciable loss of n-3 PUFA and minimal *cis- trans* isomerization.

2.3.2.3. Micella refining

This refining technique was developed commercially for the processing of cottonseed oil to remove a toxic pigment called gossypol (Bhosle and Subramanian 2005) though it was used in the refining of soyabean, sunflower, palm, coconut and tallow oils. In this process the oil is initially mixed with the solvent (hexane) in the holding tank for a while before it is contacted with a necessary quantity of caustic soda. The crude micelle caustic mixture is then mixed for some time to allow efficient contact between caustic soda and the micelle. This mixture is centrifuged at suitable temperature to obtain a light coloured refined micella which is separated from the soap stock by centrifugation. The main purpose of adding hexane to the oil is to reduce the viscosity of the oil and to enhance the density difference between oil and soap phase, which in turn improves separation efficiency and reduces oil loss.

Though this technique offers several advantages like the use of dilute caustic soda, reduced energy requirements and neutral oil loss for the production of refined oil with excellent color properties, it is not used widely in the industries due to higher fixed capital requirement to procure expansive explosion proof equipment and high-shear mixers. A laboratory scale micella refining with cotton seed oil was performed with crude oil of varying FFA contents to which 2.5 parts of sodium hydroxide was added. The test was conducted at room temperature and resulted in the reduction in the colour of crude oils comparable to the commercially refined oils (Wan et al. 1996).

The conventional deacidification methods were not suited for oils which contained high quantities of FFA and resulted in large neutral oil losses (Bhosle and Subramanian 2005). Since these conventional techniques pose disadvantages in the deacidification of oil, the search for new approaches in deacidification are gaining a lot of interest.

2.3.2.4. Deacidification by supercritical fluid extraction

In the recent years, two different approaches are being followed to reduce FFA content in oil. First approach employed was supercritical fluid extraction, solvents and membrane processes to remove FFA from oil and secondly, by using chemical catalysts / enzymes to convert FFA to ester form by reesterfication with added glycerol.

Preferential selectivity shown by supercritical fluid towards FFA present in oil is exploited to separate FFA from oil due the differences in the smaller molecular weights of FFA. The underlying mechanism of supercritical fluid assisted deacidification involves the use of supercritical fluids as extracting solvents such as carbon dioxide at temperatures and pressures above the critical point of the solvent. In the SCF state, the solvents exhibit the properties which are intermediate between the liquid and the gaseous states. Unique feature of solvent capacity of SCF solvents lies in the sharp variability of density with pressure and temperature. At this stage they impart high solvent power, good transport properties, good diffusivity and nil surface tension. By far carbon dioxide is used as SCF solvent because it is cheap, nontoxic

and non-flammable, available in abundance with high purity. The most frequently used supercritical fluid is carbon dioxide which is modified by co solvents such as methanol or ethanol. These co solvents have volatility that is intermediate to SCF solvent and FFA without changing the properties of density and compressibility of SCF solvent (Mukhopadhyay 2000). Some of the recent reports on deacidification of oil are as mentioned here for vegetable and marine oils containing high quantities of FFA. Chen et al. (2008) have reported the deacidification of rice bran oil at 250 bar and 353K, achieving 97.8% reduction in FFA using super critical CO_2. Rodriguez et al. (2012) have reported the extraction of FFA and other impurities including arsenic from Salmon oil, without decomposing n-3 PUFA glycerides by carrying out supercritical CO_2 extraction at 25MPa and 313K.

This process is highly advantageous since it involves simultaneous extraction and fractionation of the oil along with the elimination of additional steps for the removal of FFA and other impurities. The advantage presented by the process makes it to be used specifically for removing the high quantities of FFA along with off flavours that is produced in large quantities in marine oils. However, due to cost considerations, this process could be applied to only those oils where higher cost of production is justified.

2.3.2.5. Deacidification by solvent extraction

Difference in solubility of polar FFA in polar solvents as against non-polar glycerides is exploited for the effective removal of FFA from the oil. This process is one of the critical steps in refining which depends on the selection of appropriate solvents for the extraction of various molecules in the oil. Various studies suggest that the use of short chain alcohols as solvents offers efficient extraction of FFA. Long chain alcohols are not generally preferred due to the fact that an increase in the chain length of the alcohols causes it to become hydrophobic in nature which results in an improper separation of FFA from the oil phase (Rodrigues et al. 2007). Ethanol, methanol and acetone have been recommended as solvents for extraction of FFA for deacidification of vegetable oils (Turkay and Civelekoglu 1991). Most of the reports suggest that deacidification with such solvents could be only partially successful, with the

concomitant loss of neutral oil. Thus one more step of deacidification may be necessary to eliminate FFA from vegetable oils completely. Hamm (1992) has claimed efficient solvent deacidification of fish oil to enrich the EPA and DHA using short chain alcohols. Very few reports on the solvent deacidification of marine oils have been reported because it suffers from a drawback that the traces of solvents have to be removed from the oil which is tedious, energy intensive and takes place at high temperatures which could affect the sensitive nutritive components of the oil. Hence, the stability of the various nutraceutically important components in the oil has to be studied before subjecting the oil to various refining methods. Table 2.7 summerizes recent studies performed on deacidification of vegetable oils using various solvents.

Table 2.7: Deacidification of edible oils by solvent extraction

Oils	Solvents used	Conditions	FFA reduction mass (%)	Research highlights	References
Palm oil	Ethanol	Oil/solvent =0.74 Water content=6 mass%. Extraction done using perforated rotating disc contactor (PRDC).	97.36	Low losses of neutral oil and carotenoids preservation.	Goncalves et al. 2016
Soya bean oil	Ethanol	Extraction at different temperature of 40, 50, 60° C and hydration levels (0 and 5.98 mass % water).	-	Increasing the hydration level in ethanol increases the FFA extraction while it suppresses the soybean oil extraction.	Toda et al. 2015.

Oils	Solvents used	Conditions	FFA reduction mass (%)	Research highlights	References
Rice bran oil	Ethanol	Continuous solvent extraction using PRDC	100	Low losses in neutral oil, tocopherols and γ-oryzanol	Rodrigues et al. 2014
Macuba pulp oil	Ethanol/water	Oil/solvent =1:2 4% of water content in ethanol	31.81	Good portioning between TAG and FFA content in the oil	Mariano et al. 2011

(Adapted from Belur et al. 2017)

2.3.2.6. Deacidification by membrane processes

This process works on the principle based on the size exclusion mechanism of FFA and triglycerides present in the oil. However, this technique shows ineffectiveness in deacidification of FFA from oil due to the minute molecular weight differences between FFA and triglycerides. Further complications in the use of membranes arise when there is an insufficient separation of trace amounts of FFA from the bulk. To address these issues, membranes with precise selectivity towards FFA are highly preferred. As a result, development of the membranes which provide strong interactions with FFA molecules becomes a prerequisite.

Several attempts have been made with membrane technology such as subjecting the oil to direct deacidification with solvents (hexane and acetone) and without solvents, pretreating the oil with ammonia, sodium hydroxide and a combination sodium hydroxide and isopropanol before passing this pre-treated oil through membrane. These attempts led to a lot of limitations such as improper separation between FFA and triglycerides due to the absence of suitable membranes, partial deacidification and very low permeate flux. So, efforts are being made by researchers to combine membrane technology with solvent extraction. In this method, appropriate solvents like the polar solvents were chosen to reduce the viscosity of the oil and simultaneously dissolve the FFA (polar in nature). The phase separated oil was passed

through a membrane which selectively separates the FFA along with the solvent to obtain the oil rich in triglycerides.

Since FFA has a carboxyl group, membranes made up of hydroxyl groups could show high selectivity and affinity for FFA in the oil due to the hydroxyl and carboxyl group interactions. Also, Raman et al. (1994) proposed the ideal use of hydrophobic membranes to effectively separate FFA from triglycerides. The use of hydrophobic membranes in deacidification of FFA from oil/ solvent mixture is highly recommended probably due to its non-reactive nature to any of the solvents. Moreover, polar solvents have lower fluxes through hydrophobic membrane compared to hydrophilic membranes which facilitates not only the efficient separation of FFA along with the solvents (retentate) from triglycerides in the oil (permeate) (Bhanushali et al. 2001), but also helps in the reduction of solvent content in the permeate. This excludes the need for the tedious and cost intensive step adopted for the removal of solvents during solvent mediated deacidification of oil. Another advantage is the losses of neutral oil is minimal during the separation since the membrane processes are pressure driven which drives the oil from the solvent phase due the intervention of hydrophobic membrane.

Raman et al. (1996) extracted FFA from a model vegetable oil using methanol, and employed NF membrane for the separation of FFA from methanol with a high rejection of more than 90% and flux higher than 25 $L/m^2.h$. Krishna Kumar and Bhowmick (1996) treated the mixtures of triacylglycerols and FFA with alcohol using both cellulosic and noncellulosic type membranes. Polyamide membranes showed better selectivity towards fatty acid separation than cellulose and polysulphone membranes. Kale et al. (1999) studied deacidification of crude RBO by extracting with methanol followed by membrane separation. At the optimal ratio of 1.8:1 methanol/oil, the concentration of FFA in the crude RBO was reduced from 16.5% to 3.7%. A second extraction at 1:1 ratio reduced FFA in the oil to 0.33%. Similar studies were conducted on deacidification of Indian Sardine oil by using methanol as the extracting solvent in the presence of a hydrophobic PTFE membrane. This membrane in the presence of polar solvent resulted in 50% rejection in the two stages of deacidification with a less oil loss and reduced solvent traces in the deacidified oil

(Charanyaa et al. 2017). A recent study by Azmi et al. (2015) proposed the use of PVDF membrane which is hydrophobic in nature to remove the FFA from the crude palm oil. Further modifications to the membrane were made by crosslinking the membrane with PVA in the presence of glutaraldehyde as the crosslinking agent. It was found that 100 ppm of PVA on PVDF membrane resulted in 5.93% rejection of FFA along with the reduction of phosphorus content and colour intensity after 3 hours of operation. This study provides a platform to modify the properties of the membranes to best suit the requirement.

2.3.2.7. Reesterification using chemical catalysts

Chemical catalyst is used to catalyze re-esterification of FFA present in the oil with the added glycerol or any glycerides which has free hydroxyl group/s. The reaction leads to the formation of monoglycerides, diglycerides and triglycerides with the generation of water molecules. Liberated water generally tends to establish equilibrium with the reactants and hence should be removed by maintaining the reaction mixture under vacuum or under the influence of the inert gas. The main factors which affect the process are reaction temperature, quantity and type of the catalyst and the amount of glycerol (Kombe et al. 2013a). Various catalysts such as zinc dust, zinc chloride and naphthalene-beta-sulphonic acid are used. Ebewele et al. (2010), reported successful deacidification of rice bran oil containing 37.96% of FFA, by esterifying with 4.3% glycerol in the presence of zinc dust at a temperature of 200°C. The FFA content reduced to 1.5%, while the triglycerides increased from 40.32% to 80.62%. Though this process is feasible, the use of high temperatures, large quantities of glycerol and chemical catalysts could raise safety concerns especially for the production of oils for pharmaceutical purposes also making it a costly process. Also, the use of high temperatures cannot be employed for oils which are heat sensitive (Fournier et al. 2007). Deacidification of high acid rice bran oil by reesterification with monoglyceride under 10mm Hg pressure at 210°C has also been reported (De and Bhattacharyya 1999). Couple of reports are available on the efforts made to perform the reesterfication process in low temperature conditions. Kombe (2015) studied the deacidification of castor oil at low temperatures of 56°C for 85 minutes in the presence of 2.34 g/g glycerol to oil mass ratio. This method caused a

reduction in FFA of the oil from 6.50% to 0.06%. Under these optimized conditions the reesterfication efficiency was found to be 99.01%.

2.3.2.8. Enzymatic reesterification

The unique ability of some microbial lipases to synthesize a glyceride from FFA and glycerol has been exploited to deacidify vegetable oils. It is a catalytic reaction that occurs when the enzymes facilitate the rearrangement of the FFA on the glycerol backbone of triglycerides. This rearrangement in turn provides the structure and functions to the glycerides so formed at the room temperature. In view of the need for low-energy processes, microbial lipase-catalyzed esterification appears to be more advantageous for deacidification than chemical catalyst mediated esterification, which is invariably carried out at higher temperatures (180–200°C) (Bhosle and Subramanian 2005). There are quite a few reports on successful practice of lipases for deacidification in the lab scale. Bhattacharyya and Bhattacharyya (1989) successfully brought down the FFA content of rice bran oil from 30 to 3.6% by esterification of the FFA with added glycerol, using a 1,3-specific *Mucor miehei* lipase. Sengupta and Bhattacharyya (1996) have reported successful deacidification of rice bran oil having 5-17% FFA using *Mucor miehei* lipase. They also showed that the FFA of mohua oil was reduced from 24.5% to 3.8% when treated with 10% *Mucor miehei* at 60°C for 20 hours under a pressure of 267Pa in the presence of glycerols. It was proposed by Sengupta and Bhattachayya (1996) that re-esterification using monoglycerides instead of glycerol to reduce the FFA content of rice bran oil containing 8.6%-16.9% to 2-4%. Diglycerides were also used for the esterification of crude palm oil in the presence of *Pseudomonas fluorescens* (Kurashige,1988). Synthesis of acylglycerols from a reaction between glycerol and n-3 fatty acid concentrate from sea blubber oil with seven different lipases were studied and lipase from *Chromobacterium viscosum* showed the highest activity (He and Shahidi 1997). Makasci et al. (1996) reported superior performance of *Candida* sp. lipase immobilized on macroporous acrylic resin in deacidification of degummed and dewaxed hyperacidic olive oil, under reduced pressure. Enzymatic deacidification yield neutral glycerides which is an advantage. However, due to long process duration and high cost of enzyme, the process is yet to draw attention of oil industries.

It is observed that chemical, physical and miscella deacidification methods followed in the industries have several drawbacks. Newer approaches either independently or in combination with the present techniques could overcome many of the drawbacks. Among the various deacidification techniques mentioned above, membrane assisted solvent deacidification seems like an interesting alternative from technical point of view because of the low loss of neutral oil and nutraceutical compounds and the efficient removal of FFA with the selection of appropriate solvents like ethanol. However, an in depth analysis of final quality of the oil in terms of FFA content and the presence of traces of solvent in the deacidified oil must be investigated along with the economic analysis for the industrial purposes.

2.3.3. Bleaching

Bleaching is a mass transfer operation that generally is performed after degumming and neutralisation stages in order to remove the traces of detrimental compounds like metal ions, pigments, oxidised glycerides (Pohndorf et al. 2016, Acquah et al. 2016). The overall quality of the oil is known to improve during bleaching as some of the acid complexed phospholipids from the degumming step and residual FFA get adsorbed on the bleaching agent and is removed during filtration (Dijkstra 2013). The aesthetic appearance of oil is also improved in this stage by removing the colour implicating substances in vegetable oil. The main colour contributing pigments in vegetable oils are the carotenoids and chlorophyll. β-carotene, a type of carotenoid, is abundantly found in vegetable oils. Even though they are known to impart oxidative stability to oil, they are removed to improve the visual appearance for consumers (Pohndorf et al. 2016). However, it is essential to remove other pigments like chlorophyll as they tend to decompose to render oil which is dark and opaque.

Bleaching generally follows the adsorption process in the presence of adsorbents like clays or bleaching earths. Adsorption transpires through a series of complex interactions between the bleaching clay and the impurities. It can be a simple physical interaction, where impurities get trapped inside the pores of clay due to the effect of Van der Waals forces (Sabah et al. 2007), or it could be due to the chemical reaction between the bleaching agent and particles occurring via ionic bonds (Ahmad et al. 2009, Kuuluvainen et al. 2015, Aachary et al. 2016). Four different adsorbents are

generally used for the successful bleaching of oil. They are active carbons, naturally active clays/earths, activated clays and other common adsorbents like silica gel or alumina powder. For bleaching of vegetable oils, clays like bentonite, sepiolite, kaolinite, attapulgite and palugorskite are specifically used (Rossi et al. 2003, Sabah et al. 2007, Liu et al. 2008, Aung et al. 2015).

Bleaching earth is the most common and frequently used adsorbent for refining many edible oils due to its low cost. But the drawback of using this adsorbent is that it results in 20-40% loss of oil due to absorption by bleaching earth. Another problem encountered is the adverse environmental impacts due to the disposal of spent bleaching earth (Loh et al. 2013). To overcome this issue, several recent studies involving application of new bleaching agents like oil palm ash (Acquah et al. 2016), use of ultrasound treatment along with bleaching clays (Abedi et al. 2015, Aachary et al. 2016) and use of high voltage electric field (Abedi et al. 2016) have evolved. Apart from the type adsorbent, the various attributes of bleaching agent that affect the efficiency of the process are porosity and capillary structure, particle size distribution of clay material and acidity of the clay material. Although activated bleaching earth clays still have the highest market share, oil refiners are showing a growing interest in nonactivated bleaching earths, especially for the bleaching of palm oil (Hamm et al. 2013). The main reason is the possible catalytic effect of highly activated bleaching earths on the formation of potentially toxic 3-monochloropropane-diol (3-MCPD) esters during palm oil refining (Greyt 2012).

The effectiveness of colour reduction in oil and the acidity of the bleaching agent used go hand in hand. The acid activated adsorbent has the ability to act as a solid support, catalyst, adsorbent, and filter aid unlike neutral bleaching agent that acts only as adsorbents (Silva et al. 2013). Since, the remnant FFA removal during bleaching determines the quality of the end product; many studies involving the acidity and load of adsorbent on the adsorption efficiency of FFA were studies along with time of contact, temperature and water content. A kinetic study conducted by Lin and Lin (2005) on FFA reduction as affected by these parameters indicated a reduction for the initial 15 min of reaction after which it increased. This could be attributed to the hydrolysis of glyceride by the acid on adsorbent or by the adsorbent itself. Another

possible reason could be due to the soap decomposition by the sodium ions associated. It was also noted that the adsorption of FFA by regenerated clay was totally different from the adsorption of β-carotene. For majority of oils, the Langmuir isotherm and Freundlich isotherm were found to better interpret the effects of these parameters on β-carotene adsorption.

Although majority of the literatures of carotenoids adsorption suggested best fit with Langmuir and Freundlich isotherms, several other models were also proposed due to the variation in the type of oil and adsorbent. In the process of understanding the kinetic and thermodynamic adsorption of pigments during rice bran oil refining, it was identified that adsorption kinetics followed pseudo first order and pseudo second order model, especially for bleaching at higher temperatures and to some extent followed Elovich model for adosption of carotenoids (Silva et al. 2013, Pohndorf et al. 2016).

The clays are usually acid activated using hydrochloric and sulphuric acid to improve their adsorption capacity. Though acid activation leads to increase in the adsorption efficiency, there is a possibility of change in the crystalline structure resulting by the formation of amorphous silica. The change in the structure leads to occurrence degradation reactions including isomerisation of PUFA and decomposition of several other unsaturated fatty acids and sterols. To avoid this particular drawback a recent study has successfully used ultrasound technique to activate the bleaching clay for the refining of olive oil. It was noted that this technique effectively reduced the undesirable components with reduced oil loss and successful preservation of glycerides in oil (Essid et al. 2016).

An understanding of the effect of temperature on bleaching efficiency revealed that use of low temperatures below 60°C failed to show significant reduction in the colouring pigments. Though high temperatures guarantee the refining efficiency, unfortunately it also results in the removal of most of the bioactive compounds. For instance, it was noted that the bleaching efficiency of degummed and neutralised tea tree oil at temperature above 85°C significantly increased pigments removal. Similarly, in a study involving the refining of Indian Sardine oil, bleaching beyond

80°C led to an increase in the acidity of the oil with a reduction in the iodine value (Charanyaa et al. 2017). Furthermore, on analysing the bioactive compounds such as sterols, tocopherols, squalene and vitamin, a drastic decrease were registered (Wei et al. 2015).

Another major parameter that influences the adsorption capacity is the particle size of the adsorbent. However, literature regarding this is inconsistent, as some reports suggests smaller particle size with low porosity increases oil-particle interactions (Girgis 2005), while some reports suggest the need for larges pores to increase adsorption and retention capacities (Acquah et al. 2016). The next critical parameter that determines the bleaching efficiency is the adsorbent dosage. Generally, there is an increase in colour reduction with increase in adsorption load due to the availability of larger surface for interaction. This observation seems to contradict with the study conducted by Charanyaa et al. (2017); an increase in the adsorbent dosage increased the intensity of colour of the oil along with the increase in FFA. After a particular load, the efficiency in bleaching remains unchanged due to the adsorption equilibration between adsorbent and oil (Acquah et al. 2016).

Removal of interfering components from the oil (e.g. phospholipids) prior to effective bleaching can reduce bleaching earth consumption. This can be achieved by adapting prefiltration with silica hydrogels. Silica hydrogels (also named ‘silica’) are free-flowing white powders consisting of silicon dioxide (all or not acid activated) and high amounts of water (50–65%). These adsorbents have little or no affinity for colour pigments present in oil but are very efficient for the removal of polar impurities like phospholipids and left over soaps coming from alkali neutralization stage (Hamm et al. 2013).

Another adsorbent that found extensive usage is activated carbon, processed at very high temperature (up to 1000°C). Traditionally it was used for colour removal, but due to its high oil retention property, bleaching earth has replaced it in most of the oil refineries. Today, activated carbon is mainly used for the removal of heavy polycyclic aromatic hydrocarbons (PAHs) from vegetable oils (Kemeny et al. 2011). At the end of the 1990s, it was also introduced in fish oil refining for the adsorption of dioxins

and polychlorinated biphenyls (Maes et al. 2005). Typical dosing for the removal of dioxin/PCB from fish oil is 1–3 kg/ton, while for the removal of PAH from coconut oil it can be 5 kg/ton or higher (Hamm et al. 2013).

For marine oils, bleaching is a crucial stage as it removes oxidation products that predominates other contaminants. This oxidative instability is caused by the higher content of unsaturated fatty acids, as a result of which the induction time of oxidation in marine oils is sooner than vegetable oils (Vaisali et al. 2016). In case of bleaching of fish oil, the most critical parameter that determines the quality is the temperature. Use of high temperatures might result in the unwanted hydrolysis of triglycerides resulting in increase of FFA concentration. The high viscous nature of the fish oils also makes the process complex making the mass transfer operation slow (Garcia-Moreno et al. 2013). Further, fish oil presents itself in a dark colour, more due to oxidative changes (Monte et al. 2015). Hence, bleaching process for fish oil has to be designed to target more of oxidation products than carotenoids which provide oxidative stability to fish oil.

2.3.4. Dewaxing

Waxes are generally present in oils as monoesters of long chain fatty acids and alcohols. Such an esterification process which results in these wax formation is spontaneous and is affected by storage conditions like temperature and time. Hence, waxes are found predominantly in fish oils oils due to the high degree of unsaturation. Some of the common vegetable oils which show high wax content are corn, rice bran and sunflower oil. If the wax content of the oil is not removed appropriately, the oil presents itself as cloudy after deodorisation. Due to this, dewaxing is usually performed prior to deodorisation stage. Usually dewaxing is performed by maintaining the oils at low temperature 6-8°C. This provokes wax crystallisation which can later be removed by centrifugation or filtration (Pestana-Bauer et al. 2012). The cooling process is carried out slowly so that proper crystallisation is obtained. Though this is a relatively simpler process, not all refining industries perform this step as they claim high reduction in wax content during conventional degumming and neutralisation stage. Further, membrane technology was also found to be suitable for

coupling dewaxing with either membrane degumming or deacidification (Manjula and Subramanian 2009).

2.3.5. Deodorization

Deodorization is the last step in the refining of oils, where volatile components of oil are removed. Primary objective of deodorization is to remove compounds responsible for undesirable odours and flavours, such as residual FFA (especially low molecular weight fatty acids), aldehydes, ketones, alcohols, light polycyclic aromatic hydrocarbons, dioxins and pesticide residues. Aldehydes, ketones and alcohols are generated due to hydrolytic and oxidative instability of oil during production and storage. It can be used to directly refine several high quality oils such as corn oil, palm oil, rice bran oil where FFA is the major contaminant. For oils like soyabean oils and marine oils which also have phospholipids as contaminants usually pre-treatment is carried out prior to the deodorization process. During deodorization, some losses of monoglycerides, sterols, sterol esters and natural anti-oxidants such as tocopherols also result. Deodorization is essentially performed at high temperatures (170°C–270°C) and under high vacuum (3–8 mmHg absolute pressure) by sparging steam. The amount of stripping steam ranges from 10 to 50 kg of steam per 100 kg of oil (Akoh and Min 2008). Deodorization is a multistage process which includes deaeration, heating, deodorization and cooling. A briefing on the role of every stage of deodorization is given below

1. Deaeration: Air is removed from the oil and the container by applying vacuum, so as to protect oil from potential oxidation during deodorization process.
2. Heating: Oil is heated to the predetermined value (170°C–270°C) by indirect heating using thermic fluids.
3. Deodorization: In this step, a stripping agent is injected into the oil to remove the volatile components. The most commonly used stripping agent is steam which is a condensable gas. Nitrogen is also used extensively, but the disadvantage is that it is noncondensable and the vacuum system used for nitrogen is much more expensive as compared to steam. Nitrogen stripping prevents hydrolysis of oil and produces high quality of oil.

4. Cooling: The oil is cooled by using heat exchangers to 60-65°C. Further cooling of the oil to 30°C is essential for stability purposes. In this stage, citric acid maybe added to remove the trace metals from the oil along with the addition of anti-oxidants if required.

Deodorization consisting of the above units can be performed in different ways (batch, semi continuous and continuous mode). The kind of process to be used for deodorization depends on the type of feedstock used and the plant capacity.

1. Batch unit: This unit is used for processing small quantities of oil. This unit is higly preferred when small batches of varying quality and quantities are to be processed because of the flexibility of adjusting the process parameters. The disadvantages of the process are the higher operating costs and longer processing time.
2. Semi continuous unit: These units are similar to the batch systems which are designed to process large capacities of feed compared to the batch process. Minimum processing time is one of the major advantages that the unit offers. The oil flow from the plant is discontinuous.
3. Continuous unit: They are the best option for processing huge quantities of feed in a single run. The process is very simple and is suitable for processing few oil types for long periods of time. Vertical tray type deodorizers are the most commonly used deodorizers in the industries.

Factors which affect deodorization process in a continuous unit are the vapour pressures of the volatiles in oil, oil flow rate, the mixing efficiency, the absolute pressure maintained in the stripping vessel, the temperature (which controls the vapour pressure of the materials being removed), the steam sparging rate, and the time of deodorization. Deodoriser design and process conditions need to be optimised to ensure minimal formation of trans fatty acids, maximal removal of volatile contaminants and a controlled stripping of valuable minor components (tocopherols, sterols etc.) (Hamm et al. 2013).

The disadvantage of deodorization is in the use of high temperatures. Many of the nutritive components in the oil are lost because they are either volatile (tocopherols and sterols) or are highly heat sensitive like n-3 PUFA in the marine oils. There is a possibility of formation of isomerization and cyclization products in the presence of

high temperatures. Deodorization should be designed in such a way that the reduction in the impurities should not be at the cost of the natural anti-oxidants or nutritive properties of the oil. Therefore, evolutions towards milder process conditions during deodorization by lowering the heat load (residence time at the high temperatures). The thermal effects can be lowered by using packed columns or dual temperature reactors. These reactors operate at two different temperatures for simultaneous deodorization and stripping of the volatiles components. This concept is successfully used in the industries. A study by Laoretani and Iribarren (2017), addressed the potential of an alternative process aimed at improving the batch deodorizers by coupling them with small continuous desorption packed column. It was proposed that the system increased the efficiency of separation by reducing the steam consumption by 16.5% and processing time by half (3-1.8 h). In their experiments, semi batch system showed a better performance than batch system in terms of cost and flexibility whereas continuous system was better economically but proved to be worse in flexibility when compared to both the designs. A recent publication by Riyadi et al. (2016) described a method of preserving the carotenes in the palm oil. They employed moderate temperatures of deodorization to remove the impurities in the oil. It was seen that at a temperature of 140°C for one hour was recommended to produce palm oil with high carotene retention. But this technique resulted in the partial removal of the impurities from the oil. Schols et al (2013) had described three stages of deodorization comprising a flash vessel, packed column and cross-flow tray system (US patent US: 2013/0287925 A1).

In an effective deodorization process it is inevitable that some amount of thermal degradation is witnessed. Hence, development of new technologies in deodorization is driven by the constant need for lower operating cost, higher refined oil yield and better vaporization of the side streams without compromising on the nutritional quality of oils.

Since all oils need to be (atleast partially) refined for applications in food industry there is a wide expansion of edible oil refining industry. Refining is a necessary process that ensures the quality of the edible oils for human consumption. The removal of undesirable components while stabilizing the essential portion of oil is

critical. Though conventional methods like alkali treatment and physical refining gives good results, destruction of desirable components due to harsh chemicals and high temperature warrant further improvement in technology. Use of eco-friendly techniques like enzymatic treatment was effective, but because of the expensive enzymes, it could not be applied on large scale processes. Conceptually, membranes could be used for all stages of refining of vegetable oils, but no literatures are available to state the process is industrially feasible. Although research on use of membranes has been going on for the past 30-40 years, very few commercial applications have been reported.

The complexity in the composition of fish oils has made the application of membrane process difficult for fish oil refining. As not many reports are available regarding the refining of fish oils, there is further scope in designing the refining process for fish oil. Novel methods regarding deodorization and deacidification of fish oil are yet to be explored. The future prospect would be to design a sustainable integrated refining process with the consideration of cost factor. Several reports are available on investigation of single variety membranes for one or two steps of refining, but use of different set of membranes and modules in combinations is yet to be assessed. Future industrialists and researchers are thus challenged to develop new processes with improved cost efficiencies, consistent (nutritional) quality of oil keeping and with sustainability aspect (minimal use of chemicals and processing aids).

2.4. STORAGE STABILITY OF EDIBLE OILS

Several studies are being undertaken in order to improve the quality of the oil by upgrading the raw material freshness and minimising the use of high temperatures during the extraction process. Edible oils characteristic taste, colour, nutritive properties, aroma and stability are a matter of great concern to the industry. One of the primary losses in the oil quality is oxidation of the lipids. Among the various technological factors that impact the quality of the oil in terms of its stability and composition, extraction methods and storage conditions play a very pivotal role in the quality deterioration. Since fish oils are a rich source of n-3 PUFA content, a feature which sets it apart from vegetable oils, is also the reason for its proneness to

oxidation. The oxidation of the fish oil is undesirable due to the release of various off flavours which negatively impacts the nutritional value of the oil. Primary oxidation products are lipid hydroperoxides (generally referred as peroxides), decomposes promptly to a range of secondary oxidation products like ketones and alcohols which prove to be highly undesirable in oils. High levels of these oxidation products in the oil ensue poor flavour in the final product. Since there is evidence which suggests the oxidation in the oil due to the presence of physical factors and volatile oxidation products that deteriorate the flavour of the oil (Shahidi and Zhong 2010), and the fact that the bulk oils are not homogenous, research to seek an explanation of lipid oxidation by various factors has intrigued interest in the recent past. Many attempts have been adopted to protect the unsaturated fatty acids from auto-oxidation however, no satisfying solutions were found to protect the lipid products (Vaisali et al. 2015). Oxidation can take place either in the presence of light (photo-oxidation), dark (auto-oxidation) and/or due to the enzymes (enzymatic oxidation). Auto-oxidation and photo-oxidation of fish oils can easily occur in mild conditions (Indrasena and Barrow 2010). The processes involved in auto-oxidation of the oil have been studied for decades and is known to occur in three different phases namely, initiation (induction period), propagation and termination (Shahidi and Chandrasekara 2010). Considering all these factors, there is a need in understanding the storage conditions of the oils to ensure an increase in its shelf life.

The presence of n-3 PUFA and its oxidation leads to the production of secondary oxidation products such as the aldehydes, ketones, alcohols and hydrocarbons. This not only contribute to the negative effects on the odour, flavour, colour and the texture of the oil but also leads to the loss of nutritional qualities such as amino acids, vitamins, polyphenols, phytosterols squalene and n-3 PUFA (Eder et al. 2010). Oxidized oils, if consumed, are highly toxic and can lead to various biological problems. Diarrhea, increased depression, tissue and organ damage due to the ingestion of oxidation products from unsaturated fatty acids (Ching 2007). It was observed that the rats fed with oxidized oil developed severe liver damage as opposed to the rats fed with fresh unoxidized oils (Totani et al. 2008).

Thwarting the oxidation of EPA and DHA in the fish oils has been a major quest in maintaining the quality in fish oils and food products. Care and protection against oxidative deterioration of fish oil while handling, processing, packaging, transferring and transporting and storage is extremely necessary. Also, the oils should not be exposed to high temperature, air and light. It must be taken care that the processed and refined fish oils should be stored in darkness, below -20°C in the presence of inert atmosphere (nitrogen) to avoid oxidative deterioration. Addition of anti-oxidants is the most common practise to retard oxidation of EPA and DHA in the oils. However, different anti-oxidants exhibit diverse inhibitory effects on the lipid oxidation of the n-3 PUFA in the oil (Indrasena and Barrow 2010). Apart from the anti-oxidants, focus on several factors which influence the quality has been laid in the next section which could help in retarding the undesirable oxidative processes in the oil.

2.5. FACTORS AFFECTING THE LIPID OXIDATION

The quality of the oil is affected by the fatty acid composition of the oil, oil processing, intensity of the heat or light, temperature, moisture in the oil (environmental factors), FFA, mono- and diacylglycerols, phospholipids, enzymes, transition metals ions, peroxides, thermally oxidized compounds, pigments, and anti-oxidants (intrinsic factors). The oxidation of the oil takes place due to the combined effects of all the factors and differentiation of the effect of individual factors is not easy (Choe and Min 2006).

2.5.1. Extrinsic (environmental) factors

2.5.1.1. Methods of oil extraction

The oil-processing method affects the oxidative stability of oil to a large extent. Crude soybean oil was the most stable to the oxidation followed by deodorization, degumming and bleached oil during 6 days of storage period at 55°C in the dark (Jung et al. 1989). Higher oxidative stability of crude oil than refined oil was suggested to be partly due to higher concentrations of tocopherols in crude oil (1670 ppm) than refined oil (1546 ppm) while the same may not apply to fish oils due to the variation in the composition of fatty acids and the presence of minor components which

accelerate the oxidative instability in the oil (Chakraborthy et al. 2015). Oxidative instability was significantly higher in supercritical carbon dioxide extracted walnut oil than in pressed oil (Crowe and White 2003). Roasting of safflower and sesame seeds before oil extraction improved the oxidative stability of the oils (Yen and Shyu 1989, Lee and others 2004), which could be due to the production of maillard products during roasting which were reported to have anti-oxidant properties. It was observed that the oxidative stability increased as the roasting and expelling temperatures of the seeds increased. Chantachum et al. (2000) published a study about the separation of oil from tuna head by wet reduction method. The yield and the quality were compared between the oils extracted from precooked fish at 100°C for 60 min and non-precooked fish. It was concluded that the yield and the quality of the oil was worthy when the heating at 85°C for 30 min was carried out. Aidos et al. (2003) focused a study on the by-product freshness of herring under the influence of different process parameters such as cooking temperature, pumping speed and decanter speed. It was concluded that the by-products were stable and the variables influencing the oil quality were separation speed of the decanter and the pumping speeds. Vaisali et al. (2015) in their recent review of various methods on refining of oils have discussed the need to recognize the composition of the oil since this provides a useful insight in making an effective choice on the kind of method specific for particular oil.

2.5.1.2. Light and temperature

The exposure of oil to light can lead to the formation of initiators. UV light is particularly harmful. The absorption of light can cause the excitation of the minor components. Sattar et al. (1976) suggested in their studies that the light of shorter wavelengths had more negative effects on the oils than longer wavelengths. Consequently, it becomes important to prevent photo-oxidation during storage to improve the quality of the oil. It has been suggested that dark glass bottles or glass bottles coated with UV absorbers improved the oxidative stability of soybean oil under light (Pascall et al. 1995, Azeredo et al. 2003).

Auto-oxidation of oils and the decomposition of hydroperoxides increase as the temperature increases (Shahidi and Spurvey 1996, St. Angelo 1996). The formation of

oxidation products is slow at low temperature (Velasco and Dobarganes 2002). The hydroperoxide decomposition rate of crude herring oil stored at 50°C in the dark was higher than the formation rate of hydroperoxide whereas, the opposite behaviour was observed in the same crude herring oil at 0 or 20°C in the dark (Aidos and others 2002). Guillen et al. (2008) showed in his study on sunflower oil that the oil samples in closed containers at room temperature, formed large quantities of monocyclic and polycyclic aromatic hydrocarbons proving that at 30°C the oxidation rates in the oil were high. Furthermore, Boran and group (2006) contends that there was a maximum resistance in the oxidation and FFA release in the various fish oils at -18°C compared to 4°C.

2.5.1.3. Moisture in oil

The availability of water strongly impacts the reactions in the oil be it enzymatic hydrolysis, non-enzymatic reactions, lipid oxidation. The formation of monolayer and the reactions involved due to the formation of monolayer is said to be highly temperature dependant (Iglesias and Chirife 1976, Almasi 1978). The moisture in lipid oxidation plays both protective and pro-oxidant roles. The protective role is presumably due to the presence of a barrier between the lipid components in the oil and the oxygen (Nawar 1996) and also due to the formation of hydrogen bonds to the lipid hydroperoxides, thereby increasing their relative stability (Labuza et al. 1970). Another study of stable water-peroxide complexes was supported by Chen et al. (1992), who found the increasing concentration of water (0-2%) declined the decomposition of methyl linoleate hydroperoxides. In contrast to these reports, moisture can also be detrimental as it solubilizes certain metal ions. Further, researchers suspect that moisture leads to the formation of secondary products (Prabhakar and Amla 1978). It was found that a high proportion of carbonyl formation of different classes (ketoglycerides, saturated aldehydes) from walnut oil varied proportionally with the increase in the moisture content in the oil. Similar effects were not observed, however, in the samples which were relatively fresh i.e. with low hydroperoxide concentrations. This would mean that moisture acts as a catalyst and induces oxidative instability in the oil. The oxidation reactions which are primarily occurring in the lipid phase would not be affected by the presence of moisture

whereas; moisture is known to play a major role in those oxidation reactions which are chiefly promoted by the water soluble pro-oxidants like the metals (Labuza 1971, Nawar 1996). Therefore, the study of moisture in the oil becomes an important study to know its effects on the lipid oxidation kinetics in the oil. A recent study by Kim et al. (2014) has shown the involvement of moisture for the formation of volatiles and that moisture acts as a substrate for lipid oxidation. The inherent moisture in the oils was able to hydrolyze the triacylglycerol molecules to mono- and di-glycerides with the release of FFA. Kittipongpittaya et al. (2016) suggested in their studies that water itself did not significantly impact lipid oxidation in the bulk oils. However, the combination of water molecules and some minor components significantly drastically increased the oxidative instability of oil. The addition of water brought the minor components close to metal ions and accelerated the oxidation. Few reports also suggest that the water molecules have the tendency to behave as a sink, by attracting the secondary oxidation products and FFA into the micelles. The reverse micelles formed by moisture in the bulk oil tend to decrease in particle size due to dispersion in the oil over the course of storage time which increases the interfacial contact for hyderoperoxides. Hence the incorporation of hydroperoxides and FFA into the micelles, led to the reduction in the oxidation of the oil (Ambrosone et al. 2006). Similar observations by Ambrosone et al. (2002) on extra virgin olive oils proposed that the peroxide values of the oil declined with the reduction in the droplet radius.

2.5.2. Intrinsic factors

The composition of fats and oil differs greatly based on the species, strain, environmental conditions and also the extraction procedure used. Before 19th century, many oils were used without refining which included olive, lard, tallow, linseed and certain cold pressed oil (Dijkstra 2013). After the advent of solvent extraction, many seed oils are extracted by this technique while boiling and expression methods are used for fish oil extraction (Bhosle and Subramanian 2005). The oil processing industry is a large scale operation, using bulk raw material from different seasons and with varying freshness and composition and thus contains essential components along with impurities, such as free fatty acids (FFA), phospholipids and volatile compounds (Table 2.8).

Table 2.8: Components in edible oils and their effects on the quality of the oil

Components	Character	Quantity		Effect on oil quality
		Crude	Refined	
Acylglycerols	Desirable	90%	>99%	Important component to be maintained
Tocopherols, Squalene, Sterols	Desirable	200-800 ppm	50-300 ppm	Improve oxidative stability of oil
Phospholipids	Undesirable	100-500 ppm	<10 ppm	Settling at the bottom during storage
FFA	Undesirable	5-20%	<1%	Pro-oxidant leading to the oxidation of acylglycerols
Metal ions and complexes	Undesirable	2-15 mg/kg	<1 mg/kg	Pro-oxidants and harmful for consumption
Oxidized products	undesirable	2-6 meq/kg	<1meq/kg	Leads to rancidity in the oil and should be removed
Moisture	undesirable	1-3%	<1%	Prooxidant and should be minimised in the oil.

(**Adapted from Vaisali et al. 2015**)

2.5.2.1. Composition of fatty acids in the oil

Oils containing more unsaturated fatty acids are oxidized rapidly than less unsaturated oils (Parker et al. 2003). As the degree of unsaturation increases, the rate of formation and the concentration of the accumulated primary oxidation compounds at the end of the induction period increase. The vegetable oils like soyabean, sunflower and safflower oils with iodine values more than 130 stored in dark oxidized faster as compared to the palm and coconut oil (iodine value less than 20) (Tan et al. 2002). The rate of auto-oxidation depends on the radicle formation from the acylglycerols which mainly depends on the types of fatty acids that are present in the oil. The difference in oxidation rate among fatty acids is lower than that for auto-oxidation. The relative reaction rates of oxygen with oleic, linoleic, and linolenic acids are 1.0: 1.4: 1.9, respectively (Vever-Bizet et al. 1989). The type of polyunsaturated fatty acids, nonconjugated or conjugated dienes or trienes, has little effect on the reaction between the lipid and oxygen (Rahmani and Csallani 1998).

2.5.2.2. Metal ions

Crude oil tends to contain transition metals such as iron, copper. Extra virgin olive oils and sesame oils manufactured without refining consists of relatively high quantities of transition metals (MAFF 1997). The catalytic effect of trace transition metals on oxidation is due to the reduction in activation energy of the initial step in the auto-oxidation process (Jadhav et al. 1996). They interact directly with the lipids to release alkyl radicals, reactive oxygen species (ROS), hydroxyl radicals and hydrogen peroxide, respectively (Andersson 1999). Copper is known to accelerate the hydroperoxide disintegration 50 times faster than ferrous ions. Ferrous ions are 100 times faster than ferric ions in decomposing the lipid hydroperoxides (Mei et al. 1998). The dissimilarities in the ability of the metal ions to promote oxidation are due to the type and the chemical state of the metals (Chen et al. 2012, Benedet and Shibamoto. 2008). Metals can also quicken the process of auto-oxidation by decomposing the hydroperoxides (Benjelloun and group 1991). It was reported by Keceli and Gordon (2002) that the ferric ions degraded the phenolic compounds such as caffeic acid in olive oils thus decreasing the oxidative stability of the oil. Shiota and group (2006) stated that the pro-oxidant activity of iron was arrested by lactoferrin in fish oil at 50°C and in soyabean oil at 120°C, which is thought to be due to the iron binding ability of lactoferrin. Studies by Chen et al. (2012) in soybean oil containing phospholipid reverse micelles suggested that iron behaves as a pro-oxidant by binding to the phospholipids concentrating the iron at the oil-water interface thus increasing the ability of iron to decompose the lipid hydroperoxides. A comparison of the catalytic activity of Cu(II) and Fe(III) cylcohexanebutyrates in the refined olive oil suggested that copper was more catalytically active than iron. It was noticed that the induction time was halved by the addition of 120 ng/g of copper in the oil versus 9000 ng/g of iron. Further, the oil was unstable due to the pro-oxidant activity of copper in spite of the addition of caffeic acid proving the strong pro-oxidant ability of copper over iron (Leonardis and Macciola 2002).

2.5.2.3. Phospholipids

The impact of phospholipids in the stability of bulk oil has been debatable. This is because of intermediate hydrophilic-lipophilic balances (~8) that they possess due to

which it forms lamellar structures or reverse micelles (Chaiyasit et al. 2007). Phospholipids which form reverse micelles exhibit pro-oxidant activity verses the lamellar structures. They have the ability to concentrate FFA at the interface resulting in the decomposition of lipid peroxides into free radicles. In addition, phospholipids tend to reduce the surface tension and increase the oxygen diffusion from headspace to the oil so accelerate the lipid oxidation (Kittipongpittaya et al. 2016). Phospholipids acts either as anti-oxidants or pro-oxidants depending on its concentration in the oil and the presence of other minor components. Nitrogen group containing phospholipids such as phosphotidylcholine and phosphotidylethanolamine demonstrate anti-oxidant characteristic under most scenarios (King et al. 1992). Yoon and Min (1987) found that the phospholipids acted as anti-oxidants in the presence of ferrous ions whereas, while the reverse phenomena was observed in the presence of various FFA (myristoleic, oleic, elaidic, linoleic and eicosenoic acids) (Kittipongpittaya et al. 2013). The results showed that FFA could impact the structural changes of phospholipids by decreasing the pH. Oxidation of Soybean oil in the dark at 60°C was the least with phosphatidic acid and phosphatidylethanolamine, followed by phosphatidylcholine, phosphatidylglycerol, and phosphatidylinositol (Yoon and Min 1987). A study by Chen et al. (2011) recorded the formation of micelles on addition of PL which had a pro-oxidant effect and shortened the lag phase of stripped soyabean oil.

2.5.2.4. Free fatty acids (FFA)

FFA is more susceptible to auto-oxidation than esterified fatty acids (Kinsella and others 1978). FFA acts as pro-oxidants in edible oil (Choe and Min 2006). The presence of hydrophobic and hydrophilic groups in the molecules confines FFA on the surface of the oils. The FFA decreases the surface tension of the oil increasing the diffusion rates of oxygen from headspace and accelerates the oxidation (Choe and Min 2006). A study by Paradiso and group (2010) on the effect of increasing amounts of FFA (0.25%, 0.5%, 1%, and 3%) on olive oil confirmed the pro-oxidant activity of FFA at 60°C. It was reasoned that the FFA led to the decomposition of the peroxides when present in higher amounts. A comparison of the effects of FFA on filtered virgin olive oil and virgin olive oil (cloudy) were analyzed and it was hypothesized that the

FFA bonded to the dispersed particles in the cloudy oil and precipitated as brown coloured particles, thus, playing stabilizing roles and lessening the oxidative and hydrolytic degenerations in the oil. The opposite effect of FFA was observed in the filtered virgin oils. The pro-oxidant effect was exhibited by FFA, independent of lipidic substrate, but the intensity of this effect was related to FFA concentration, and the unsaturation in the chain. Therefore, it was concluded that the avoidance of filtration is highly desirable in order to extend the shelf life of oil (Frega et al. 1999). The FFA (different chain lengths and unsaturation degrees) effect on the oxidative stability of marine lipids was studied by FFA interacting with the cod liver oil. It was witnessed that the pro-oxidant effect of FFA increased with time, temperature and the FFA content in the reaction mixtures. The pro-oxidant activity of FFA with the increasing degrees of fatty acid unsaturation was observed whereas, at a lower temperature of -10°C, the activity of FFA was negligible (Aubourg 2001).

2.5.2.5. Oxidized products

The processing of oils under various conditions could produce oxidized products such as dimers, trimers, hydroxyl trimers. At high temperatures or in the presence of metals, hydroperoxides decomposes to alkoxy radicals that are again converted to low-molecular weight aldehydes, ketones, acids, esters, alcohols, and short-chain hydrocarbons. Among these products, 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-flavours. 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 auto-oxidation of lipids (Akoh and Min 2008). The oxidized products of hydroperoxide decomposition contain hydrophobic and hydrophilic groups which increase the surface tension and accelerate the oxidation by the introduction of oxygen into the oil (Jung et al. 1989, Davis et al. 1993, Choe and Min 2006 and Hemery et al. 2015). A study by Guillen et al. (2008) reported an increase in the oxidative instability of sunflower oil maintained at 30°C due to the release of large quantities of monocyclic and polycyclic aromatic hydrocarbons. A recent study by Chakraborty and Joseph (2015) testified the release of copious amounts of unfavourable volatile compounds

from sardine oil which generated fishy odours which were removed successfully by distillation (100°C) under vacuum.

2.6. PRODUCTION OF n-3 PUFA CONCENTRATE

n-3 PUFA concentrated oil is a refined oil which is subjected to modification using various methods to increase the quantity of n-3 PUFA like EPA and DHA. Industries in general produce n-3 PUFA concentrates involving an initial step of oil extraction from the species followed by concentration. In the recent past, industries have focussed on concentration n-3 PUFA in the oil in the form of ethyl esters formed by esterification or saponification of triglycerides with ethanol. Concentration up to the level of 30% EPA+DHA can be prepared directly from the oils without splitting the fat by careful selection of various methods such as winterization, molecular distillation, solvent crystallization etc. Enhancement of EPA and DHA in the oil beyond 30% is difficult, since it requires the fatty acids to be cleaved off the acylglycerols, either as di- or monglycerides. Various methods like molecular distillation, supercritical fluid extraction and urea complexation are frequently used to enhance the EPA and DHA contents upto 50-85% level. Specific methods of separation and purification like HPLC can concentrate the EPA and DHA upto 90% levels (Breivik et al. 1997). A wide range of monoglyceride (as ethyl esters) forms of EPA and DHA are commercially available, some of which have been registered as drugs in various countries (Haraldsson et al. 2000).

The synthesis of EPA and DHA enriched oil is not a simple task to achieve by the traditional chemical esterification techniques. The labile n-3 PUFA are very sensitive to the drastic conditions offered by the traditional methods. The presence of all cis n-3 PUFA configuration makes them extremely disposed to oxidation, *cis*- *trans* isomerization, double bond migration or polymerization. These methods adopted by the industries unveil a major drawback of the loss of natural triglyceride form of fatty acids from oil. Several authors such as Mishra et al. (1993), Wanasundra and Shahidi (1998) and Rubio-Rodriguez et al. (2010) have described a conspectus of the various conventional methods to concentrate n-3 PUFA in the oil.

2.6.1. Molecular distillation and Urea complexation

The most frequently used methods to enhance the n-3 PUFA content in the oil on a large scale are molecular distillation and urea complexation. Molecular distillation works on the separation of different fatty acids due to the differences in their boiling points. It effectively separates squalene, organic environmental pollutants like polycyclic aromatic hydrocarbons, halogenated organic compounds and volatiles, diacylglycerol ethers with the concentration of n-3 PUFA from oils. This technique can be used to separate carbon atoms (C20 and C22) acids/ esters generally after these have been separated from the less unsaturated acids/ esters by urea fractionation or other techniques. Due to the sensitivity of n-3 PUFAs (highly disposed to oxidation), this technique involving reduced pressure, low temperature and minimal residence time proves to be advantageous for the concentration.

Urea complexation is the simplest and effective technique which involves the alkaline hydrolysis of the oil. The FFA generated during the hydrolysis is further mixed with the solution of urea. During the process, the complexation of the glycerides by urea takes place after which the entire solution is cooled down and the glycerides are crystallized out. The crystallized product is separated by the process of filtration. The final filtrate is the n-3 PUFA concentrate. The process is considered easy, inexpensive, fast, robust and eco-friendly. But using urea for concentrating n-3 PUFA is considered a disadvantage due to the probable formation of ethyl carbonate (animal carcinogen) which should be avoided for human consumption (Canas and Yurawecz 1999).

Many researchers have used molecular distillation and urea complexation as techniques for the enrichment and purification of n-3 PUFAs. These chemical methods, however, are not specific for the different fatty acids. Also, these methods produce the LC-PUFA concentrates in the form of FFA or alkyl esters. These techniques are known to involve extremes of pH and temperature which may destroy the natural n-3 PUFA by oxidation, *cis-trans* isomerization and double bond migrations (Halldorsson et al. 2004). Hence, techniques involving its unique ability to specifically concentrate the targeted fatty acids like EPA and DHA in the triglyceride

fractions of the oil with the involvement of mild conditions during the concentration process are the most favoured.

2.6.2. Enzymatic concentration of oil with EPA and DHA.

Lipases have been introduced to concentrate n-3 PUFA to help solve the problems posed by the above mentioned techniques. They are hydrolases which act on carboxylic ester bonds resulting in the separation of fatty acids from the glycerol backbone (Houde et al. 2004). One of the unique features of lipases is that they tend to remain active in the aqueous and the non-aqueous interface which differentiates them from esterases (Vakhlu and Kour 2006). These enzymes offer high efficiency and mildness with the advantage of its application in organic media (Chakraborty et al. 2010). They offer concentrates of EPA and DHA with high purity, efficiency and with excellent yields. This process requires neither chemicals nor organic solvents at high concentrations and are therefore, highly feasible from an industrial as well as hazards point of view. The application of lipases is based on the fatty acid selectivity. Concentration levels of 50-70% EPA+DHA can be obtained in the oils by hydrolysis or alcoholysis reactions. Higher levels of concentration above 90% can be achieved by adopting two-step enzyme processes by the usage of high purity of lipases. Inordinate amount of enzymatic methods followed by a separating the products by using processes like membrane filtration, urea complexation and molecular distillation have been proposed in the literatures to obtain EPA and DHA in various forms (FFA, ethyl esters and glycerides) (Oliveria-Carvalho et al. 2003, Wanasundra and Shahidi 1998, Solaesa et al. 2016). Among the various available methods for the production of n-3 PUFA concentrates in glyceride forms, enzymatic enrichment using lipases, involving interesterification and hydrolysis, have been extensively studied.

Lipases catalyze the hydrolysis of fatty acid ester bonds in the triglycerides and release FFA in the presence of water molecules (Sheldon 1993). Various factors are suggested to affect the reaction rates of the lipases in hydrolysis like the positional specificity of the enzyme, the rate of the reverse reactions, the specificity of lipase for fatty acids, concentration of lipase, substrate concentration, temperature, pH, and the presence of metallic ions (Sun et al. 2002, Tanaka et al. 1992, Uhlig 1998a, Uhlig

1998b). The mechanism of concentration of n-3 PUFA in the presence of lipase is based on the fatty acid specificity towards SFA and MUFA, keeping the n-3 PUFA unharmed on the glyceride moiety (Wanasundra and Shahidi 1998). The presence of cis carbon-carbon double bonds in EPA and DHA creates a bend in the chains which brings the terminal methyl groups of these fatty acids close to the ester bond causing a steric hindrance effect on the approaching lipase molecules. This results in the protection of EPA and DHA from lipase catalyzed hydrolysis (Carvalho et al. 2002, Wanasundara and Shahidi 1998).

Hydrolysis is said to be a simpler and a faster process over the other competing methods such as interesterfication reactions which require organic solvents in the reaction mixture and caution with handling and discarding procedures (Mohapatra and Hsu 1999). An enzymatic lipid hydrolysis system in general consists of oil, buffer (optimum pH for lipase) or water and lipase enzyme as a catalyst dissolved in a buffer of optimum pH. After a defined period of hydrolysis of oil, the lipase is inactivated by the addition of alcohol. FFA released during hydrolysis is removed by neutralization or solvent extraction. The solvent layer strips the FFA from the hydrolyzed oil and is separated by a separating funnel, and the n-3 PUFA concentrated oil is evaporated to remove the traces of solvent (Chakraborty et al. 2010). If immobilized enzymes are used for the hydrolysis, the lipase from the system is removed either by filtration or centrifugation with no requirement of organic solvents.

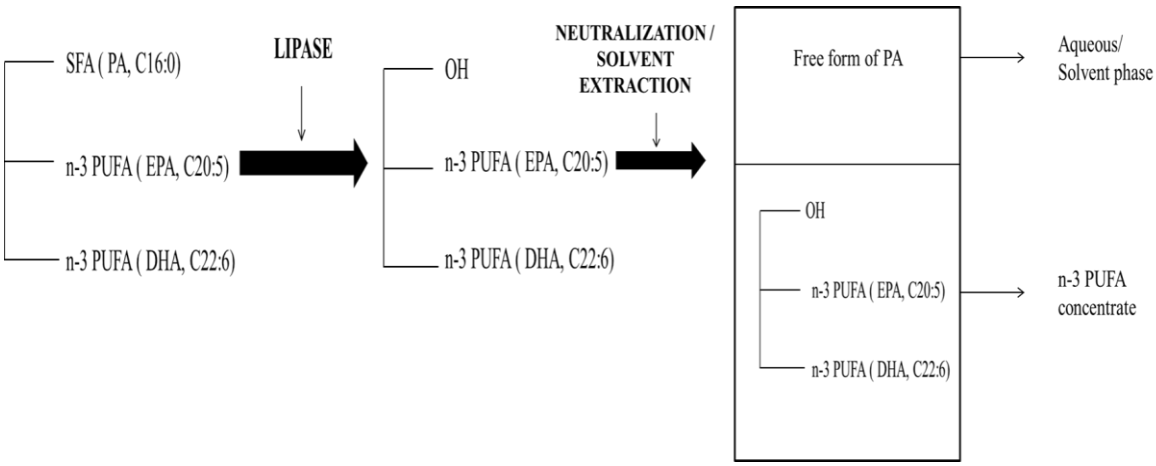


Figure 2.4: A representation of hydrolysis and concentration of n-3 PUFA by lipase

A survey of the literature revealed a diverse range of methods for the enrichment and purification of LC-PUFAs. However, most of the existing methods for purifying individual PUFAs and PUFA concentrates are non selective for different fatty acids. The unique specificity of the lipases for the enrichment of PUFAs in th oil is effectively exploited. Several research groups have worked on this aspect and below are the literature devoted to the hydrolysis studies of various fish oils through free commercial lipases hydrolysis which includes the examination of the conditions of hydrolysis, research highlights and their respective references from the years 1990 till date (Table 2.9).

Table 2.9: Various studies on hydrolysis of edible oils using different lipases

Oil used	Lipase used	Conditions for hydrolysis	Research highlights	References
Cod liver oil / refined sardine oil	*Humicola lanuginose,Candida cylidracea, Aspergillus niger, Rhizopus delemar, Geotrichum candidum*	6 mL lipase enzyme + 4 mL of fish oil. Agitation :700 rpm. Temperature: 20°C Time: 100 hours	The extent of n-3 PUFA enrichment were about 50% for both *Candida Cylindracea* and *Aspergillus niger*	Hoshino et al. 1990
Tuna oil	*Candida cylidracea*	50g of tuna oil + 50g of distilled water + 200 U of lipase per 1g of oil Agitation: 500 rpm Temperature: 37°C.	The enhancement of DHA from 25% to 53%. The EPA content remained very close to the original oil.	Tanaka et al. 1992.

Oil used	Lipase used	Conditions for hydrolysis	Research highlights	References
Tuna oil	*Pseudomonas sp.*	2.5 g tuna oil +2.5 g water + 500 U of Lipase-AK for 1 g of the reaction mixture Temperature: 40°C Time: 48 h	83% of DHA in tuna oil was recovered in the FFA fraction at 79% hydrolysis. The DHA content in the hydrolyzed oil could be raised from 24% to 72 wt% in an 83% yield.	Shimada et al. 1997.
Seal blubber oil/ menhaden oil	*Candida cylindracea*	4.0 g of oil + phosphate buffer (6.0 mL of a 0.1 M solution; pH 7.0) containing *C. cylindracea* lipase Agitation: 200 rpm.	SBO: 54.3% total ω-3 fatty acids were obtained. Enzyme concentration: 308 U/g oil reaction time: 40 h, Temperature: 37°C. MHO: 54.5% total ω-3 fatty acids were obtained. Enzyme concentration: 340 U/g oil Time: 45h temperature: 38°C.	Wanasundara and Shahidi 1998.

Oil used	Lipase used	Conditions for hydrolysis	Research highlights	References
Brazilian Sardine Oil	*Candida cylindracea lipase, Rhizopus delemar lipase, Aspergillus oryzae lipase and Chromobacterium viscosum lipase*	500mg of sardine oil+150 U lipase powder (3.5 mL of pH 7 phosphate buffer), agitation: 200 rpm Temperature: 35°C.	Sardine oil was treated at 35 °C with this lipase for 16 h resulted in 60.0% hydrolysis , with an increase in the DHA content from 10.2% in the original oil to 22.5% in the unhydrolysed acylglycerol. There was no significant increase in EPA by any of the four lipases tested.	Carvalho et al. 2002
Salmon oil	*Asperigillus oryzae*	1000g of oil+ 200 kilo LU+ 1000 mL of distilled water temperature: 37°C pH: 7 Time: 12 h The hydrolyzed oil was subjected to membrane filteration to separate FFA from glycerides.	The n-3 PUFA content increased from 41.6% to 46.5% in hydrolyzed oil. The DHA increased from 9.9 to 11.6% and EPA from 3.6 to 5.6%.	Linder et al. 2005.

Oil used	Lipase used	Conditions for hydrolysis	Research highlights	References
Fish oil (unspecified species)	*Penicillium expansum, P. cyclopium, Candida lipolytica, A. oryzae and Pancreatic Lipases*	-	*A. oryzae* showed the highest efficiency in PUFA concentration increasing the EPA from 3-9% and DHA from 4.3% to16.5% for 24 h hydrolysis.	Zheng et al. 2005.
Sardine oil	*Candida Rugosa* (CRL), *Candida cylindracea, Mucor javanicus and Aspergillus niger*	2 g of sardine oil+ (250U and 500U of lipase in 8 mL phosphate buffer) agitation: 500rpm Temperature: 37° Time: 1.5,3,6,9 h.	*Candida Rugosa* was the most effective in hydrolysis of oil. 250 U of lipase increased the EPA content from 26.86% to 33.74% in 1.5 h. The lipase of 500 U enhanced the DHA from 13.62% to 29.94% in 9 h. in comparison with *Candida Rugosa, Candida cylindracea,* the other two lipases resulted in a low n-3 PUFA enhancement.	Okada and Morrissey 2007.

Oil used	Lipase used	Conditions for hydrolysis	Research highlights	References
Salmon oil	*Aspergillus niger, Rhizopus javanicus and Penicillium solitum.*	RSM was performed for all the four lipases to obtain maximum PUFA content.	*Aspergillus niger* lipase was the most effective in obtaining 60% hydrolysis degree and concentrating DHA from 14.4% to 34% in the residual acylglycerol in optimum conditions of 500U/g oil at a temperature of 45°C, water/ oil mass ratio rate 2:1(m/m) for 24h.	Carvalho et al. 2009
Sardine oil	*Bacillus circulans*	100 mL of the reaction mixture containing triglycerides + 500 U of lipase agitation: 500rpm temperature: 37±1°C.	The purified *Bacillus circulans* lipase enriched the sardine oil with EPA (37.7 ± 1.98%) in the triglyceride fraction after 3 h of hydrolysis which was followed by urea complexation which resulted in free fatty acids containing EPA (51.3 ± 4.65%).	Chakraborty et al. 2010.

Oil used	Lipase used	Conditions for hydrolysis	Research highlights	References
Salmon oil	*Candida rugosa* (CRL)	3g of oil+9.5 mL of distilled water heated Temperature: 45°C Agitation: 300rpm. Repeated hydrolysis was performed by using 3g of oil obtained from short path distillation under the same reaction conditions.	n-3 PUFA content after the first round of hydrolysis was the double the initial amount and found to be 38.71 mol%. The FFA free oil was subjected to second round of hydrolysis and to obtain and enhancement of 50.58% of n-3 PUFA.	Kahveci and Xu 2011.
tuna oil	*Yarrowia lipolytica, Thermomyces lanuginosus and Candida rugosa (CRL1, CRL3 and CRL4)*	0.75 mL of oil+ 0.75 mL of lipase	*Yarrowia lipolytica* was the most effective lipase for DHA purification. Using this enzyme in an open reactor process resulted in the highest concentrations of DHA ethyl ester (77%) and n-3 PUFA esters (81%) with a recovery of 94% and 77% respectively.	Casas-Godoy et al. 2014

2.6.2.1. Strategies for Catalytic improvement of lipases

Numerous efforts have been dedicated to the use and development of commercial free lipases for the hydrolysis of oil. However, the perfect functioning of these enzymes for hydrolysis in the first attempt is very limited because of certain precincts related to the process parameters or enzymes like the moderate stability (Garcia- Galan et al. 2011, Zhang and Cui 2010), substrate and product inhibitions, inefficient recycling and high production cost (Andexer et al. 2009, DiCosimo et al. 2013, Rodrigues et al. 2011). To avoid these limitations, various strategies have been endeavoured to improve the biocatalytic performances in terms of product yield such as directed evolution (Cherry and Fidantsef 2003, Muller et al. 2013), rational enzyme design (Frushicheva et al. 2010), enzymatic modifications (Sonawane 2006), immobilization of enzyme (Betancoran and Luckarift 2008) and adjusting reaction conditions (Henzler- Wildman et al. 2007). Amongst the above listed strategies, enzyme immobilization and bioimprinting of the enzymes have been regarded as the most promising techniques since it offers advantages like improved operational stability, enhanced enantioselectivity, reusability of the enzymes, easier reactor operation and product separation (Brady and Jordaan 2009, Garcia-Galan et al. 2011, Iyer and Ananthanarayan, 2008, Liese and Hilterhaus 2013). Enzyme immobilization is categorized into two major techniques i.e. carrier bound and carrier free immobilization (Garcia-Galan et al. 2011). Although the enzymes immobilized on the non-catalytic matrices (carrier bound immobilization) delivers certain advantages, its offers disadvantages like the volumetric activity of the biocatalyst and reduced productivity of the reaction due to the presence of carrier which is non-catalytic in nature (Cui and Jia 2013). Whereas, in the carrier free immobilization there is no requirement of an extra carrier which makes this process more advantageous than carrier bound immobilizations. In this process, the preparation is done directly by cross-linking the enzyme preparations. Carrier free immobilization includes cross-linked enzyme (CLE), cross-linked enzyme crystals (CLECs), cross-linked spray-dried enzyme (CSDE) and cross-linked enzyme aggregates (CLEAs). Among the various carrier free immobilization techniques listed above, CLEAs are attracting a lot of attention due to its simplicity and robustness (Cao et al. 2000). For the preparation

of CLEAs, the enzyme solutions under consideration are aggregated using precipitants which are followed by cross-linking of the resulting aggregates using cross-linking agents. The produced CLEAs are proved to be stable to the extremes of heat, solvents and proteolysis (Ju et al. 2013, Sheldon 2007, Yang et al. 2012). Moreover, CLEAs exhibits increased operational stability, volumetric productivities and recoverability (Sheldon 2011).

An inherent disadvantage of other cross linking methods, which is often laborious procedure requiring a very high enzyme purity led to the preparation of the enzymes by simply precipitating the enzymes and further cross linking the physical aggregates of the enzyme molecules. Few research groups have worked on this aspect and below are the literature devoted to the preparation of the modified lipases which includes the examination of results and their respective references from the years 2002 till date.

Table 2.10: Studies on cross-linked enzyme aggregate preparations of various lipases

Lipases	Result	Reference
Candida antarctica lipase A (CALA), *Candida Antarctica* lipase B (CALB), *Thermomyces lanuginosa*, *Rhizomucor miehei*, *Aspergillus niger*, *Pseudomonas alcaligens, Candida rugosa*.	Hyperactivation of the produced CLEAs were tested in the presence of surfactants like SDS, triton X-100, crown ether, dibenzo-18-crown-6. *Thermomyces lanuginosa* and *Rhizomucor miehei* in the presence of SDS were more than 2 and 3 times active in comparison with the native enzyme. Lipase from *Candida antarctica* dissolved in dibenzo-18-crown-6 showed a considerable activation. The effects of surfactants on *Aspergillus niger, Pseudomonas alcaligens, Candida rugosa* were negligible.	Lopez-Seranno et al. 2002
Candida antarctica lipase A (CALA), *Candida antarctica* lipase B (CALB), *Rhizomucor miehei*,	The optimization of the crucial parameters such as precipitant and its concentration, cross-linker and its concentration and protein concentration were established. One of the most appealing features of this	Schoevaart et al. 2004

Lipases	Result	Reference
Candida rugosa	method is the use of small amounts of enzyme for the initial preparation. The SEM analysis gave an insight of the CLEA behaviour.	
Candida antarctica lipase B	Is completely stable in aqueous media. Performed admirably in supercritical carbon dioxide and organic solvents.	Hobbs et al. 2006
Candida rugosa	The two major factors which played an important role in their effects on particle size of the lipase were enzyme and glutaraldehyde concentration. Lipase with particle size 40-50µm showed the highest activity compared to the oter particle sizes while maintaining 86% of its original activity. The enatioselectivity the CLEA produced was 1.8 times as compared to the free lipase in the kinetic resolution of ibuprofen racemic mixture.	Yu et al. 2006
Combination of α-amylase and phospholipase A_2	Increased thermal stability at 50°C as compared to the free enzyme. The lipase activity was retained until three cycles of use.	Dalal et al. 2007
Pseudomonas cepacia	The immobilized lipase for enantioselective acylations of 1-phenylthanol, 1-(2-furyl) ethanol and N-acylated 1-amino-2-phenylethanol with vinyl acetate in organic solvents. The preparation was highly reusable at room temperature. Upon immobilization, there was an increase in activity (174%). However, the recyclability of this lipase was limited to three uses.	Hara et al. 2008
Pseudomonas sp.	The CLEA was prepared using acetone as the optimal precipitant with the immobilization efficiency and activity retention as 70.6% and 45.1%, respectively. CLEA requires only 12 h to obtin 50%	Zhao et al. 2008

Lipases	Result	Reference
	conversion of N-(2-ethyl-6-methylphenyl) alanine while free enzyme required 48 h. The residual activity of the CLEA and free enzymes were 72.2% and 23.%, respectively at 60°C for 24 h of hydrolysis. The immobilized enzyme could be reused upto ten cycles with the loss of efficiency of 19.1% only.	
Burkholderia cepacia	Three CLEAs were prepared with varying concentrations of glutaraldehyde of 10mM (CLEA A), 40mM (CLEA B), 60mM (CLEA C). The tranesterifcation of β- citronellol was highest for uncrosslinked enzyme. CLEA A was best suited for the enantioselective synthesis. The thermal stability of the enzyme at 55°C in aqueous media was the highest for CLEA C as is reflected by the half-life of the enzymes. SEM confirmed that morphology of CLEA was mainly dependant on the extent of cross-linking.	Majumder et al. 2008
Thermomyces lanuginose	Ammonium sulphate showed the best precipitation of the lipase with a two fold increase in its activity in the presence of SDS. The micrographs of SEM and TEM revealed the large sizes of the aggregates compared to the free lipase which is due to the cross-linking with glutaraldehyde. The CLEA showed more than 90% residual activity even after 10 cycles of repeated use.	Gupta et al. 2009
Candida rugosa	CLEAs from *Candida rugosa* were prepared using glutaraldehyde as the cross-linking agent. They were stable at 50°C and 60°C with	Kartal et al. 2011

Lipases	Result	Reference
	good reusability. They were able to retain 40% of the intital activity after 15 cycles of reuse in aqueous media with the maintenance of constant activity thereafter. Higher esterification in cyclohexane catalyzed by the produced CLEA in the presence of long fatty acids and alcohols as substrate molecules was witnessed.	
Geotrichum sp.	Cross-linking of the lipase in the presence of acetone and polyethyleneimine (PEI) was applied for hydrolysis of fish oil to enhance the n-3 PUFA content. 65% of relative degree of hydrolysis was maintained after the incubation in the range of 50-55°C for 4 h. They increased the DOH from 12% to 42%. After five batches of reuse, PEI-CLEAs still maintained 72% of DOH.	Yan et al. 2012
Thermomyces lanuginose (TLL)	CLEAs from this lipase were prepared by using ammonium sulphate (precipitant) and glutaraldehyde (cross-linker). The effect of hydrolytic activity of the CLEA was studied in the presence of various additives. Traditional non-layered CLEAs showed recovered activities ranging between 3 and 31% compared with native lipase. Novel TLL layered CLEAs consisting of a protein cofeeder core and successive layers of target lipase showed an improvement in the retained activity. One-layered non-additivated CLEAs of the enzyme showed the highest recovered activity of 75%.	Torres et al. 2013.
Candida Antarctica lipase B (CALB)	Highly active CALB-CLEAs were synthesised using layered methodology These CLEAs were characterized for their catalytic activity in three different	Torres et al. 2014.

Lipases	Result	Reference
	reaction conditions. It was found that the amount of cross-linker is the key parameter for the increased catalytic activity during storage. The hydrolytic activity of the matured layered CLEA retained an activity of 68%.	

Bioimprinting on the other hand, is prepared by the polymerization of the functional group and the cross-linking in the presence of template molecule. The template is made to imprint within the enzymes by polymerizing with the cross-linkers into a rigid enzyme. The template is then removed from the enzyme by continuous washing. This modified enzyme is known to have an imprint containing the functional group similar to the molecules in the substrate. These enzymes are known to increase the activity by the increased electrostatic and hydrogen bonding interactions between the surface residues of the enzymes (Fishman and Cogan 2003). They can be easily regenerated without any loss of affinity and therefore are highly specific to a particular reaction system. They are stable towards a wide range of solvents, metal ions and acid treatments (Kandimalla and Ju 2004). Unfortunately, this method of imprinting is successful in conditions when the enzymes are restricted to anhydrous environment or when dissolved in organic solvents. It so happens that when the enzymes are dissolved in aqueous conditions, the memory of the enzymes are lost or in other words the enzymes returns back to its usual conformation unless additional stabilization measures are adopted to prevent this change.

This technique was first studied on subtilisin described by Russel and Klibanov (1988) where subtilisin exhibited a 100 fold rate enhancement after lyophilisation in the presence of competitive inhibitor. Imprinting of chymotrypsin with D-amino acids resulted in an enzyme which was able to esterify the enantiomers successfully (Stahl et al. 1991). Nine different lipases when imprinted with n-octyl-β-D-glucopyranoside as an imprint molecule, increased the rate of acceleration and reaction yield (Navarro and Braco 1997). Okahata et al. (1995) described a method

for the modification of CRL which involved a combination of imprinting and coating with amphiphiles. The enzyme was coated with a range of coating molecules and imprinted with (R)-1- phenylethanol after which enantioselectivity of the enzyme increased by 14 fold. When *Geotrichum* sp. lipase was bioimprinted using palmitic acid as the imprint molecule and immobilized by crosslinking and applied to hydrolyze fish oil, the EPA and DHA content increased from 22 to 41 % in 8 h, compared to 30.2 % in 14 h obtained by free lipases (Yan et al. 2010). In another study using *Geotrichum* sp. lipase by Yan et al. (2009), a combined modification method including bioimprinting, pH tuning, lipid coating, salt activation, and immobilization improved the activity of the lipase in anhydrous organic solvents. The lipase thus modified exhibited 18.4 fold increase in the esterification activity for methyl oleate synthesis and retained 90% activity with the resuse in 10 cycles. The bioimpriting and immobilization of *Candida antarctica* lipase A (CALA) was optimized for various conditions by reponse surface methodology of which the enzyme to support ratio was found to be the most critical factor in the recovery of omega-3 PUFA in the glycerides fraction with the increasing amounts of enzyme. When applied for the ethanolysis of salmon oil, the decrease in the content of n-3 PUFA in the ethyl ester fraction reduced from 3.76 mol% to 1.47 mol% under the optimized conditions (Kahveci and Xu 2012). CRL and CALA lipases were prepared by imprinting and immobilization for the use in organic solvents. CRL bioimprinted with oleic acid exhibited a 8 fold increase in transesterification in hexane. The enzyme coated with lecithin after bioimprinting of the lipase did not improve the activity of the lipase. CALA was immobilized and imprinted, which did not result in any improvement in the activity of the lipase (Kahveci and Xu 2011).

CHAPTER 3

REFINING OF CRUDE INDIAN SARDINE OIL

3.1 RATIONALE BEHIND THE PROPOSED RESEARCH WORK

Due to the high demand for nutritionally rich fish oils, it becomes essential for the fish oil processing industries to come up with techniques to remove the undesirable components like phospholipids, FFA, glycerides, sterols, tocopherols, pigments, toxic substances such as heavy metals, dioxins or PCBs and metal salts (like copper, iron and mercury), which makes the oils unfit for human consumption (Morais et al. 2001). A refining process that removes the objectionable impurities from oil with the least possible effect on the nutritionally important components and loss of neutral oil have become significant since the research community is focusing on the quality of the fish oil for human consumption, pharmaceutical applications (Vaisali et al. 2015) and the source of high value dietary nutrient input for aquaculture (Tacon and Metian 2008).

Refining of crude oil performed by physical and chemical treatments, is frequently followed in the industries as they give good results by knocking out the impurities, but the destruction of desirable components due to harsh chemicals and high temperature seeks further improvement in technology. Enzymatic treatment employed to refine the crude oils is effective and eco-friendly, but could not be applied for industrial purposes since it is cost intensive. Although membranes could be used for all the stages of refining of oils, not much work has been done on it and very few applications have been reported (Vaisali et al. 2015).

Since limited reports are available on the refining of fish oil (Charanyaa et al. 2017, Vaisali et al. 2015) and a hunt for newer and gentler processes to replace the conventional oil refining methods, have necessitated the effective choice of refining methods specific to Indian Sardine oil. The present study inspects the practicality of the different stages of refining involving degumming, conventional and membrane based solvent extraction and bleaching for removing the impurities and consequently concentrating the n-3 PUFA content from the Indian Sardine oil. An effort was made to develop, design and optimize a sustainable integrated refining process with a further investigation and analysis of the effects of refining on physicochemical characteristics of oil after every stage of refining.

3.2 MATERIALS AND METHODS

3.2.1 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 -21°C in the dark. Orthophosphoric acid (OPA), lactic acid (LA), acetic acid (AC), methanol, ethanol, propanol, butanol, granulated activated charcoal (GAC), bentonite, activated earth, iso-octane, glacial acetic acid, p-anisidine, potassium iodide, sodium thiosulphate, potassium hydroxide, phenolphthalein indicator, wijis solution, chloroform were purchased from Merck, India. Membranes were purchased from Axivia, India. All the reagents (analytical grade) and solvents (chromatographic grade) were used without further purification.

3.2.2 REFINING METHODOLOGY OF CRUDE SARDINE OIL

The fish oil was refined through the steps, namely degumming, deacidification by conventional liquid- liquid extraction and membrane assisted deacidification and bleaching at various conditions (Figure 3.1). 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 200g of crude sardine oil with varied concentrations (% wt. basis) of the individual acids like OPA, lactic acid and acetic acid with the crude oil and stirred for 30 minutes. The oil was then centrifuged at 6000 xg for 20 minutes in order to remove the precipitated gums (Cmolik and Pokorny 2000). The effectiveness of the individual acids and optimum conditions were established by studying the various physicochemical characteristics of oil before and after the degumming process.

Deacidification is employed to remove the FFA from fish oils through solvent extraction. The gum free oil obtained after degumming (154g) was subjected to solvent extraction using various solvents such as methanol, ethanol, propanol, 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 stirred for an hour and then transferred to a separating funnel and left overnight for the phase separation (Kale et al. 1999). The phase separated oil was then analyzed for its FFA and solvent content. 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 in order to see if there was any further decrease in the level of 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 (Hamm 1992).

Membrane mediated solvent extraction was performed in the presence of hydrophobic membrane like polytetrafluoroethylene membrane (PTFE) (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 pressures up to 3 bars, was used for the present study (Figure 3.5). The phospholipid free oil was continuously stirred with methanol using a magnetic stirrer for an hour in the membrane unit. The impact of pressure on the FFA removal from the mixture was studied by varying vessel pressure by a nitrogen cylinder. The solvent free oil was obtained as permeate with the rejection of FFA, glycerides and solvent. Further, the permeate was analyzed for FFA and solvent content. The membrane performance indicators like FFA rejection in a membrane (Equation 3.1) and flux (Equation 3.2) were calculated as;

$$\text{FFA rejection in membrane} = \frac{(CR - CP)}{CR} \text{x} 100 \qquad (3.1)$$

$$\text{Flux (LMH)} = \frac{\text{volume of permeate (L)}}{\text{membrane area (square meter) x time (hours)}} \qquad (3.2)$$

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

Although, bleaching process was performed primarily to remove the coloured components from the oil, nowadays, it is modified to remove the impurities and oxidized products. The solvent treated oil (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 deacidified oil and required

amount of bleaching agent was mixed by a magnetic stirrer in a closed vessel and the process was carried out under vacuum. Further, optimization of various parameters like temperature (50°C, 60°C, 70°C, 80°C, 90°C), concentration of bleaching agents (3%, 5%, 7%, 9%, 11%, 13%) (w/w) and duration of bleaching (10 min, 20min, 30 min, 40min, 50 min, 60 min) were carried out to get good quality oil with lower FFA content and higher iodine value. Flow chart of refining process followed in this work is shown in Figure. 3.1.

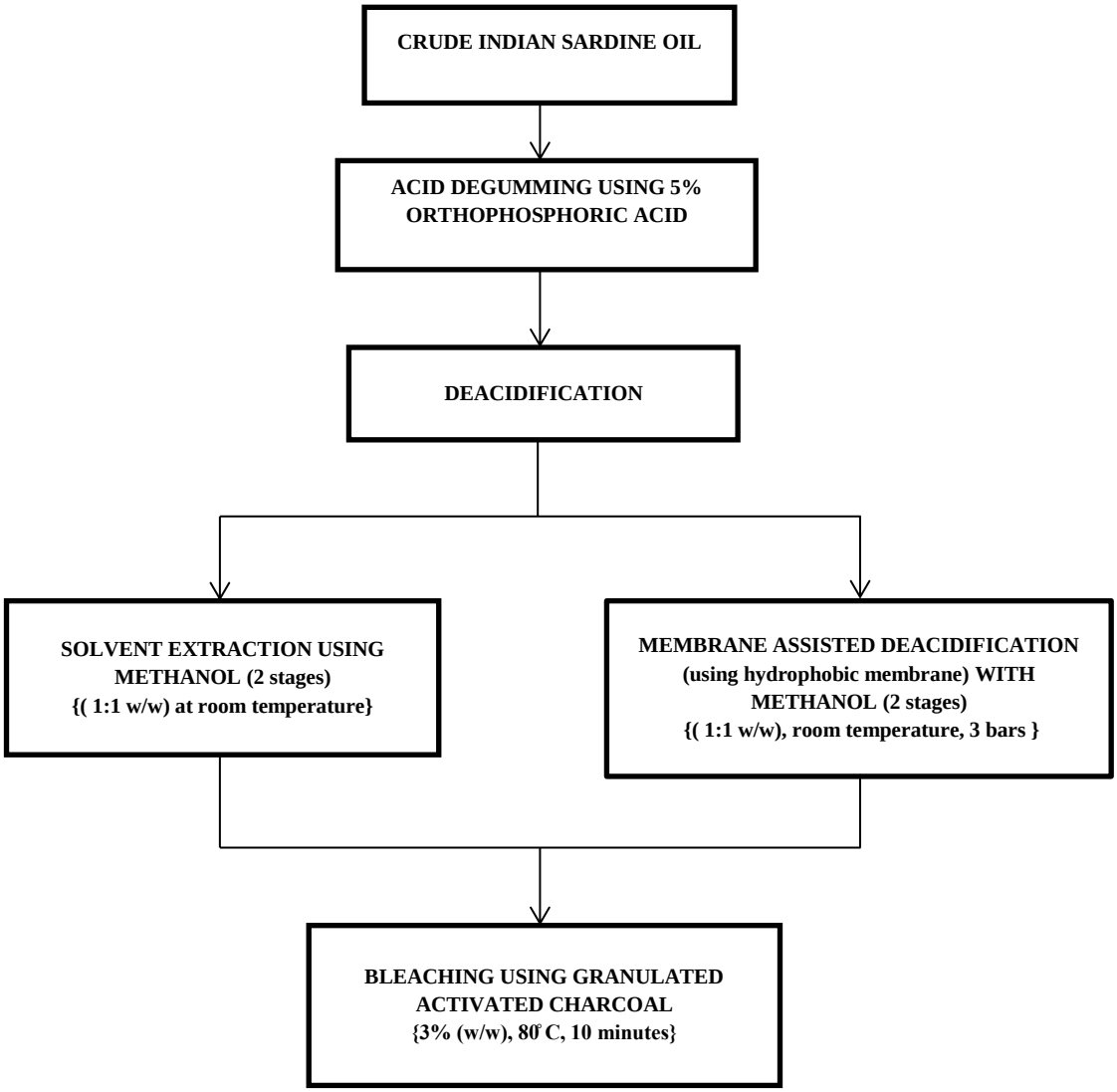


Figure 3.1: Scheme followed for refining of crude Indian Sardine oil.

3.2.3 *Characterization of the oils*

The phospholipid content of the oil during the degumming process was determined by a method adopted by Hundrieser et al. (1985) with minor modifications. The oil sample was prepared by dissolving 0.5 mL of oil in 0.5 mL of chloroform. The solvent present in the mixture was evaporated and cooled after which 0.4 mL of chloroform along with 0.1 mL of chromogenic solution (a mixture of ammonium molybdate, concentrated hydrochloric acid, mercury, concentrated sulphuric acid, methanol, chloroform and water) were added to the sample and heated in the water bath for 2 min and cooled. Further, 3mL of chloroform was added to this cooled oil sample and the oil layer was analyzed for phospholipid at an absorbance of 730 nm. The phospholipid content in the oil was found out from the standard graph which was generated using lecithin (phospholipid standard) dissolved in chloroform at various concentrations.

FFA content in the oil was determined by titration with KOH (0.1 N) with phenolphthalein as the end point indicator, according to the official method of American Oil Chemists' Society (AOCS, 2009) methodologies, (Cd 3d-63), expressed as oleic acid given by Equations (3.3 and 3.4).

$$Acid\ value, mg\ KOH/g\ of\ test\ portion = \frac{(A-B)\times M\times 56.1}{W} \quad (3.3)$$

$$FFA\ (\%\ of\ oleic\ acid) = \frac{Acid\ value}{1.99} \quad (3.4)$$

where, A is the volume (mL) of the standard alkali (KOH) used for the titration of oil (test) sample, B is the volume (mL) of the KOH used for the titration of blank sample, M is the molarity of KOH and W is the mass (g) of the test portion (oil).

Metal ion concentrations in the oil were determined by atomic absorption spectroscopy in accordance to the method followed by Aluyor et al. (2009). The oil samples which were acid digested and allowed to cool down for different metal analysis. Standards of Iron, Copper and Mercury were prepared with which the spectrometer was calibrated. The oil samples and standards were injected and analyzed by AAS. The detection limits of heavy metals in the samples were 0.0001

mg/L by AAS using GBC software. Iron, copper and mercury lamps were used for the detection of these ions, respectively. Air- acetylene gas mixture was used for generating the flame.

The peroxide value (PV) of the oil was determined according to the AOCS method (Cd 8b-90) as per equation (3.6). The p- Anisidine value (p-AV) of the oil was determined according to AOCS, (Cd 18-90) as per the equation (3.7). TV was calculated as in equation (3.5);

$$\text{TOTOX} = (2 \times \text{PV}) + \text{p-AV} \quad (3.5)$$

where, PV is peroxide value and p-AV is the p-anisidine value.

For the analysis of the peroxide value using acetic acid and isooctane method, the test oil sample (0.5g) was weighed in the flask and contacted and mixed with 3mL of acetic acid: isooctane solution (3:2) to dissolve the oil completely. 0.05 mL of saturated potassium iodide (KI) was added to the mixture and was allowed to stand for a minute by continuously shaking the solution thrice during the one minute incubation of the mixture. This mixture was immediately contacted with 3mL of distilled water and 0.1 mL of starch. Further, the titration of mixture with 0.1M sodium thiosulphate was carried out until the blue grey colour disappears in the upper layer and the lower layer exhibits a milky white appearance.

$$peroxide\ value\ (PV) = \frac{(S-B)\times M\times 1000}{W} \quad (3.6)$$

where, B is volume (mL) of the titrant for blank sample, S is the volume (mL) of the titrant for test (oil) sample and M is the molarity of sodium thiosulphate.

In order to determine the amount of aldehydes (principally 2-alkenals and 2, 4 dienals) in the oils, p-Anisidine value was found by the reaction in an acetic acid solution of the aldehydic compounds in the oil and p-Anisidine. The test sample (0.5g of the oil) was weighed and dissolved in 25 mL of isooctane. The absorbance of the oil sample was measured at 350 nm in spectrometer against pure isooctane. Further, 1 mL of p-Anisidine reagent was added to the 5 mL of oil solution (oil dissolved in pure isooctane) and was incubated for ten minutes. After exactly 10 minutes of incubation

the absorbance was noted at 350 nm against 5 mL of isooctane dissolved in 1mL anisidine reagent. The p-Anisidine value is given by the formula

$$p - Anisidine\ value\ (p - AV) = \frac{25\times(1.2\times Eb - Ea)}{W} \quad (3.7)$$

where, Ea is the net absorbance of the oil solution, Eb is the net absorbance of the oil-anisidine solution and W is the weight of the oil sample (g).

The fatty acid composition was estimated using GC (Trace 3330 GC Ultra, Thermoelectron Corporation) after converting the fatty acids to their respective esters (Ichihara and Fukubayashi 2010). Oil samples (50 mg) were collected from each stage of refining and each of these samples were subjected for an hour of incubation at 70°C in the presence of diethyl ether (200 µL) and hydrolyzed using 0.5 M methanolic sodium hydroxide (2 mL). The reaction mixture was acidified using 2M of 0.6 mL of hydrochloric acid to initiate the esterification reaction. The FFA released during the hydrolysis reaction was extracted with 1mL of hexane. The hexane was evaporated in vacuum and methylated with 10% of boron trifluoride in methanol (2.2 mL) for 20 min. The reaction mixture was cooled and further treated with 1 mL of hexane and water to extract the FAMEs generated during the course of reaction. The FAME enriched sample was injected into the GC for analysis using a DB-5 column with dimensions of 30m*0.25 mm*0.2µm and a flame ionization detector (FID) equipped with a split/splitless injector. FAME analysis was performed with an oven temperature of 160°C and maintained at this temperature for 1 min, after which the temperature was increased at the rate of 5°C/min until it reached 185°C. This temperature was maintained for 10 min with a further increase of temperature to 240°C at the rate of 8°C/ min. The third ramp was conditioned at a temperature of 240°C with a hold time of 10 min. The right inlet and the detector temperatures were 280°C and 300°C, respectively. Samples were prepared and analyzed in triplicates. FAME peaks of the oil sample were identified by comparing the retention time of FAME standards from Sigma Aldrich expressed as per cent of total fatty acids (%). Quantification of fatty acids was done by integration of peak area. Chromatograms were analyzed using Chrom Cad software.

3.2.4 *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 $p<0.05$.

3.3 RESULTS AND DISCUSSION

The sardine oil is an important source of n-3 PUFA, as EPA and DHA together represent one third of the total fatty acids (Gamez-Meza et al. 1999). The sardine oil procured from Mukka fish oil industry 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 TV (Table 3.1). Also, crude oil obtained by the traditional heat processing causes the dark coloration in the oil. The presence of such a high concentration of impurities and n-3 PUFA causes the oil to oxidize rapidly making refining a mandatory process. 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 stages to obtain good quality oil. A comparison of the compositions of various fish oils like capelin, Norway pout, mackerel, sardines, anchovy was well documented by Pike and Jackson (2010) which showed that the n-3 PUFA contents (EPA and DHA) were predominantly found in sardines followed by mackerel oil. Likewise, the 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.

3.3.1 Refining of crude sardine oil

3.3.1.1 Degumming

Degumming is the initial step in edible oil refining which removes phospholipids and some portion of trace metals and mucilaginous substances (Vaisali et al. 2015). Sardine oil was degummed through an acid degumming process by considering various acids like OPA (Orthophosphoric acid), LA (Lactic acid) and AC (Acetic acid). 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 (Dijkstra and Opstal 1989). 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 (Figure 3.2). This is because of the fact that OPA is much stronger acid (more polar) as compared to that of LA and AC. These acids are very reactive and will drop the pH much more than the other two acids. At low pH, hydratable and non hydratable phospholipid become hydrophilic and is easily separated by centrifugation. OPA helps in the removal of both hydratable and non hydratable phospholipids simultaneously. OPA is also known to be much cheaper and easily available than the organic acid (Cmolik and Pokorny 2000). 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 (Kanamoto et al. 1981). A slight increase in the phospholipid content beyond 5% could be due to the fact that the raise in pH was not sufficient for the dissociation of phosphatidic acid. Hence, 5% OPA was chosen for degumming of crude sardine oil. It was reported that OPA was capable of removing both the hydratable and non hydratable phospholipids simultaneously (Cmolik and Pokorny 2000). The results were consistent with the findings of De and Patel (2010) pertaining to degumming of rice bran oil. A similar result was reported by Eshratabadi et al. (2008) for the degumming process of soyabean crude oil, containing a phospholipid content of 454 ppm, using 2% of OPA. The analysis of phospholipid content in the degummed sardine oil was reduced by

57.31%. It was also noticed that degumming using OPA reduced copper, iron and mercury content by 92.65%, 38.6%, and 89.82%, respectively (Table 3.1). It has been observed that the metal ions tend to bind to phospholipids under low pH and gets precipitated along with phospholipids after getting hydrated. Similar trend was observed during the development of "Total degumming process" for soybean oil (Dijkstra and Opstal 1989).

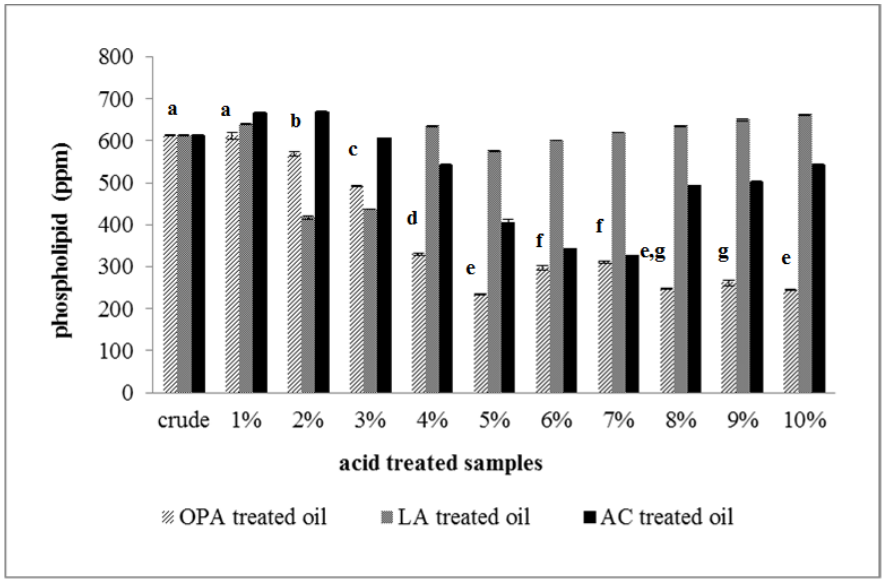


Figure 3.2: Effect of various acids and its concentrations on degumming of sardine oil. Statistical comparison was made among different concentrations of OPA (%) and means with the same letters are not significantly different at 0.05 probability level.

3.3.1.2 Deacidification by solvent extraction

3.3.1.2.1 Conventional solvent extraction

The solvent extraction of FFA was an alternative to the neutralization step in the conventional refining methodology with the reduction in the heavy loss of oils due to soap formation. This method removes the FFA at ambient condition without much loss of neutral oil and triglycerides. Besides, solvent stripping from oil, the recovery of solvent is easily done by vacuum evaporation or distillation at low temperature. Rodrigues et al. (2007) suggested that the short chain alcohols are the most suitable

solvents for the deacidification of palm oil and enhances flavor and aroma with reduced amounts of diacylglycerols and FFA in the palm oil. Hamm (1992), 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 3.3 depicts the performances of various solvents on the removal of FFA from the oil. It was observed that the highest removal of FFA was achieved by methanol when compared to the other three alcohols. As the chain length of alcohol increases, the hydrophobicity of the alcohol increases which results in an incomplete separation of solvents from the oil phase. The lowest chain length alcohol, methanol, hence provides a better phase separation along with the higher extraction selectivity for FFA (Raman et al. 1996). Cherukuri et al. (1999) also reported a similar finding for the deacidification of rice bran oil with a short chain alcohols namely methanol, ethanol and iso propanol.

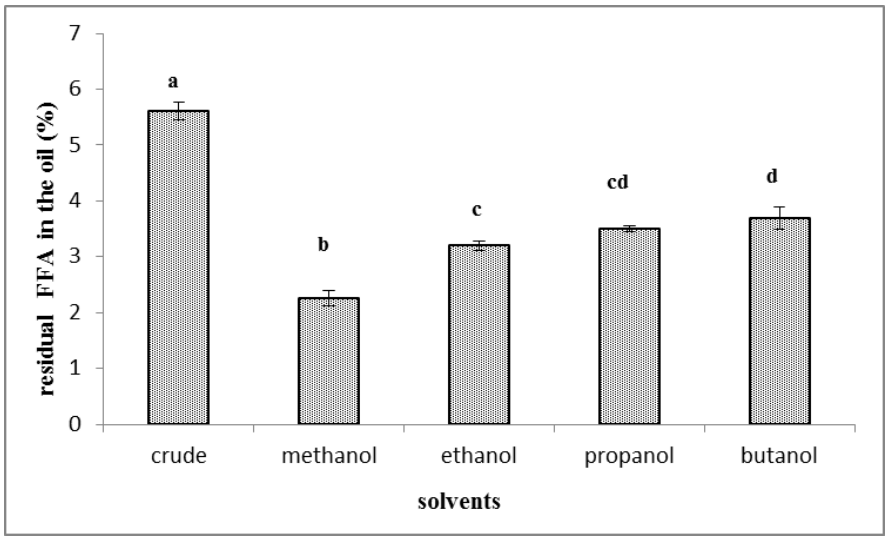


Figure 3.3: Performance of various solvents on deacidification of oil at 1:1 solvent to oil ratio. Statistical comparison was made among different solvents and means with the same letters are not significantly different at 0.05 probability level.

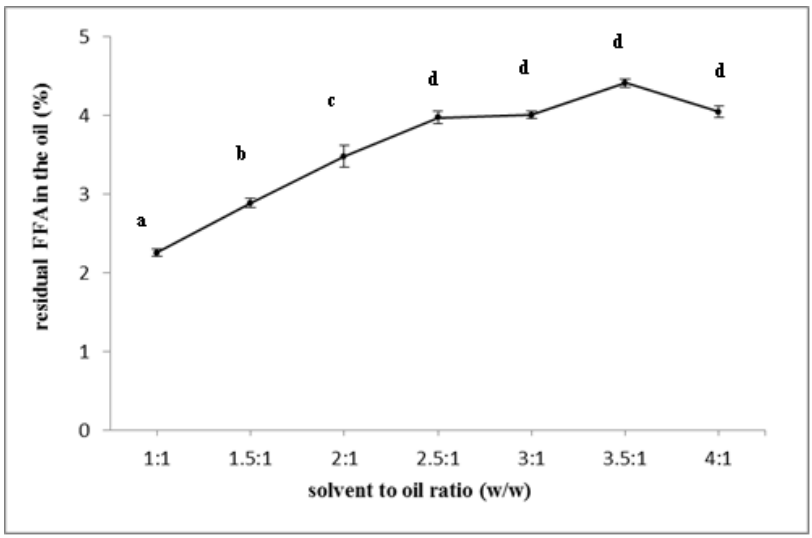


Figure 3.4: Effect of solvent to oil ratio on deacidification during the first stage solvent extraction. Statistical comparison was made among different solvent to oil ratios and means with the same letters are not significantly different at 0.05 probability level.

The amount of methanol required for the removal of FFA was studied by varying the solvent to oil ratio (w/w) (Figure 3.4). 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 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. Kale et al. (1999) 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. Also, the solubility of triglycerides increases in direct proportion to the FFA contents in the oil (Mariano et al. 2011). Higher oil loss was also observed as the number of extraction stage increases with the simultaneous reduction in FFA. Mariano et al. (2011) 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) (Rodrigues et al. 2007). 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 FFA of 5.64 %.

3.3.1.2.2 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 (Rodrigues et al. 2007). The solvent having dissolved FFA (extract) can be efficiently separated from glycerides using micro porous hydrophobic membranes (Raman et al. 1994). 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 (Manjula and Subramanian 2009). Membrane assisted solvent extraction of FFA from soya bean oil has been reported by Raman et al. (1996). Below is the schematic representation of the membrane unit adopted to deacidify the sardine oil in the present work.

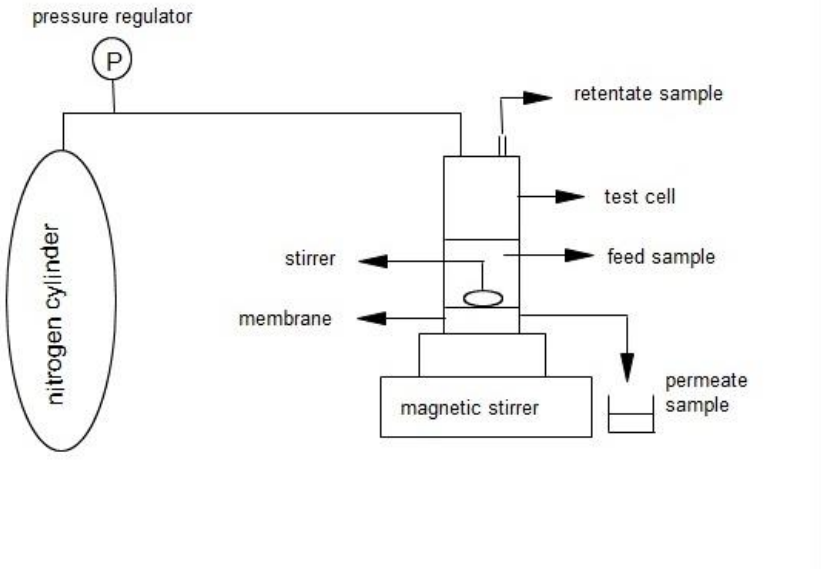


Figure 3.5: Schematic representation of experimental test cell

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 (Bhanushali et al. 2001). Since methanol has a very high selectivity for the FFA as compared to glycerides and due to the similar polarity (Raman et al. 1996), the FFA in the oil is entrapped within the micelles of polar methanol (Rao et al. 2013). A hydrophobic membrane, polytetrafluoroethylene (PTFE), was used in a flat sheet membrane separation module (Figure 3.5) to study the separation of micelle phase (extract) from the useful oil comprising glycerides (Raffinate). The hydrophobic nature of the membrane which is due to the presence of high electronegativity of fluorine, 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 solvents. In addition, the polar solvents like methanol have a lower flux through hydrophobic membranes as compared to that of hydrophilic membranes (Zwijnenberg et al. 1999). 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) (Figure 3.6 and Table 3.1) with a maximum permeate flux (Figure 3.7). These results are concurring with the observation of Rao et al. (2013).

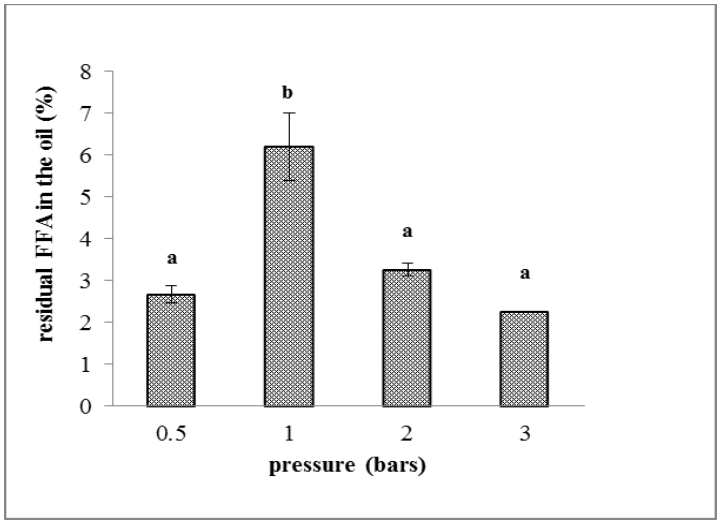


Figure 3.6: Effect of trans-membrane pressure on FFA removal from oil. Statistical comparison was made among different trans-membrane pressures

and means with the same letters are not significantly different at 0.05 probability level.

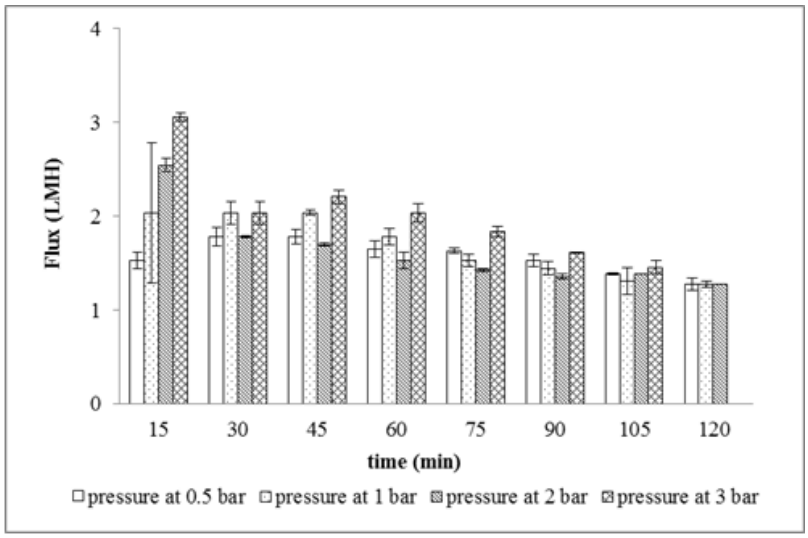


Figure 3.7: Effect of trans-membrane pressure on flux of oil at room temperature.

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 were 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 conventional solvent deacidification was found to be 25% during the first stage and 5% in second stage and it is 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 formation of presence of a small intermediate phase, perhaps due to the incomplete separation

of micelles, which forms a stable emulsion in oil-methanol mixture. Whereas in the case of membrane assisted deacidification, the repulsive behaviour of the hydrophobic membrane on the 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. The higher oil loss and the presence of solvent in the oil mediated by conventional solvent extraction compared to membrane deacidification could be because of these differences in the processing conditions of the two techniques. Hence, membrane deacidification makes the desolventisation an efficient process.

3.3.1.3 Bleaching

Bleaching process must take into consideration for the reduction of the colored pigments along with the removal of undesirable impurities from neutralized oils using bleaching agents. The use of activated charcoal as adsorbents for the bleaching process was used in soyabean, corn, palm, sunflower, rapeseed, cottonseed, peanut and linseed oil refining (Cooley et al. 1975, Moore et al. 1979 and Zosel, 1979). The success of a bleaching process depends on the ability of the bleaching agents to remove the colour impurities, metal ions and result in minimum oil retention. Therefore, a proper selection of bleaching agents plays a vital role in the bleaching process. Hence, the degummed and deacidified fish oil was treated with different bleaching agents like GAC, bentonite, and activated earth.

As FFA are known to increase the susceptibility of oils to oxidative degeneration, the efficiency of bleaching using different adsorbents, for various concentrations and durations of bleaching were selected based on the FFA content and iodine value of the oil as indicators. The deacidified oil at room temperature was used for bleaching at five different temperatures (50°C, 60°C, 70° C, 80°C and 90°C) under vacuum with 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). Temperature is one of the important process parameters that influence bleaching. It should be high enough to get a low viscosity of the oil, as it guarantees better dispersion of particles, improved

adsorbent oil interactions and flowability. Hence a temperature range from 60°C to 125°C is chosen for bleaching (Berbesi 2006).

The degummed and deacidified fish oil was treated with 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. Similar results were proposed by Habile et al. (1992) when the bleaching capability of earth was compared with GAC in bleaching of soyabean oil. Bleaching with earth led to excessive oil loss due to the presence of activated earth in the powdered form than the granulated form of activated charcoal in the mixture. Filtration/ centrifugation of oil after the bleaching with GAC are much easier than powdered form which results in reduced oil losses. 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 (Srikaeo et al. 2011, Moore and Yeates 1979, Zosel 1979). Hence, GAC was chosen as the bleaching agent for all the further studies.

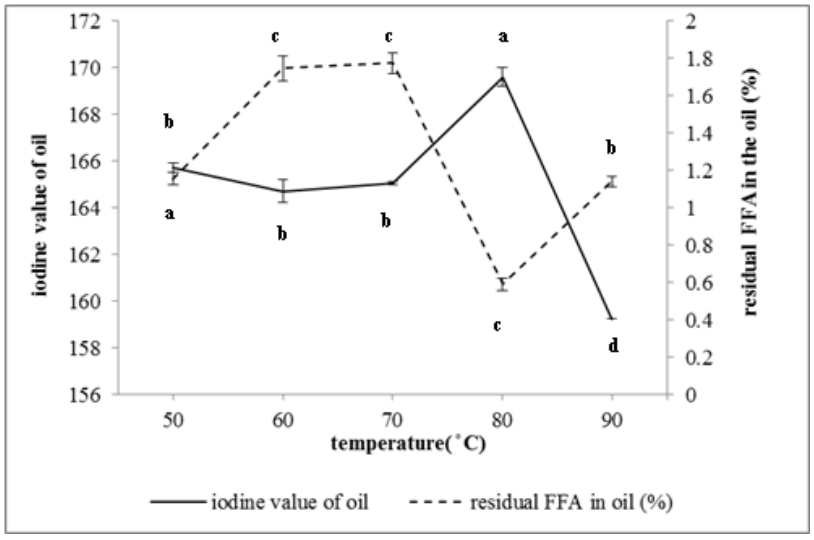


Figure 3.8: Effect of temperature on bleaching of oil under vacuum for 10 minutes. Statistical comparison of iodine value and FFA content were made among different temperatures and means with the same letters are not significantly different at 0.05 probability level.

The deacidified oil was bleached under vacuum at five different temperatures (50°C, 60°C, 70°C, 80°C and 90°C). The FFA content was observed to be the considerably low in the oil treated at 80°C (p<0.05) as compared to the oil treated at other temperatures (Figure 3.8). The reduced viscosity of the oil at high temperature helps to disperse the GAC 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 activation energy associated with the active sites of the adsorbent and resulted higher oil interaction with adsorbent and flowability (Zschau 2001). However, a sudden increase in FFA was noticed (Figure 3.8) at 90°C, could be due to the decomposition of glycerides. Further, iodine value was observed to be the highest at 80°C 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 (Fournier et al. 2007). Thus, to maintain the efficiency of the bleaching process, it is safer to maintain an upper limit of 80°C as it will minimize the probability of chemical or physical changes in the triglycerides in the oil. Similar results were reported by Jung et al. (1989) where the FFA content of olive oil increased at 90°C with the reduction in iodine value. Rich (1964) reported that the treatment of soyabean and cotton seed oil at 80°C resulted in reduction in color of the oil. Most of the oils are therefore treated in the temperature range of 80°C-100°C (Berbesi 2006).

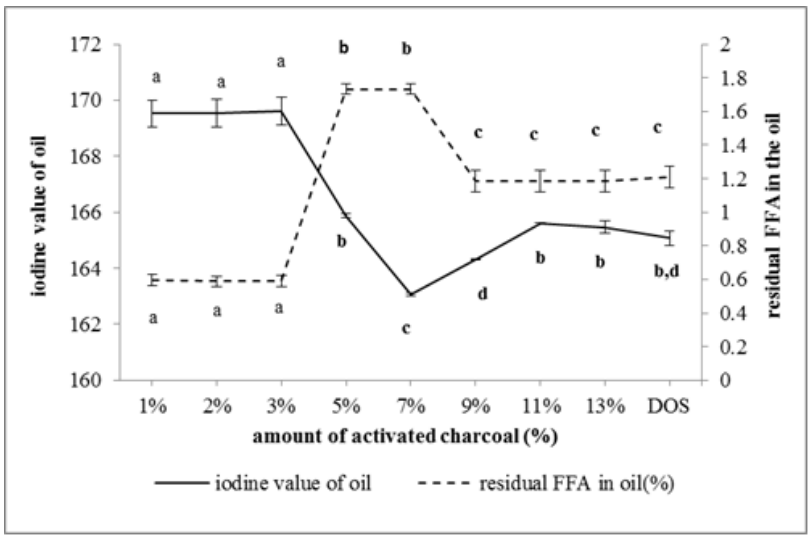


Figure 3.9: Effect of concentration of GAC on bleaching of oil at 80°C for 10 minutes in vacuum. (DOS-Deacidified oil sample). Statistical comparison of iodine value and FFA content were made among different concentrations of GAC and means with the same letters are not significantly different at 0.05 probability level.

The bleaching was carried out at 80°C 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) (Figure 3.9). 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 had not only led to an 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 (Mag 2007). Since the FFA in the oil increases, it results in the oxidation of oil by the addition of double bonds in the color pigments which led to the increase in color of the oil, a phenomenon called color reversion (Cmolik and Pokorny 2000). Hence 3 % (w/w) GAC is 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 (Diaz and Santos 2001).

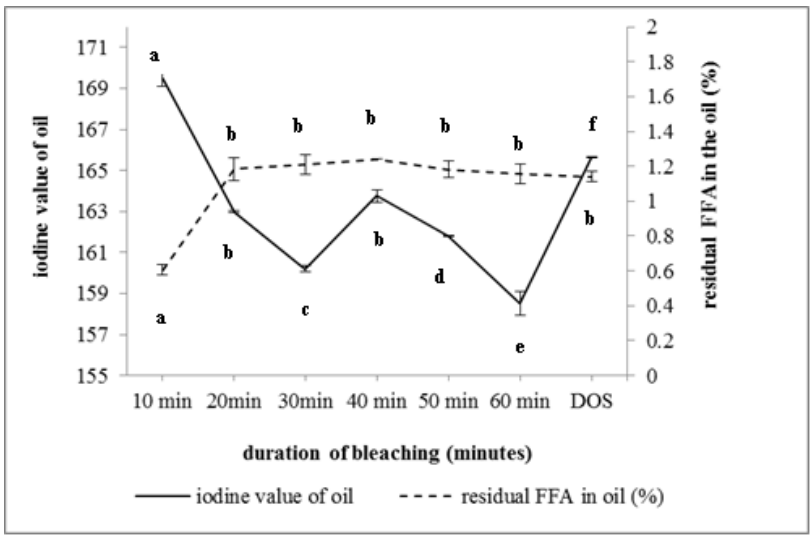


Figure 3.10: Effect of duration of bleaching on oil at 80°C with 3% (w/w) of GAC. (DOS- Deacidified oil sample). Statistical comparison of iodine value and FFA content were made among different time intervals and means with the same letters are not significantly different at 0.05 probability level.

The bleaching was then carried out for different durations at 80°C with 3% (w/w) GAC. It was observed (Figure 3.10) 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 (Berdeaux et al. 2007). Fournier et al. (2007) found that residence time and temperature has a maximum impact on trans-fatty acid formation in fish oils. Berbesi (2006) stated that the contact time for effective bleaching typically ranges from 10 to 45 minutes in palm oil. Hence bleaching was performed for 10 minutes at 80°C in the presence of 3% (w/w) of GAC. The obtained results concur with Berbesi's observation that contact time for effective bleaching typically ranges from 10 to 45 minutes for palm oil and 20 to 30 minutes being the most common (Berbesi 2006).

3.3.1.4 Characteristics of oil at different stages of refining

Table 3.1 shows the characteristics of the oil at each refining stage. It provides the trend of variation in the quantities of the FFA, phospholipids, and metal ions during the refining steps of sardine fish oil. The oil obtained after the complete refining of fish oil was analyzed for its physical quality and chemical composition (Table 3.2). Oil had the specific gravity of 0.92, a density of 0.91 g/cc 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 (Bimbo 1998). The chemical quality of the oil was also observed to be in the range as mentioned by Bimbo (1998). The yield after degumming the crude oil was 77%. The subsequent yields of oil for the 1^{st}, 2^{nd} 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 (Rodrigues et al. 2007) 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 (Zschau 2001).

Table 3.1: Characterization of Indian Sardine oil during various stages of refining

Samples	% FFA	Phospholipid (ppm)	TV	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

Samples	% FFA	Phospholipid (ppm)	TV	Metal ions (ppm)		
				Copper	Iron	Mercury
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.

From Table 3.1, 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 (Patterson 1992). The refining process reduced the TV by 75.68% of crude oil. However, in the second stage of solvent extraction, the TV increased. This may be due to the increase in iron content (Fe^{++}), maybe because methanol acted as an electron donor to produce ions (Fe^{++}) which acts as the pro-oxidant and hence there is an increase in the TV. But, TV 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. The moisture content of the crude sardine oil was reduced from 0.22% to 0.12%, which lies within the standard quality value (<0.2%) (Bimbo 1998). The improvement in the oil quality after refining thus reduces the susceptibility of the oil to rancidity and improves its stability.

Table 3.2: 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^{ab}	48.9±1.49^{b}	46.87±1.84^{ab}
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}
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}
DHA	5.20±0.07^{a}	5.46±0.08^{b}	5.81±0.03^{c}	5.89±0.02^{cd}	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 $p<0.05$.*

Predominant fatty acids present in oil were estimated at every stage of refining to understand the change in their concentration (Table 3.2). 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 the 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 3.2 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) (Tengku-Rozaina and Birch 2013). 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 progressive loss of EPA during refining along with the rest of the FFA. Whereas 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 will not take place, and thus a concomitant enrichment was witnessed. This result concurs with the findings of Noriega-Rodriguez et al. (2009) on sardine (*Sardinops sagax* caerulea) oil refining. In this discussion, more emphasis was 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 (Table 3.2 and Figure 3.11).

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°C) deodorization step using steam (Biernoth and Rost 1967). It also effectively removes undesirable metal ions, FFA and phospholipids without causing destruction to n-3 PUFA.

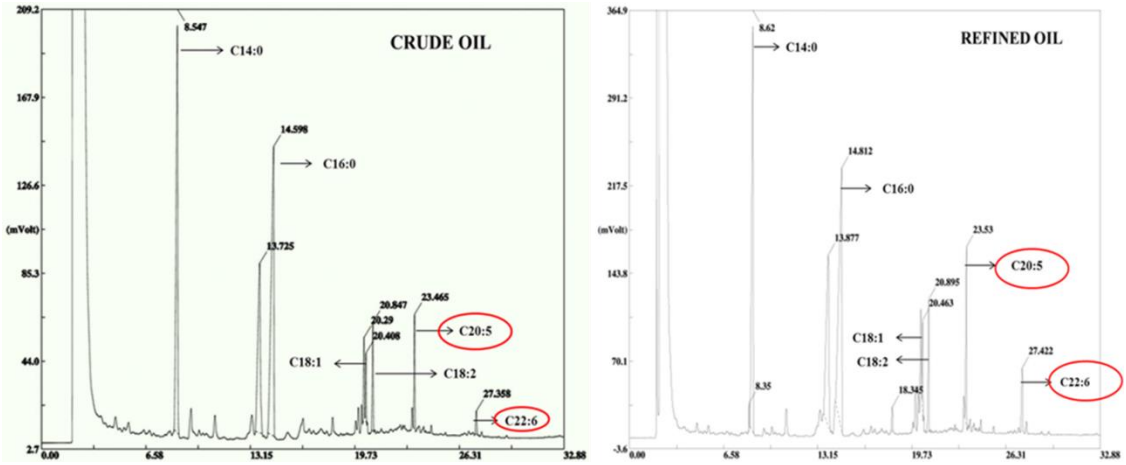


Figure 3.11: GC chromatograms of crude and refined sardine oil

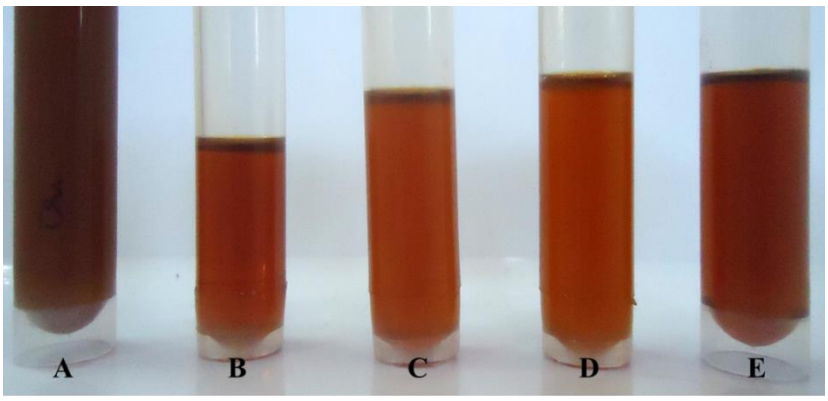


Figure 3.12: Visualization of the oil during different stages of refining. Crude Sardine oil (A), OPA degummed oil (B), 1st stage methanol deacidified oil (C), 2nd stage methanol deacidified oil (D), GAC assisted bleached oil (E).

3.4 SUMMARY AND CONCLUSIONS

Table 3.3: Summary of refining process and characteristics of refined oil

Stage	Materials/ reagents used	Effective materials / reagents	Conditions involved in the study	Optimized conditions	Characteristics of the oil at optimized conditions.			
					PL (ppm)	FFA (%)	EPA (%w/w)	DHA (%w/w)
1	-	-	-	-	612.66	5.64	11.19	5.20
2	OPA, LA, AC	OPA	(1-10% OPA) stirred for 30 min and centrifuged at 6000xg for 20min	5% OPA	261.5	4.5	10.09	5.46
3	Methanol,ethanol, propanol, butanol	Methanol	1:1, 1.5:1, 2:1, 2.5:1, 3:1, 3.5:1, 4:1 (w/w) and stirred for an hour at room temperature	1:1 mathanol to oil ratio	156.6	2.26	11.28	5.81

Stage	Materials/ reagents used	Effective materials / reagents	Conditions involved in the study	Optimized conditions	Characteristics of the oil at optimized conditions.			
					PL (ppm)	FFA (%)	EPA (%w/w)	DHA (%w/w)
4	Methanol, ethanol, propanol, butanol	Methanol	1:1, 1.5:1, 2:1, 2.5:1, 3:1, 3.5:1, 4:1 (w/w) and stirred for an hour at room temperature	1:1 mathanol to oil ratio	79.66	1.13	11.56	5.89
5	Methanol, ethanol, propanol, butanol	Solvent: Methanol Membrane: PTFE	(0.5 bar, 1 bar, 2 bar, 3 bar) at room temperature	3 bar	-	2.25	-	-
6	Methanol, ethanol, propanol, butanol	Solvent: Methanol Membrane: PTFE	(0.5 bar, 1 bar, 2 bar, 3 bar) at room temperature	3 bar	-	1.09	-	-
7	GAC, bentonite, activated earth	GAC	GAC load: (1%, 2%, 3%, 5%, 7%, 9%, 11%, 13%) Temperature : (50°C,60°C,70 °C, 80°C and 90°C) Duration: (10, 20, 30, 40, 50, 60 min).	GAC load: 3% (w/w) Temperature : 80°C Duration : 10 min	5.66	0.56	11.81	6.1

**1-Crude oil; 2- Stage of Degumming; 3- 1st stage of conventional solvent extraction; 4- 2nd stage of conventional solvent extraction; 5- 1st stage of*

membrane deacidification; 6- 2nd stage of membrane deacidification; 7- stage of bleaching.

- 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 an essential process in order to guarantee the quality of the oil. The current refining strategy employed sheds light on the optimization of the various parameters with the view to either maintain or enhance the n-3 PUFA content of the oil.
- The tailor made strategy in the present work includes degumming, deacidification by solvent extraction (which replaces the conventional alkali neutralization) and bleaching.
- 5% OPA resulted in the maximum removal of phospholipids. The nature of OPA caused degumming of hydrophobic and hydrophilic phospholipids simultaneously.
- The improvement in deacidification was achieved by employing PTFE membrane assisted solvent extraction. Lesser oil loss and solvent content in the oil, the better efficiency of FFA separation through the membrane were found to be some exquisite attributes revealed from this process.Bleaching in the presence of GAC with the exclusion of high temperatures was successful in effectively removing undesirable metal ions, FFA and phospholipids without causing any damage to n-3 PUFA.
- During our studies, we observed a distinguished enhancement in terms of stability of the oil and the n-3 PUFA content from 16.39 % (11.19 Eicosapentaenoic acid (EPA) + 5.20 Docosahexaenoic acid (DHA)) to 17.91 % (11.81 EPA + 6.1 DHA) under the optimized conditions of refining.
- The strategy employed produced satisfactory results of physical and chemical quality of sardine oil.
- This approach 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. The efficiency of the formulated refining strategy was thus vindicated.

CHAPTER 4

STORAGE STABILITY OF REFINED INDIAN SARDINE OIL

4.1 RATIONALE BEHIND THE PROPOSED RESEARCH WORK

The crude oil extracted from Sardines by physical methods consists primarily of triacylglycerides (TAGs), which contain fatty acids of various chain lengths (including EPA and DHA) in addition to 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. 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, photo-oxidation, 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 and 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 and 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. The extent and the rate of spoilage strongly depend upon storage conditions (extrinsic factors), presence of several impurities and fatty acid profile.

Environmental conditions like air, heat, light, moisture and other intrinsic factors like phospholipids (PL's), phospholipases (PLS), FFA and metal ions have found to enhances oxidative and hydrolytic spoilage (Vaisali et al. 2015, Chaiyasit et al. 2007, Naz et al. 2004). The rate and mechanism of oxidation in fish oils containing n-3 PUFA is considerably different from the other oils and also the oxidative and hydrolytic stability vary greatly, depending on fish species (Boran et al. 2006). Several studies on the effects of individual factors on the oxidative stability of the vegetable and marines oils are available. However, no reports are available on the holistic effects of environmental conditions, intrinsic factors and storage time on the quality of the n-3 PUFA rich Indian Sardine oil. It is therefore the thrust of this work to study the effects of extrinsic factors and intrinsic factors on the quality of the

Indian Sardine oil, which is rich in n-3 PUFA with the ultimate goal of identifying the most important factor/s which affects the storage stability of n-3 PUFA in the oil. The findings of this study could reveal the oxidative properties of the Indian sardine oil of different varieties in long term storage and also the changes in the n-3 PUFA compositions.

4.2 MATERIALS AND METHODS

4.2.1 MATERIALS

Crude Indian Sardine oil was refined by the strategy adopted by Charanyaa et al. (2017). The crude oil was degummed using 5% 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. Repeated solvent extraction and bleaching under the same conditions were performed to get the impurities below detection limits (Table 4.1). 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.

The oil samples considered in this work were divided into two groups; one set of oil samples were kept under the influence of environmental conditions (extrinsic factors) namely light intensity, temperatures, moisture and combination of all extrinsic factors (C1) (a total of 16 flasks) and another set of oil samples (a total of 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 the sections. All the experiments were conducted in duplicates along with control flasks (in duplicate) to eliminate experimental error.

4.2.2 METHODS

4.2.2.1 Effect of environmental factors on the stability of the oil

4.2.2.1.1 Effect of light exposure

10g each of refined sardine oil was subjected 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 analyzed for TV and FFA values.

4.2.2.1.2 Effect of temperature

10g each of refined sardine oil was stored 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.

4.2.2.1.3 Effect of moisture content

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.

4.2.2.2 Effect of intrinsic factors on the quality of the oil

4.2.2.2.1 Effect of metal ions, PLS, PL, FFA

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 and purged with nitrogen and sealed. The samples were collected for chemical analysis every week till the end of five weeks.

4.2.2.3 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).

4.2.2.3.1 Combined effect of prominent extrinsic factors (sunlight, moisture and temperature (30°C)) (C1)

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).

4.2.2.3.2 Combined effect of intrinsic factors (ferric chloride, Phospholipase-A, Phosphotidylcholine, Oleic acid) (C2)

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.

4.2.3 *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 terms of TV and FFA 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. This was done by weighing 0.1 g of oil and neutralised with 5 mL of iso propanol to make sure that the oil is completely dissolved before titrating.

The test portions are shaken vigorously by adding phenolphthalein indicator and titrated it with standard alkali (0.1 N KOH) until a permanent pink colour of the same intensity is produced. The calculation of the acid value and FFA were performed according to the Equations (4.1 and 4.2);

$$Acid\ value, mg\frac{KOH}{g} of\ test\ portion = \frac{(A-B)\times M\times 56.1}{W} \quad (4.1)$$

$$FFA\ (\%\ of\ oleic\ acid) = \frac{Acid\ value}{1.99} \quad (4.2)$$

where A is the volume (mL) of the standard alkali (KOH) used in titrating the oil (test) sample, B is the volume (mL) of the KOH used in titrating the blank sample, M is the molarity of KOH and W is the mass in grams of the test portion (oil).

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). The oil samples which were acid digested and allowed to cool down for different metal analysis. Standards of Iron, Copper and Mercury were prepared with which the spectrometer was calibrated. The oil samples and standards were injected and analyzed by AAS. The detection limits of heavy metals in the samples were 0.0001 mg/L by AAS using GBC software. Iron, copper and mercury lamps were used for the detection of these ions, respectively. Air-acetylene gas mixture was used for generating the flame.

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, after drying it in an oven at 100°C. The results were expressed as percentage of wet weight (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 (2009) methods. TV is given by the Equation (4.3);

$$TV = (2 \times PV) + p\text{-}AV \quad (4.3)$$

The procedures and equations related to peroxide value and p-anisidine values are detailed in Chapter 3 under section "Characterization of oils" in Materials and Methods section.

Fatty acid methyl esters (FAME) were prepared by the method followed by Ichihara and Fukubayashi (2010) by 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) by maintaining the GC at conditions as mentioned by Charanyaa et al. (2017). 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. FAME peaks of the oil samples under the influence of prominent extrinsic and intrinsic factors were identified and quantified by comparing the retention time of the samples with FAME standards from Sigma Aldrich and expressed as per cent of total fatty acids (%).Chrom Cad software was used for the analysis of the chromatograms.

4.2.4. *Statistical analysis*

TV and FFA 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. The statistical significance is specified by appropriate letters within all the figures.

4.3 RESULTS AND DISCUSSION

The presence of higher quantity of polyunsaturated fatty acids in the fish oil, 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 Indian Sardine oil were determined under different storage conditions with the addition of various impurities commonly found in crude Indian Sardine oil. The aim

of the current investigation was to identify the 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.

Refined Indian Sardine oil with the initial characteristics as shown in Table 4.1 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 environmental conditions and the intrinsic factors typically affecting the evolution of FFA and TV in sardine oils are presented in the Figures 4.1- 4.4. Figure 4.1 (A and B), 4.2 (A and B), 4.3 (A and B) and 4.4 (A and B) displays the development in the FFA and TV of oil samples under the influence of the various factors. The values represented here are obtained at 95% confidence level.

Table 4.1: Composition of Refined Indian Sardine oil

Parameters	Values
Free fatty acid (% oleic acid)	BDL*
Phospholipid (ppm)	BDL*
Iron (ppm)	BDL*
Copper (ppm)	BDL*
Mercury (ppm)	BDL*
TV	24.36
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

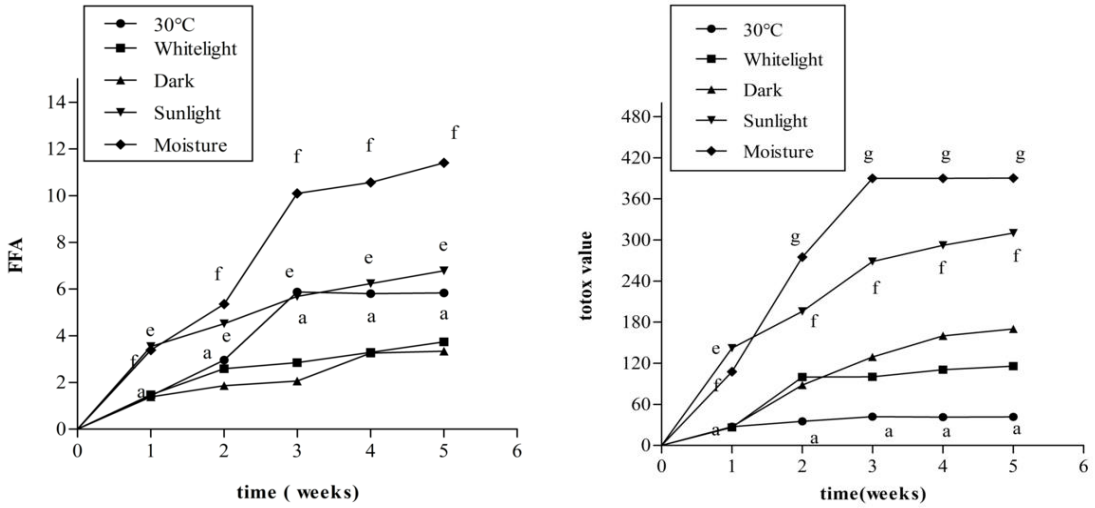
4.3.1 Effect of extrinsic parameters on the storage stability

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. Possibly, the first critical observation that can be deduced from Fig.4.1- 4.4 is that there is an increase in the FFA and TVs for prolonged storage times which are in accordance with Naz et al. (2004), Boran and others (2006).

Sunlight was verified to show highest effect on storage instability compared to white light and darkness. Sardine oil exposed to sunlight was found to attain highest FFA and TV (p<0.05) indicating higher hydrolytic and oxidative instability compared to the oil samples maintained under white light and dark conditions during five weeks storage (Figure 4.1(A) and 4.1(B)). It was found that PV change was minimal in all the oil samples, whereas p-Anisidine value 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 and TVs of 115.76, 170.24, 310.06 in the fifth week for samples placed in dark, white light and sunlight respectively were recorded. The obvious variations in the FFA and TV for three samples could be due to the preferential choice of light by the oil samples and the intensity of the source. These results indicate that as the light intensity increased, hydrolytic and oxidative instability also increased. Also, the exposure of oil to sunlight increased the temperature of the oil (by few degrees), leading to 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 and Min 2006 and 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.

Oil samples stored at 30°C in the dark conditions recorded the highest FFA and TV (p<0.05) followed by -21°C (Figure 4.1(A) and 4.1(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 pAV, 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 and 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.

Thirdly, the presence of micelles formed by moisture in the bulk refined oil impacted the stability of the oil by increasing the FFA and TV during five weeks storage (Figure 4.1 (A) and 4.1(B)) (p<0.05). The FFA content and TV increased by 1.95 and 9.32 times the control values. 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 and Decker 2000). These association colloids act as reaction site for lipid oxidation and forms volatiles (Kim et al. 2014). The increase in FFA and TV is due to the pro-oxidant property of moisture which acts as the substrate and hydrolyze the oils to produce off flavours. (Meng et al. 2012 and 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.



(A) **(B)**

Figure 4.1: Experimental data on the influence of extrinsic factors (30°C, light and moisture) on FFA release (A), Experimental data on the influence of extrinsic factors (30°C, light and moisture) on the TV in the oil for five weeks (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 in one particular week are not significantly different (*P*<0.05) by Tukey's test.

4.3.2 Effect of intrinsic parameters on the storage stability

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

Indian 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. It becomes a prerequisite to attain a proper knowledge on the role of transition metals in the bulk oil to develop strategies to protect the oil from oxidation for the effective extension of shelf life of the oil. Comparative analysis of FFA and TV of the oil samples containing them shows that iron displayed the strongest pro-oxidant activity in the oil (p<0.05). 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 and Shibamoto. 2008). A marginal increase in TV (1.25 and 1.2 times) was recorded in oil samples containing Cu^{3+} and Hg^{3+} compared to control (Figure 4.2(B)), whereas FFA decreased by 39.4

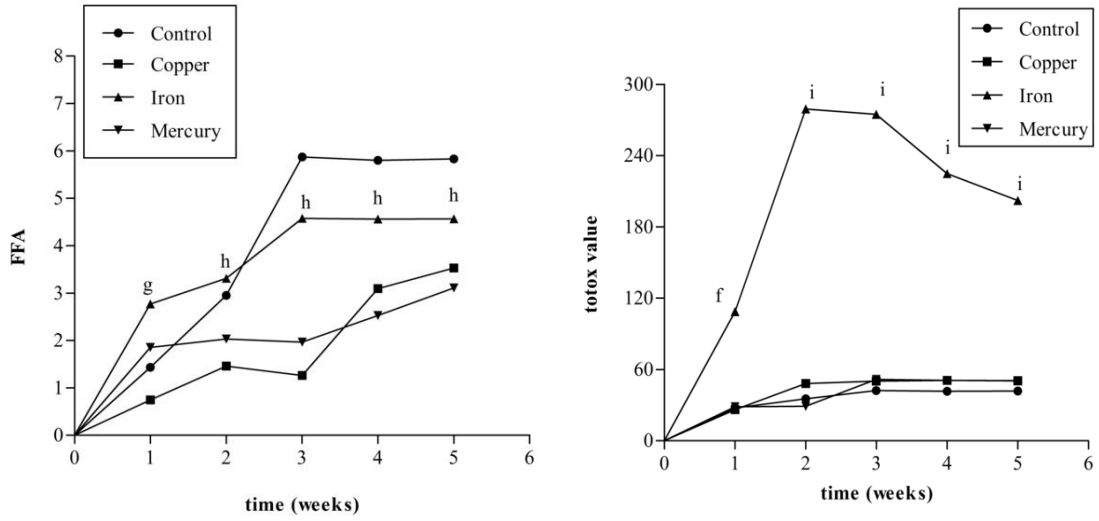
% and 46.6 % respectively (Figure 4.2(A)). Significant increase in TV was recorded in oil samples containing Fe^{3+} (4.83 times) (Figure 4.2(B)) whereas the FFA decreased by 21.7 % similar to other metal ions studied. This indicates these transition metal ions did not lead to the hydrolytic instability of oil during five weeks storage. 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. The increase in the TV 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 Fe^{3+} 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. To account for the dip in TV in the oil, it is possible that the free iron concentration for the interaction with the oil droplets decreased due to its absorption into the micelles. (Choi et al. 2009). Another potential reason that could decrease the interaction between iron-lipid hydroproxides is because of the thick barrier at the interface. (Chaiyasit et al. 2007). Similar results were reported by Kapchie et al. (2013) in Soyabean oil system by ferric ions.

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 (Figure 4.3(A) and 4.3(B)) ($p<0.05$). 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 Phosphotidyl choline 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, Nwoso et al. (1997) have reported that PL (Phosphotidyl choline) 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 and 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. 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.

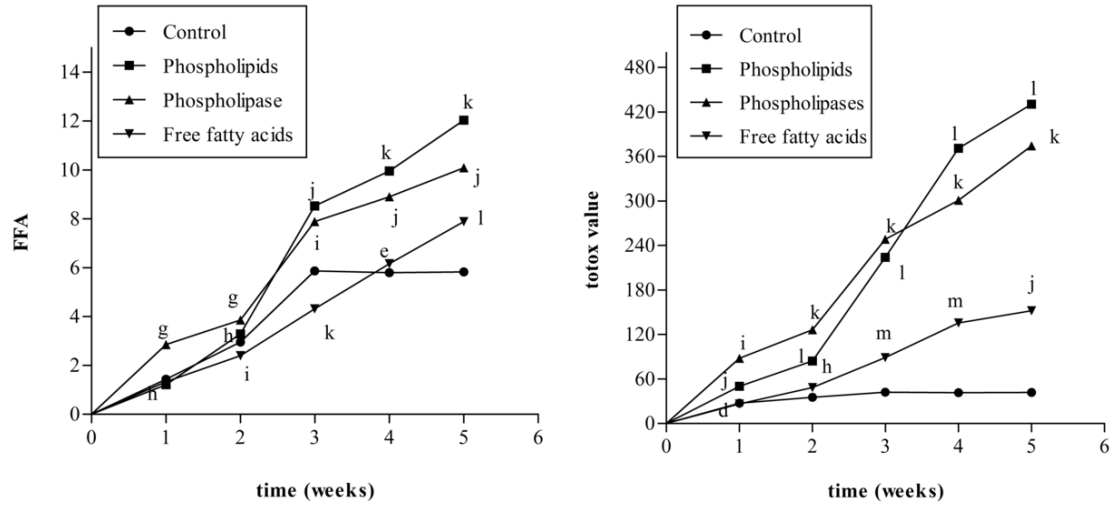
It is clear from Figure 4.3(A) and 4.3(B), that the addition of PLS also resulted in the steady increase in both FFA (1.7 times) and TV (8.94 times) as compared to control over five weeks of storage. As PLS have the ability to hydrolyze glycerides, inclusion of PLS is expected to increase FFA in the bulk oil steadily. Perhaps the release of FFA could have caused the rapid breakdown of hydroperoxides leading to the high TVs (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.

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 (Figure 4.3(A) and 4.3(B)). The FFA has exhibited pro-oxidant properties in bulk oil time and again. The pro-oxidant property is attributed to the carboxyl group, which form free radicals by the decomposition of hydroperoxides (Miyashita and 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). Paradiso and group (2010) on the effect of increasing amounts of various forms of FFA on olive oil confirmed the pro-oxidant activity of FFA. The results obtained here is in consonance with the above reports.



(A) **(B)**

Figure 4.2: Experimental data on the influence of metal ions on FFA release (A), Experimental data on the influence of metal ions on the TV in the oil for five weeks (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 in one particular week are not significantly different (*P*<0.05) by Tukey's test.



(A) **(B)**

Figure 4.3: Experimental data on the influence of PL, PLS and FFA on FFA release (A), Experimental data on the influence of PL, PLS and FFA on the TV (B) in the oil for five weeks. Statistical comparison was made among all the

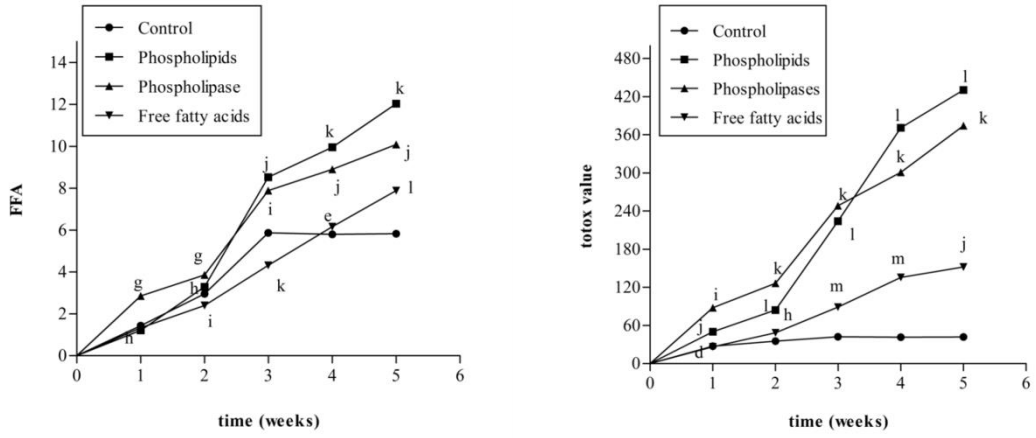
factors chosen for the study for individual weeks. The means with the same letters for the different factors in one particular week are not significantly different (p<0.05) by Tukey's test.

4.3.3 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. FFA values attained were 2.45 times the FFA noted in case of moisture alone at the end of fifth week. TV values attained by the end of fifth week were less than the TV values attained in the presence of moisture alone (Fig.4.4 (B)). Also, the pro-oxidant activity of water in bulk oil is significantly impacted by the increase in temperature of the oil influenced by sunlight because of vapour pressure of water in oil (Park et al. 2014). Interestingly, TV attained by the end of 4th week by C1 was slightly higher than the TV attained in the presence of moisture alone. TV started declining in the fifth week for C1 (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.

Intrinsic parameters showing prominent effect on storage instability of oil were chosen and experiments were conducted to ascertain their combined effect. Ferric chloride, Phospholipid (PL), Phospholipase-A(PLS) and free fatty acid (FFA) were added to oil samples (1000 ppm) (C2), 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 (Figure 4.4 (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 (Figure 4.4 (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 pAV and in both the cases PV values recorded throughout were negligible compared to pAV. 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 and Min 2008). Escape of volatile molecules and formation of epoxide groups might have resulted in a downward trend in TV.



(A) **(B)**

Figure 4.4: Experimental data on the influence of C1 and C2 on FFA release (A), Experimental data on the influence of C1 and C2 on the TV (B) in the oil for five weeks. Statistical comparison was made among the factors which led to

maximum deterioration of oil for individual weeks. The means with the same letters for the different factors in one particular week are not significantly different (p<0.05) by Tukey's test

4.3.4 Effect of prominent factors on n-3 PUFA content of fish oil during five weeks storage

Table 4.2: Impact 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±0.001	$8.23±0.74^{dc}$
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±0.002	$8.3±0.25^{cd}$
Combination 1 (C1)	7.23±0.56	2.49±0.07	$9.72±0.63^{cd}$
Combination 2 (C2)	12.06±0.16	3±0.08	$15.06±0.25^{b}$

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. A statistical elaboration by the means of ANOVA provided the factors which significantly affected the oxidation of the oil (p<0.05) (Table 4.2). 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 (Figure 4.5). The DHA and EPA content of oil samples were estimated after five weeks storage and are reported. Highest n-3 PUFA deterioration was observed pertaining to moisture (p<0.05) and lowest was observed in oil samples having phospholipases, phospholipids and C2 (p<0.05) (Table 4.2). 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 and 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.

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 accords 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 (Figure.5). Oil samples containing PL and PLS showed very low n-3 PUFA deterioration (20 and 15 % respectively) (p<0.05) 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 (p<0.05). On the contrary, oil samples exposed to FFA and Iron independently showed maximum n-3 PUFA destruction (57 and 47 % respectively) (p<0.05). This shows that PL and PLS retarded n-3 PUFA deterioration. A study by Choe and 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. Individual PL compounds have varying oxidative stability depending on the fatty acid profile and the presence and nature of polar heads. It was indicated that phosphatidylinositol exhibited highest oxidation rates due to the presence of high PUFA percentage, followed by phosphtidylethaolamine, phosphatidylserine and lastly phosphatidylcholine (Pikul and Kummerow 1990). Nwoso 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 high 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.

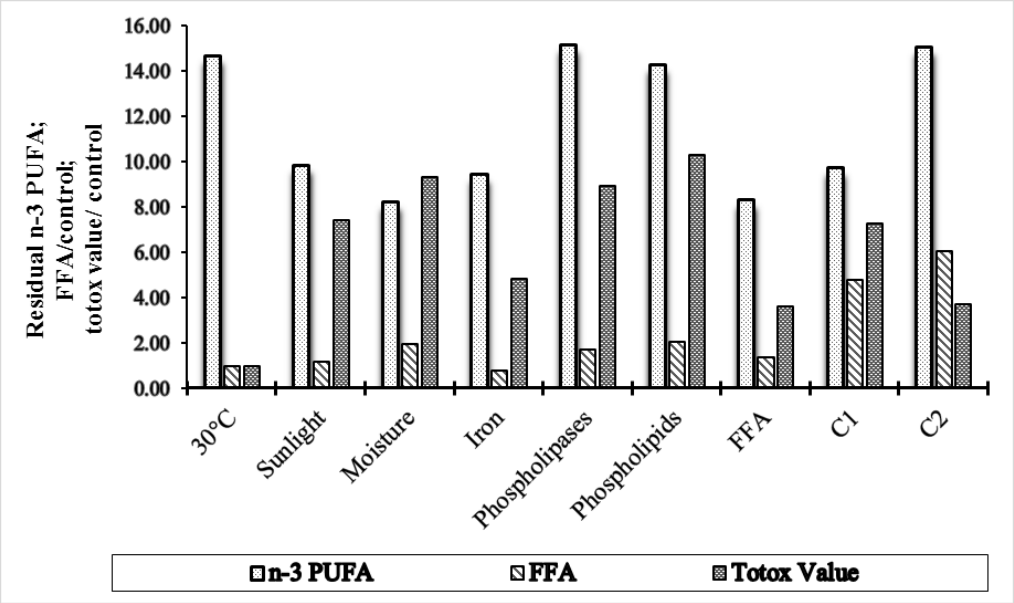


Figure 4.5: Effect of selected intrinsic and extrinsic factors which led to the maximum instability in oil, on n-3 PUFA content and storage stability after five weeks of storage at 30°C, in the dark conditions under inert atmosphere. C1- combination of 1000 ppm moisture, sunlight and temperature (30°C). C2- combination of Ferric chloride, PLS, PL, FFA (each 1000 ppm). Residual n-3 PUFA and the rate of increase in FFA and TV compared to control experiments are plotted.

4.3 SUMMARY AND CONCLUSIONS

Table 4.3: Summary of the storage stability of oil under various conditions

Parameters	Effect of storage stability of Indian Sardine oil at end of five weeks		
	FFA	TV	Total n-3 PUFA (EPA+ DHA)
Refined oil	BDL	24.36	17.91
30°C	4.28	138.99	14.68
Sunlight	6.78	310.06	9.85
Moisture	11.4	390.22	8.23

Parameters	Effect of storage stability of Indian Sardine oil at end of five weeks		
	FFA	TV	Total n-3 PUFA (EPA+ DHA)
Iron	4.57	202.1	9.43
Phospholipases	10.1	374.3	15.16
Phospholipids	12.03	430.6	14.28
FFA	7.89	152	8.3
Combination 1	27.95	303.4	9.72
Combination 2	35.2	155.3	15.06

- The aim of the current investigation was to find the most detrimental factor/s affecting the hydrolytic and oxidative stability and n-3 PUFA of Indian Sardine oil during five weeks storage. The identification of those factors which affect the quality of the oil is crucial to provide the best storage conditions to minimize the rancidity in oil.
- Reports on study of the storage stability of edible oils considering both extrinsic and intrinsic factors over a period of time is very few, particularly in the case of Indian Sardine oil.
- It was found from our studies that the presence of moisture, FFA, iron and sunlight affected hydrolytic and oxidative stability and are detrimental to n-3 PUFA.
- Though PL and PLS resulted in the hydrolytic and oxidative instability of the oil, n-3 PUFA protection was witnessed during five weeks of storage of Indian Sardine oil.
- The ability of PL to protect n-3 PUFA from deterioration is probably owing to the structure of phosphotidylcholine.
- Further investigation is necessary to evaluate the degree of protection offered by various PL and PLS and their mode of action.
- An understanding of both extrinsic and intrinsic parameters of bulk oils will contribute to the development of knowledge of lipid oxidation and help to protect the release of lipid by products in a more integrated manner. This step is essential due to the extensive use of n-3 PUFA as supplements or food products.

CHAPTER 5

LIPASE MEDIATED ENHANCEMENT OF n-3 PUFA IN THE OIL

5.1 RATIONALE BEHIND THE PROPOSED RESEARCH WORK

Review of literature suggests plenitude of methods for the production of n-3 PUFA from fish oil including urea complexation, low temperature crystallization, molecular distillation, iodolactonization, methods of salt solubility and liquid-liquid extraction and fractionation using sodium nitrate solution (Chakraborty et al. 2010). These methods, however suffer disadvantages of producing n-3 PUFA in the form of FFA which are nutritionally unfavourable, non-selective for the different fatty acids and employ extremes of reaction conditions. Compared to the chemical processes, enzyme mediated hydrolysis of oil provide diverse advantages like mild reaction conditions, increased specificity and reduced side reactions which leads to the energy and economic benefits (Zhou et al. 2015). Commercial lipases from *Candida rugosa, Geotrichum candidum, Humicola lanuginsa, Chromobacterium viscosum, Rhizomucor miehei, Aspergillus niger* and *Rhizopus delemar* have been expansively used for these purposes (Hoshino et al. 1990).

However, the use of commercial soluble enzymes leads to the wastage and limited reuse of the enzymes. To avoid this, designing of enzyme preparations with high activity and stability has attracted consideration over native enzymes. Enzyme immobilization, lipid coating and bioimprinting are the recently employed techniques to modify lipase (Yan et al. 2010). As already described, several researchers reported good performance of bioimprinted lipases in non-aqueous environment. Yet, reports on the successful use of bioimprinted enzyme in aqueous environment are rather scarce. Thus, an effort was made in the present work to maintain the imprinted characteristics of the CRL in an aqueous milieu by cross-linking the CRL with glutaraldehyde in the presence of co-aggregates, to produce an enzyme for the use in aqueous systems by laying focus on the positive aspects of both bioimprinting and immobilization.

This chapter deals with the study of hydrolysis of oil to enhance the EPA and DHA using *Candida rugosa* lipase (CRL) and *Pseudomonas cepecia* lipase (PCL). An understanding of their mode of action on the enrichment have been also been discussed. Also, optimization of the preparation of cross-linked enzyme aggregates

(CLEA) along with its characterization and optimization of parameters to enhance degree of hydrolysis (DOH) so as to get n-3 PUFA enriched oil. Furthermore, the physical properties and performance of CRL-CLEA was studied and compared with free-CRL. Finally, the reusability of CRL-CLEA was also investigated.

5.2 MATERIALS AND METHODS

5.2.1 MATERIALS

Crude fish oil procured from Mukka Fish Oil Industries Ltd. (Mangaluru, India) was refined according to the method of Charanyaa et al. (2017). The crude oil was degummed using 5% 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. *Candida rugosa* lipase (CRL) and *Pseudomonas cepacia* lipase (PCL) was purchased from Sigma Aldrich, India. Iso-propanol, propanol, butanol, acetone, polypropyleneglycol, tert-butanol, n-hexane, ethanol, diethyl ether, sodium hydroxide, hydrochloric acid, boron trifluoride in methanol (10%), ammonium sulphate, acetone and phenolphthalein indicator (analytical grade) were purchased from Merck, India and used without further purification. Surfactants like tween 20, tween 80, triton x 100, gelatin and SDS were purchased from Merck, India. Bovine serum albumin (BSA), polyethyleneimine (PEI), glutaraldehyde and oleic acid (OA), sodium borohydride were purchased from Himedia, India, Sigma Aldrich, India, Merck, India, and Spectrum, India, respectively. Solvents like iso-propanol and acetonitrile of chromatographic grade were purchased from Merck, India and were used for chromatographic analysis without further purification.

5.2.2 METHODS

5.2.2.1 Modification of lipases (CRL and PCL)

Modification of CRL and PCL was performed by dissolving various quantities of lipase (2mg, 4mg, 6mg, 8mg, and 10mg) in 1 mL of various solvents (hydrophobic

and hydrophilic), followed by the addition of various surfactants (non-ionic, anionic and cationic) to the enzyme solution.

5.2.2.1.1 Effect of mixed surfactants

This experiment was performed to study the effect of contact of two surfactants with lipase on the degree of protection against inhibition. The degree of hydrolysis of oil was measured by three different methods as proposed by Kawase et al. (1985).

Method 1 (Ma): Lipase was contacted with anionic surfactants first for 10 min followed by addition of non-ionic surfactants for another 10 min. The lipase hydrolysis was started by adding the emulsion to the oil.

Method 2 (Mn): Lipase was contacted with non-ionic surfactants first for 10 min followed by addition of anionic surfactants for another 10 min. The lipase hydrolysis was started by adding the emulsion to the oil.

Method 3 (Mm): Both the surfactants were mixed simultaneously in the beginning and allowed to contact with the lipase solution for 10 min and operated similarly as the above procedures.

5.2.2.2 Preparation of bioimprinted cross-linked CRL aggregates

Bioimprinting of CRL with OA was carried out as per Kahveci and Xu (2011) with slight modifications. 75 mg of CRL was mixed with 0.5 mL of 0.1 M of phosphate buffer (pH 7). The imprint molecules like OA (0.5 mmol) were dissolved in 1 mL ethanol and 100 mg of tween 60 and was then added to the enzyme solution for imprinting and stirred for half an hour. The cross-linked aggregate of this bioimprinted enzyme was prepared by the method described by Vaidya et al. (2012) with minor modifications. Commercial CRL (75 mg) was bioimprinted by incubating 820 U of CRL (200 µL) with varying quantities of OA and incubated at 18°C for 30 min with continuous stirring for half an hour (Fishman and Cogan, 2003). To this enzyme mixture, BSA was added and stirred for an hour by incubating the mixture at 18°C, 300 rpm. Polyethyleneimine (PEI), a well-known co-aggregator of enzymes was added (5% of 25 mg/mL) and stirred for an hour. Then glutaraldehyde (25 %,

v/v), which is the cross-linking agent was added and stirred for an hour. 1 mL (100mM) of sodium borohydride was added to this enzyme mixture and allowed to react for 15 min to remove the schiffs bases that might have formed during the course of cross-linking. Thereafter, the mixture was centrifuged at 3600 x g for 15 min to separate cross-linked enzyme aggregate pellets. The pellet obtained was washed thrice with distilled water and the product obtained was lyophilized for 16 h at -30°C to get CRL-CLEA. Figure 5.1 describes the process of preparation of the hydrolyzed oil enriched with enhanced quantities of EPA and DHA through various steps involving imprinting of CRL in the presence of oleic acid followed by co aggregation in the presence of BSA and PEI and cross linking with glutaraldehyde. It also represents the gas chromatograms of hydrolyzed oil in the presence of free CRL and CRL-CLEA.

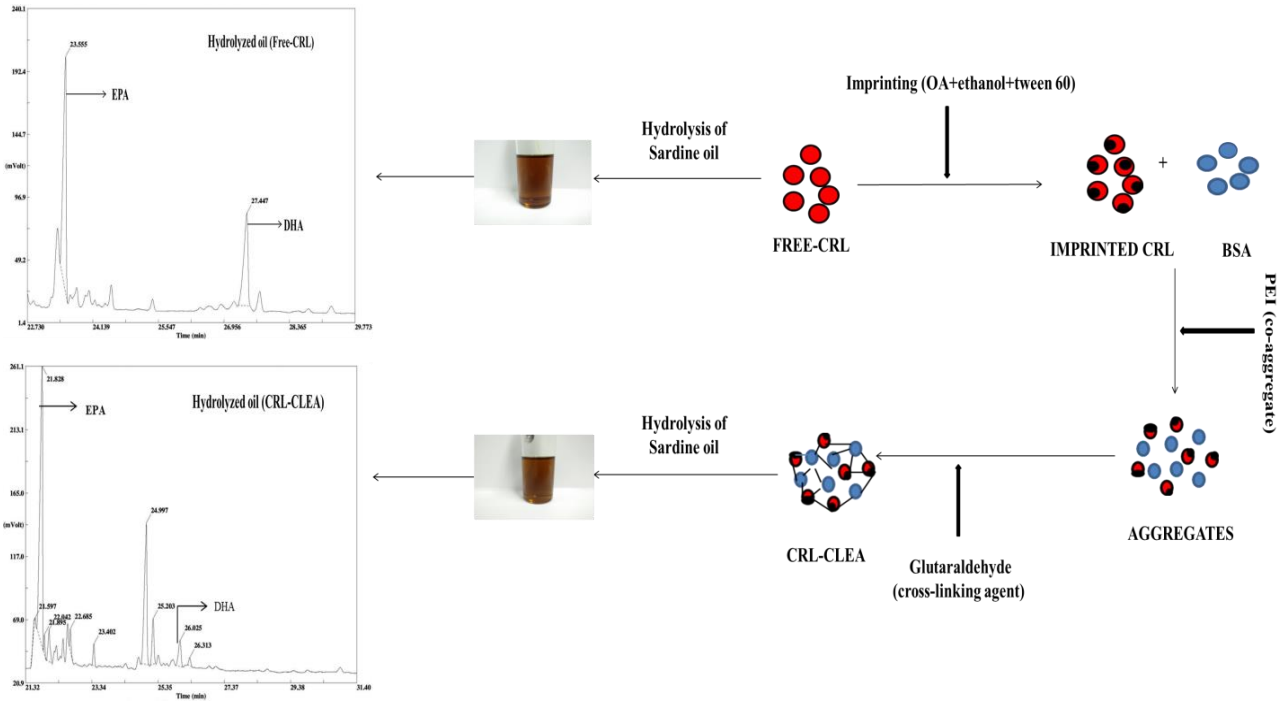


Figure 5.1: A diagrammatic representation of the steps involved in the preparation of CRL-CLEA from free-CRL.

5.2.2.2.1 Optimization studies

Indian Sardine oil was refined as reported by Charanyaa et al. (2017) and the obtained high purity oil (Table 5.1) was taken for optimization of parameters for enzyme catalyzed hydrolysis. Different reaction parameters such as CRL-OA volume ratio

(1:1, 1:2, 1:3, 1:4, 1:5), CRL-BSA volume ratio (1:1, 1:2, 1:3, 1:4, 1:5), CRL-PEI volume ratio (1:1, 1:2, 1:3, 1:4, 1:5), glutaraldehyde quantity (40, 100, 160, 220, 280, 340, 400 µL) and cross-linking time (15, 30, 45, 60, 120, 180 min) were optimized by one factor at a time approach. Two assessment parameters, activity recovery (Equation 5.1) and aggregate yield (Equation 5.2) were considered in arriving at an optimum condition for the production of CRL-CLEA, as described by Vaidya et al. (2012).

$$Activity\ recovery = \frac{A_{CLEA}}{A_{Free} \times V_{Free}} \times 100 \quad (5.1)$$

$$Aggregate\ yield = \left(100 - \left[\frac{A_{Residual} \times V_{Residual}}{A_{Free} \times V_{Free}}\right]\right) \times 100 \quad (5.2)$$

where A_{CLEA} is the activity as depicted by CRL-CLEA; A_{Free} is the activity of free-CRL; V_{Free} is the volume (mL) of the free-CRL involved in the preparation of CRL-CLEA; $A_{Residual}$ is the activity of the residual CRL supernatant; $V_{Residual}$ is the volume (mL) of the residual CRL remained after the formation of the CRL-CLEA. The experiments were performed in duplicates and the mean values of the results were presented.

Table 5.1: Composition of refined Indian Sardine oil

Parameters	Values
Phospholipid (ppm)	5.66
Iron (ppm)	BDL*
Copper (ppm)	0.1
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

5.2.2.3 Determination of lipase activity

Lipase activity was determined by performing controlled hydrolysis of tributryin substrate by mixing 1:1 (w/w) ratio of tributyrin to water. The buffered enzyme solution was then added to the emulsion for 15 min and the hydrolyzed substrate was analyzed for the amount of free fatty acids of tributyrin released by titrating hydrolyzed tributyrin against 0.1 N potassium hydroxide. The end point (pink colour) was observed by adding phenolphthalein solution as the indicator. One unit of enzyme activity (U) was expressed as the amount of CRL/PCL/ CRL-CLEA required to liberate one µmole of FFA of tributyrin per min at the respective optimum temperatures.

5.2.3 Comparison of modified CRL and PCL

5.2.3.1 Optimization of Sardine oil hydrolysis using modified CRL and PCL

The reaction system containing 1 g of refined sardine oil and 1 g of water (1:1 w/w) was taken and homogenized using hand held continuous homogenizer (IKA T18 basic, Ultra- Turrax) for 15 min. It was then mixed with modified CRL and PCL incubated with a constant stirring on a magnetic stirrer at 300 rpm to start the hydrolysis reaction. The DOH of CRL and PCL was determined after the incubation period and were compared. Various parameters like temperature (30°C, 40°C, 50°C, 60°C), enzyme load (2 mg/mL, 4 mg/mL, 6 mg/mL, 8 mg/mL, 10 mg/ mL), oil-water ratio (1:1, 1:2, 1:3, 1:4, 1:5) and time of hydrolysis (15 minutes, 30 minutes, 45 minutes and 60 minutes) were optimized by changing one parameter at a time, while keeping all the other parameters constant.

The DOH was determined by measuring the acid value of both hydrolyzed and unhydrolyzed oils as well as the saponification value of the unhydrolyzed oil according to the equation (5.3).

$$Degree\ of\ hydrolysis\ (DOH) = \left(\frac{AV_t - AV_0}{SV_0 - AV_0}\right) \times 100 \qquad (5.3)$$

where SV_0 and AV_0 are the saponification value and the acid values of refined sardine oil before hydrolysis, respectively, and AV_t is the acid value of the hydrolyzed oil at the optimized reaction time.

The hydrolysis was performed under the optimized reaction conditions of both CRL and PCL at optimal time, after which the reaction was stopped by adding 15 ml of methanol. The hydrolyzed oil was subjected to methanol extraction, to eliminate the FFA released during the process. The solvent extracted oil (hydrolyzed) was studied for its fatty acid composition using GC which was compared with the fatty acid composition of the refined oil (unhydrolyzed). The experiment was done under vacuum conditions in order to prevent oxidation of the oil. The acid value of oil was calculated according to the official method of American Oil Chemist's Society (AOCS) (2009) methodologies, (Cd3d-63).

5.2.4 Comparison of CRL-CLEA and free-CRL

5.2.4.1 Structural characterization by scanning electron microscopy (SEM)

The morphology of CRL-CLEA and free-CRL was obtained by studying the micrograph on JEOL JCPDS (Japan) operated under an accelerating voltage of 20 kV at a magnification of 2500x. The enzyme samples were lyophilized and coated with gold before being scanned under vacuum. The SEM images were captured in low vacuum and processed using ImageJ software.

5.2.4.2 Optimization of Sardine oil hydrolysis using free-CRL and CRL-CLEA.

The reaction system containing 1 g of refined sardine oil and 1 g of water (1:1 w/w) was taken and homogenized using hand held continuous homogenizer (IKA T18 basic, Ultra- Turrax) for 15 min. It was then mixed with lyophilized CRL-CLEA and free-CRL incubated at 18°C with a constant stirring on a magnetic stirrer at 300 rpm to start the hydrolysis reaction. The DOH of free-CRL and CRL-CLEA was determined after the incubation period and were compared. Various parameters like pH (6, 6.5, 7, 7.5, 8), temperature (30°C, 40°C, 50°C, 60°C), enzyme load (2, 4, 6, 8, 10, 12 mg/mL) and oil-water ratio (1:1, 1:2, 1:3, 1:4, 1:5) were optimized by changing one parameter at a time, while keeping all the other parameters constant.

The DOH was determined according to the Equation (5.3).

The hydrolysis was performed under the optimized reaction conditions of free-CRL and CRL-CLEA for 15 minutes according to the procedure mentioned in the section 5.2.3.1

5.2.5 Analysis of hydrolyzed oil

After enzymatic treatment using modified CRL and PCL, CRL-CLEA and free-CRL, 15 ml of methanol was added and the aqueous methanol solution containing inactivated enzyme and FFA was discarded. The oil was subjected to methanol extraction to remove released FFA (Charanyaa et al. 2017). After the solvent traces were evaporated and dried, the residue was esterified with borontrifluoride methanol agents and analyzed by GC (Trace 3330 GC Ultra, Thermo Electron Corporation) equipped with a flame ionization detector (FID), split/splitless injector and DB-5 column (30m x 0.25 mm x 0.2 µm) using the guidelines from Ichihara and Fukubayashi (2010). The right inlet and the detector temperatures were set at 280°C and 300°C, respectively. The oven temperature was initially held at 160°C for 1 min which was then increased to 185°C at the rate of 5°C/min and held for 10 min. The temperature was further increased to 240 °C at 8°C/ min and held for 10 min. The total run time was approximately 32 min. The samples were prepared and analyzed in duplicates. FAMEs were identified and quantified by comparing the retention time of the samples with FAME standards from Sigma Aldrich expressed as percent of total fatty acids (%). Chrome Cad software was used for the analysis of the chromatograms.

Triglycerides, diglycerides and monoglycerides present in FFA free hydrolyzed oil was estimated by HPLC. The oil sample (8 µL) was dissolved in 4 mL of iso-propanol (chromatographic grade) and analyzed by HPLC with a pump (LC-20AD, Shimadzu Co, Kyoto, Japan) in the presence of RPC18 column using ELSD (Gilson). The sample was analyzed by using a gradient program in accordance to the method followed by Aoki et al. (2004) employing a mixture of iso-propanol and acetonitrile mixture as the elution systems. The flow rate and the column temperature were 0.5

mL/min and 30°C, respectively. The evaporation temperature, nebulizer temperature and gas flow rate of ELS detector were 110°C, 80°C and 1 mL/min, respectively.

The purity of synthesised hydrolyzed sardine oil obtained from HPLC chromatograms were verified with LC-MS (Shimadzu Co, Japan). The samples for LC-MS were prepared similar to the method adopted for the preparation of samples for HPLC, and injected into the system with a pump (LC-20AD), UV/Vis detector (SPD-20 A), column oven (CTO-10 AS) and MS fitted with an electron spray ionization source (ESI) by full scan acquisition. The conditions of column, mobile phase, and flow rate and column temperature were the same as the conditions used for studying the contents of the refined and hydrolyzed fish oil in HPLC. The detection wavelength of PDA used was 250 nm. The ESI conditions in desolvation line temperature, heat block temperature, detector voltage, drying gas flow rate, interface current and voltage were 250 °C, 200 °C, 1 kV, 15 L/min, 0.2 µA, 4.5 kV, respectively. The analysis of the oil was made by studying both the positive and negative ion mode over the range of 100-1000 m/z.

5.2.6 Reusability of CRL-CLEA

To assess the reusability of CRL-CLEA, the enzyme was used repeatedly for multiple batches under optimal conditions of hydrolysis. After the specified optimal reaction time, the enzyme was separated by centrifugation, washed with distilled water twice and then added again to the fresh reaction mixture. The DOH was estimated after each cycle and DOH estimated in the first cycle was considered as 100%.

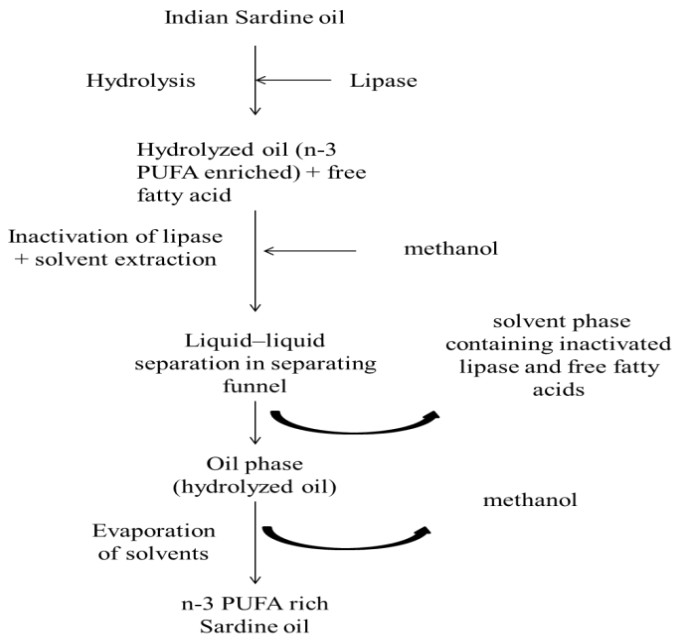


Figure 5.2: A flowchart representing the step-wise procedure of hydrolysis of Indian Sardine oil using the lipases.

5.3 Results and discussions

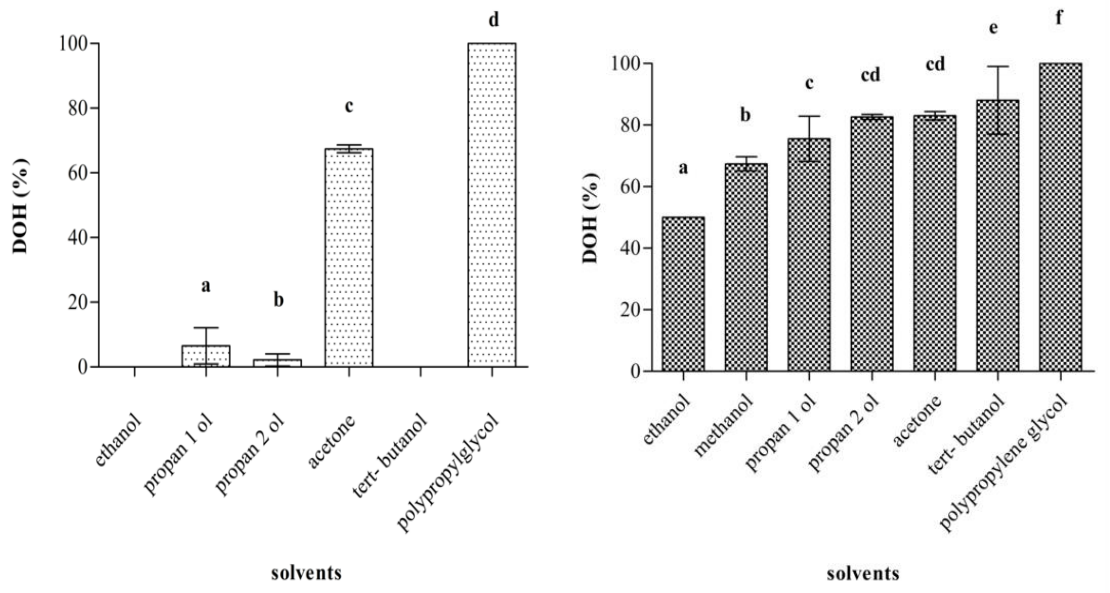
The ability of lipases for the fatty acid selectivity such as EPA and DHA has allowed the separation of these fatty acids from the remaining fatty acids in fish oils, resulting in the increased yields of n-3 PUFA rich concentrates. Therefore, in the present study, the DOH was examined under various conditions with the objective of obtaining elevated amounts of EPA and DHA in the hydrolyzed oil with the consequent removal of FFA by solvent extraction.

5.3.1 Modification of lipases

5.3.1.1 Effect of solvents on CRL and PCL

In order to study the effects of various solvents on CRL and PCL, the enzymes were treated with 1 mL of the solvents (ethanol, 1 propanol, 2 propanol, butanol, acetone, polypropyleneglycol and tert-butanol). The solvents were chosen on the basis of its low toxicity and its use in pharmaceutical and food industries. The increased hydrolysis was witnessed when the enzymes were dissolved in polypropylene glycol (Figure 5.3 (A) and (B)). This is probably due to the neutral pH of the solvent in

which the enzymes remain fairly active. The lipases are known to contain residues of amino acids at the active site and result in maximum binding of the substrate at neutral pH. The DOH exhibited by the CRL dissolved in buffer (pH 7) was 3.4% whereas; PCL in buffer (pH 7) showed 7.33%. These values confirmed that the presence of polypropylene glycol increased the DOH of the enzymes to a great extent. Studies by Iyer and Ananthanarayan (2008) showed that the addition of polyols like polypropylene glycol to the enzyme strengthens the hydrophobic interactions among the non-polar amino acid residues which lead to protein rigidification and resistance to thermal deactivation. A study by Doukyu and Ogino (2010) proved in their studies that PCL and CRL have a higher organic solvent tolerance compared to any other lipases and esterases.



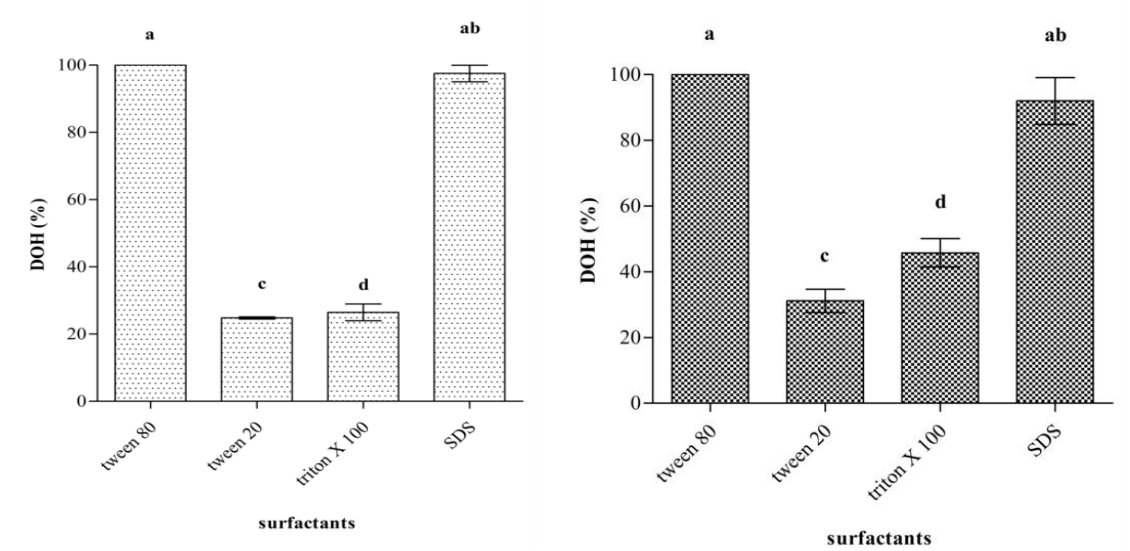
(A) **(B)**

Figure 5.3: Effects of solvents on CRL (A) and PCL (B). In case of influence of solvents on CRL, the DOH corresponds to 4.74% was taken as 100% and 9.48% for PCL was taken as 100%.

5.3.1.2 Effect of surfactants on CRL and PCL

The enzymes dissolved in polypropylene glycol were subjected to further modification using various surfactants (Tween 80, Tween 20, Triton X 100, and SDS). Tween 80 (2%) and SDS (2%) were observed to better the DOH in sardine oil in the above mentioned conditions for CRL and PCL. Tween 80, a non-ionic surfactant, composed of polyethoxyethylene sorbitan and oleic acid was known to increase the hydrolysis degree in the oil (Figure 5.4(A) and Figure 5.4 (B)) when lipase solution was treated with tween 80 because of their hydrophobic interactions with the hydrophobic amino acids in the lipases. This interaction tends to enhance the lipase catalysed hydrolysed reactions (Fadologlu 1996, Delorme et al. 2011). Lipases like CRL and PCL have relatively high content of hydrophobic amino acids on their surfaces with tend to bind to tween 80 and result in the increased hydrolysis of the oil (Fadologlu 1996). Another reason for better hydrolysis could be due to the presence of oleic acid in tween 80 which during the incubation in the enzyme solution perhaps got imprinted in the lipase leading to a better hydrolysis of sardine oil. Non-ionic surfactants in general are considered as mild detergents and they do not extensively interact with the protein structure (Salameh and Wiegel 2010).

Similarly, when a mixture of lipase solution in the presence of SDS, an anionic surfactant, was added to the oil increased the hydrolysis degree because of the unspecific binding to the protein surface which leads to the electrostatic interactions related to the charge of the lipase (Figure 5.4(A) and Figure 5.4 (B)). The explanation for these results could be that the SDS micelles bind to the lid and activate the lipase by conformational changes exposing the active site for substrate binding. Salameh and Wiegel (2010) reported similar results with the support of fluorescence and X-ray crystallography which showed that micelles certainly bound to the lid that covers the active site and promote conformational changes.



(A) **(B)**

Figure 5.4: Effect of surfactants on CRL (A) and PCL (B). In case of influence of surfactants on CRL, the DOH corresponds to 6.24% was taken as 100% for tween 80 and 6.08% was taken as 97.5% for SDS. In case of influence of surfactants on PCL, the DOH corresponds to 10.93% was taken as 100% for tween 80 and 10% was taken as 98.6% for SDS.

5.3.1.2.1 Effect of mixed surfactants on CRL and PCL

Protective effect of surfactant mixture

In order to protect the lipase from the large decrease in the hydrolysis ability, the effects of mixture of non-ionic surfactants and anionic surfactants were studied. The experiments were done in three different methods to observe the sequence of contact of surfactants with lipase for a better DOH. The relative hydrolysis efficiency of the three methods were in the order of method M_n > method M_m > method M_a. When the oil was treated with CRL in the presence of tween 80 and SDS individually, the DOH was 6.24% and 6.08%, respectively. Similarly, for PCL, the DOH in presence of tween 80 and SDS was 10.93% and 10%, respectively. It is evident that DOH of tween 80 is higher than SDS which could be because of the slight inactivation of lipase by SDS. When tween 80 and SDS were added as a mixture to CRL and PCL solutions, the DOH were found to have increased to 7% and 10.8% for CRL and PCL, respectively. This increase in the hydrolysis efficiency proves the protective effect of

tween 80 as compared to SDS in methods M_n whereas, in method M_a, the subsequent addition of tween 80 no longer reactivated the lipase activity. Therefore, method M_n was used for the rest of the studies.

5.3.2 Optimization of bioimprinted, carrier free cross-linked enzyme aggregate preparation.

5.3.2.1 Effect of CRL: OA ratio

Among the different ratios of CRL:OA considered (1:1, 1:2, 1:3, 1:4, 1:5 v/v), 1:5 ratio showed the highest activity recovery and aggregate yield of 33.8% and 71.4%, respectively (Table 5.2). Hence this was chosen as the optimal value and was used for all the further experiments. Interestingly, in the absence of OA poor values of activity recovery and aggregate yields (10.8% and 22.3%) were obtained. These results highlighted that CRL might have a high selectivity towards OA (Yan et al. 2009). The advantage of using OA as the imprint molecule is that it is a liquid at the room temperature, which on mixing with ethanol and tween 60 could have got imprinted on the enzyme and increased the DOH. This could be due to the conformational changes in the site of CRL which matches the substrate molecule. Further, to check if ethanol and tween 60 acted as the imprint molecule, a comparison of DOH by non-imprinted CRL and CRL imprinted with ethanol and tween 60 individually was performed which showed that ethanol had no contribution to the enhancement in the DOH of CRL. On the contrary, tween 60 enhanced the DOH of the oil. Hence, further validation was made by studying the DOH exhibited by OA- ethanol-tween 60 which was much higher than of OA-tween 60 or OA-ethanol. This result only confirms that the contribution of OA to the DOH of CRL (Table 5.3). These results concur with the findings of Yan et al. (2009), where they had reported imprinting with *Geotrichum* sp. lipase with OA in the presence of methanol and *tert*-butyl alcohol. As tunnel like conformation present in CRL is sterically suitable for OA (Dominguez de Marıa et al. 2006), it can be concluded that OA could successfully imprint the CRL in the presence of ethanol and Tween 60. Foresti et al. (2005) had confirmed the bioimprinting of CRL with OA for esterification of OA and ethanol.

Table 5.2: Effect of CRL: OA ratio

CRL: OA ratio (v/v)	Activity recovery (%)	Aggregate yield (%)
1:0 (control)	10.8	22.3
1:1	21.4	33.1
1:2	21.8	37.6
1:3	14.2	32.8
1:4	22.1	71.2
1:5	**33.8**	**71.4**

Table 5.3: Effect of bioimprinting and further cross linking of CRL using various molecules on degree of hydrolysis of Indian Sardine oil.

CRL with imprint molecules	Degree of hydrolysis (%)
Non imprinted CRL	3.45±0.2
Ethanol	0
Tween 60	12.52±1
OA	14.8 ±0.9
OA + Tween 60	18.62±1.1
OA + Ethanol	15.1±0.3
OA + Ethanol + Tween 60	20.51±0.25

5.3.2.2 Effect of CRL: BSA ratio

CRL: BSA ratio was found to be an important factor influencing activity recovery and the aggregate yields (Table 5.4). With the increase in the CRL: BSA ratios, the activity recovery and the aggregate yields reduced drastically. It was demonstrated that the maximal activity recovery and aggregate yields (36.2% and 72.6%) were achieved at the ratio of 1:1. Any further decrease in the ratio (increase in the BSA concentration) led to the decrease in the activity recovery, possibly due to the formation of clusters with mass transfer limitations. On the contrary, only 7.1% of the activity was recovered in the absence of added BSA. Addition of BSA is known to facilitate CLEA preparation in cases in which the protein concentration of the enzyme preparation is low. The cross-linking agent, glutaraldehyde reacts with amino groups of amino acid residues such as lysine (Torres et al. 2013). Interestingly, the CRL is found to contain a significant number of amino groups containing amino acid residues

(Dominguez de Maria et al. 2006) in the active site. Glutardehyde might have reacted with them, resulting in the reduction of activity of the cross-linked enzyme (Migneault et al. 2004, Kaieda et al. 2014). Perhaps in the presence of BSA, which has a very high number of lysine residues on the surface, glutaraldehyde reacts with them shielding CRL.

Table 5.4: Effect of CRL: BSA ratio

CRL: BSA ratio (v/v)	Activity recovery (%)	Aggregate yield (%)
1:0 (control)	7.1	15.3
1:1	**36.2**	**72.6**
1:2	15.1	64.8
1:3	15.8	60.7
1:4	17.5	50

5.3.2.3 Effect of CRL: PEI ratio

Trials were performed to screen two precipitants (ammonium sulphate and acetone) and one co-aggregator (PEI), for the preparation of CLEA from free-CRL. Precipitation with ammonium sulphate and acetone exhibited low activity recovery (15.73 and 0), which was disagreeing with the results obtained with *Pseudomonas* sp. lipase by Zhao et al. (2008). Hence, experiments with precipitants were discontinued any further. However, co-aggregation with PEI, gave the highest activity recovery and aggregate yield. Further, CRL: PEI ratio of 1:1 showed the highest activity recovery and aggregate yield (Table 5.5). The increase in quantity of PEI for cross-linking resulted in the decreased activity recovery. These results substantiate with the previous findings by Vaidya et al. (2012) where 1:1 ratio of aminoacyclase co-aggregated with PEI showed pronounced activity recovery. A similar trend was observed during the development of CLEA from *Geotrichum* sp. lipase (Yan et al. 2012). PEI is a water soluble, cationic polymer possessing high density of ionized amino groups. Perhaps, this high density of cationic groups in PEI may permit the strong ionic exchange between PEI and anionic groups on CRL and BSA aggregates and thus stabilize the protein. Simultaneously, it also participated in the cross-linking

reaction with glutaraldehyde resulting in the increased extent of cross-linking (Vaidya et al. 2012, Majumdar et al. 2008).

Table 5.5: Effect of CRL: PEI ratio

CRL: PEI ratio (v/v)	Activity recovery (%)	Aggregate yield (%)
1:1	**39.6**	**90.8**
1:2	25.2	86.7
1:3	14.3	79.3
1:4	20.4	57.3
1:5	19.2	72.9

5.3.2.4 Effect of amount of glutaraldehyde

It is already well established that the extent of cross-linking depends upon both glutaraldehyde concentration and cross-linking time (Cui and Jia 2013). Moreover, these two factors also depend upon the availability of free amino groups of lysine residues on the enzyme and availability of co-aggregators. Thus, it becomes necessary to determine optimum concentration of glutaraldehyde for CRL in the presence of co-aggregators (BSA and PEI) to recover maximum activity and yield. As is evident from Table 5.6, the activity recovery and the aggregate yields of the enzyme increased with the increase in the amount of glutaraldehyde. Use of 340 µL of glutaraldehyde for cross-linking gave the highest activity recovery and aggregate yield. To get an insight on the enzyme immobilization, lipase activity of the supernatant (after separation of cross-linked enzyme aggregate) was determined. Significant lipase activity was detected in the supernatant when the amount of glutaraldehyde used was less than the optimum (340 µL), signifying insufficient cross-linking at lower amounts of cross-linker. Also, negligible enzyme activity was found when glutaraldehyde used was higher than 340 µL. Moreover, activity recovery also reduced drastically, which may be due to the reduction in the flexibility of the enzyme required for its activity due to excessive cross-linking (Talekar et al. 2013a, 2013b). These results seem to be in agreement with the previous findings of Kartal et al. (2011) and Zhou et al. (2015).

Table 5.6: Effect of amount of glutaraldehyde

CRL: PEI ratio (v/v)	Activity recovery (%)	Aggregate yield (%)
40	21.9	80
100	25.2	82.2
160	24	81.2
220	28.6	84.4
280	28.9	86.9
340	**49.9**	**92.5**
400	14.5	70.4

5.3.2.5 Effect of time on cross-linking.

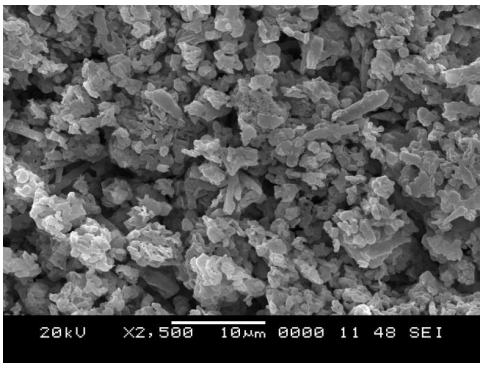
Since cross-linking is a time dependant reaction, the time required to achieve maximum activity recovery of the enzyme during the process becomes vital. The experiments were performed with the optimized amounts of PEI and glutaraldehyde. It should be noted that the initial cross-linking experiments performed till this stage was for 30 min. Now, the effect of cross-linking time was studied between 15 -180 min (Table 5.7). As can be seen from the table, the maximum activity recovery was achieved in 45 min of cross-linking beyond which there was a gradual reduction in the values. These findings seem to be in consonance with the previous reports by Vaidya et al. (2012) and Talekar et al. (2012a) which stated that the increase in the cross-linking time resulted in the increase in the activity recovery of the obtained CLEA until a particular point. After 45 min, there was a reduction in the activity recovery due to the loss of flexibility of the enzyme because of intensive cross-linking by the increased duration of contact of glutaraldehyde with CRL (Cui and Jia 2013).

Table 5.7: Effect of cross linking time

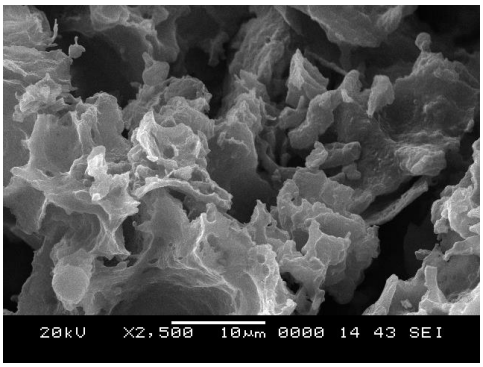
Time (minutes)	Activity recovery (%)	Aggregate yield (%)
15	32.5	95.2
30	36.8	88.7
45	**55.5**	**98.2**
60	34.9	90.6
120	29.9	83.3
180	24	83.9

5.3.3 Morphology of CRL-CLEA

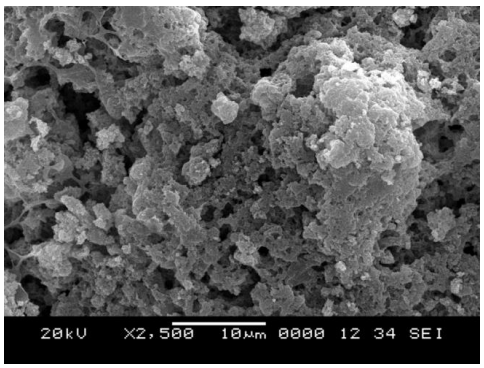
The morphology of CRL-CLEA was examined by Scanning electron microscopy (SEM). The SEM images revealed that commercial CRL showed fine grains of various sizes (Figure 5.5A), whereas CRL-CLEA has coarse grains with deep pores (Figure 5.5B). Schoevaart et al. (2004) and Gupta et al. (2009) have reported similar undefined structure of small grain size while studying the morphology of CLEA prepared using CRL and *Thermomyces lanuginosa*, respectively, in the presence of precipitant and glutaraldehyde, devoid of either BSA or co-aggregates. Enzymes, such as *Candida rugosa* lipase (CRL) or *Prunus amygdalus (R)*-oxynitrilase, which are heavily glycosylated, form smaller undefined aggregates (Schoevaart et al. 2004). It was highlighted by Schoevaart et al. (2004) that CLEAs are of two types (type 1 and type 2) based on the structures viewed in SEM. Type 1 CLEAs materialize as spherical structures whereas type 2 CLEAs are course grained structures (less defined). Due to the differences in the structures of the CLEAs, type 1 with many cavities allows better mass transfer compared to the type 2 CLEAs which have fewer cavities. The use of co-aggregates, BSA and PEI in our case has resulted in the formation of bigger aggregates, as evident from Figure 5.5B.

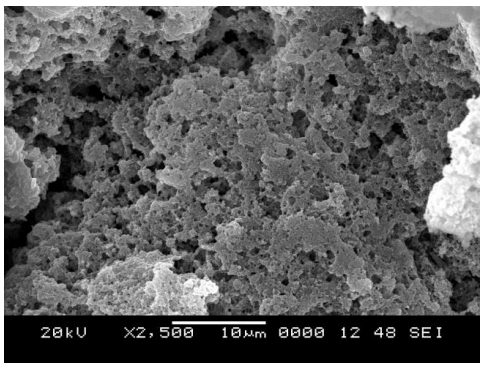


(A)



(B)





(C) **(D)**

Figure 5.5: Scanning electron micrograph of (A) commercial free-CRL (B) CRL-CLEA produced from free-CRL (C) CRL-CLEA after five cycles of use (Squeezed CRL-CLEA) and (D) image of CRL-CLEA after seven cycles of use (Squeezed CRL-CLEA).

5.3.4 Hydrolysis of Sardine oil using the four enzymes

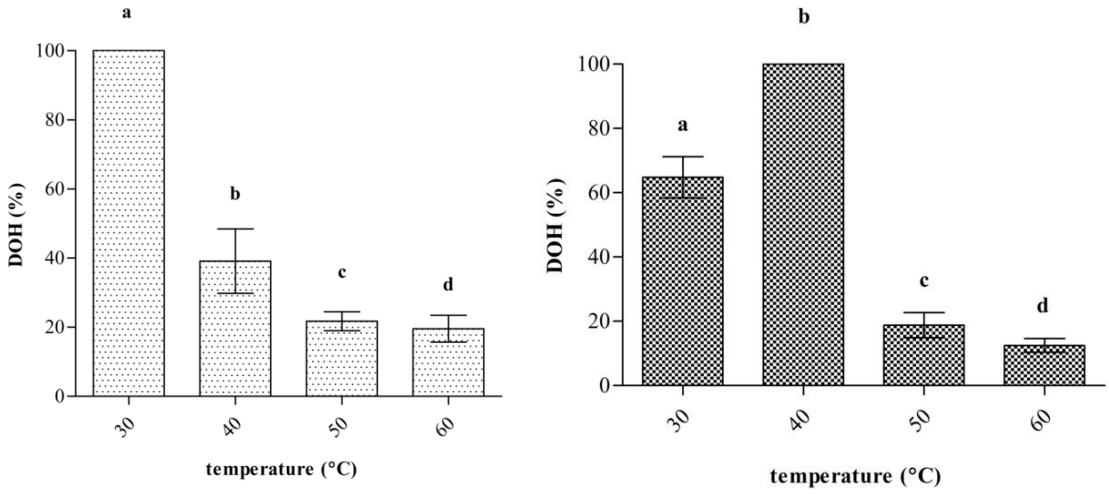
The refined Indian Sardine oil having 17.91% (w/w) of n-3 PUFA (Table 5.1) was taken for enzymatic hydrolysis to enhance n-3 PUFA content. Modified CRL and PCL, free-CRL and CRL-CLEA prepared under optimized condition, were used and compared. Important process parameters were studied for all the enzymes independently, and the DOH was evaluated to find the optimal conditions.

5.3.4.1 Hydrolysis of oil using modified CRL and PCL

5.3.4.1.1 Effect of temperature on hydrolytic activity of CRL and PCL

The selection of the suitable temperature in lipase catalysed reactions of oils is important because higher temperatures beyond 40°C may cause lipase denaturation whereas temperatures lesser than 20°C may slow down the rate of reaction (Aditi et al. 2014). Trials were performed to screen the temperature at which CRL and PCL displayed maximum DOH in the oil. It was seen that CRL showed a maximum DOH (7.12%) at 30°C and PCL showed a maximum DOH (10.87%) at 40°C. However, a rapid decrease in the DOH was observed at the rest of the temperatures (Figure 5.6 (A) and Figure 5.6 (B)). This is due to the denaturation of the enzyme structure at these temperatures. Several researchers have reported the optimum temperature of soluble CRL at 37°C (Montero et al. 1993, Xu et al. 1995). Montero et al. (1993)

found that treatment at higher temperatures led to the inactivation of the enzyme. According to Fadologlu (1996), CRL exhibits its maximal activity at 40°C. It was observed that the reaction rate of PCL increased from 25°C to 45°C, and remained constant at 50°C and decreased at higher temperatures (Pencreach et al. 1997).



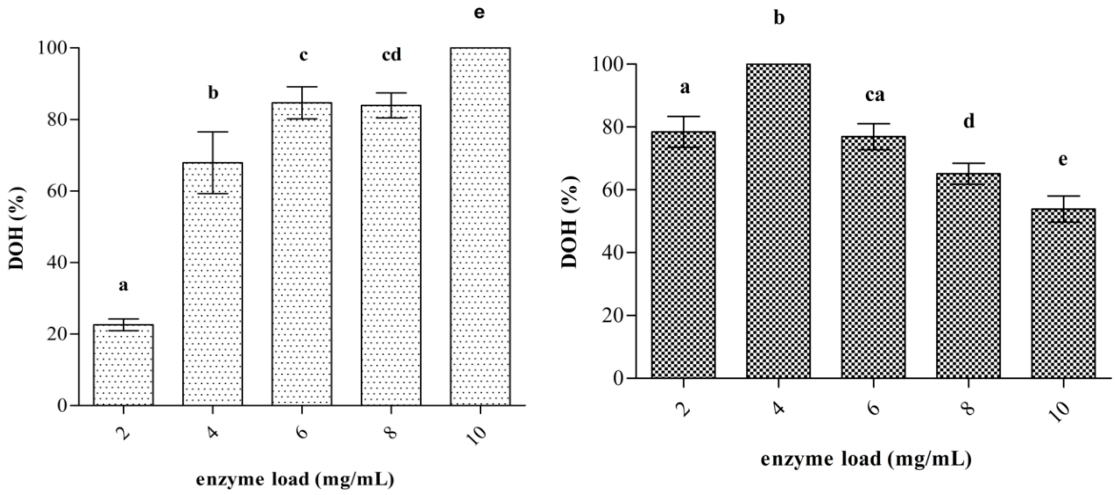
(A) **(B)**

Figure 5.6: Effect of temperature on DOH of CRL (A) and PCL (B). In case of influence of temperature on CRL, the DOH corresponds to 7.12% was taken as 100% and 10.87% for PCL was taken as 100%.

5.3.4.1.2 Effect of enzyme load of CRL and PCL on DOH

As lipase hydrolysis takes place at the interface, the amount of the enzyme at the interface is very important. The effect of enzyme concentration has been studied as shown in the Figure 5.7 (A) and Figure 5.7 (B) for CRL and PCL, respectively. Figure 5.7(A) shows that the increase in the concentration of CRL in the reaction mixture results in the increase in the yield of reaction. Tsai et al. (1991) have also reported that the increase in the rate of hydrolysis and lipase loads was linear in the hydrolysis of olive oil for the enzyme concentration lower than 0.2%. Similar to the reports of Tsai et al. (1991), the DOH increased with the CRL concentration with the maximum seen at 10 mg/mL (270 U) whereas, this trend was not observed in the case of PCL (Figure 5.7(B)). The DOH (10.9%) at 4mg/mL (340U) was seen to be a maximum after which the DOH decreased with the increase in enzyme concentration. It is possible that after

4mg/mL of PCL, the oil lipase solution interface generated under the experimental conditions gets saturated with lipase i.e there is a formation of monolayer at the interface and accumulated intermediates (Pongket et al. 2015).



(A) **(B)**

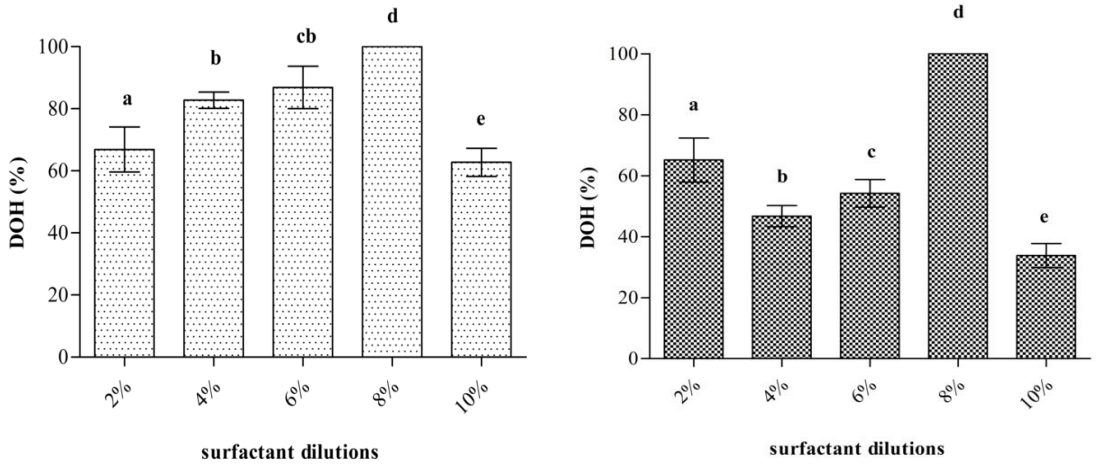
Figure 5.7: Effect of enzyme load on DOH of CRL (A) and PCL (B). In case of influence of enzyme load on CRL, the DOH corresponds to 7.32% was taken as 100% and 10.9% for PCL was taken as 100%.

Hence, a further increase in PCL concentration would not show much change in the DOH. Rooney and Weatherly (2001) have also reported that after the optimum concentration there was no change in the rate of hydrolysis for sunflower oil under similar experimental condition. Therefore, in all the further studies a CRL concentration of 10 mg/mL and PCL concentration of 4 mg/mL was considered optimum.

5.3.4.1.3 Effect of dilution of surfactants

It's evident from Figure 5.8(A) and Figure 5.8(B) that at 8% of mixed surfactants (4% tween 80 and 4% SDS) for both CRL and PCL showed a maximum DOH of 7.47% and 10.92%, respectively. Beyond 8% of mixed surfactants, a drastic reduction in DOH was observed which is due to the increased concentration of tween 80 and SDS in the system causing the partial or complete unfolding of the tertiary structure of the protein due to the additional hydrophobic interactions (Reynolds and Tanford (1970),

Otzen and Oliveberg (2002),Otzen 2002). Mogensen et al. (2005) described deactivation of *Thermomyces lanuginosus* lipase (TLL) in the presence of nonionic and zwitterionic surfactants at high concentrations.

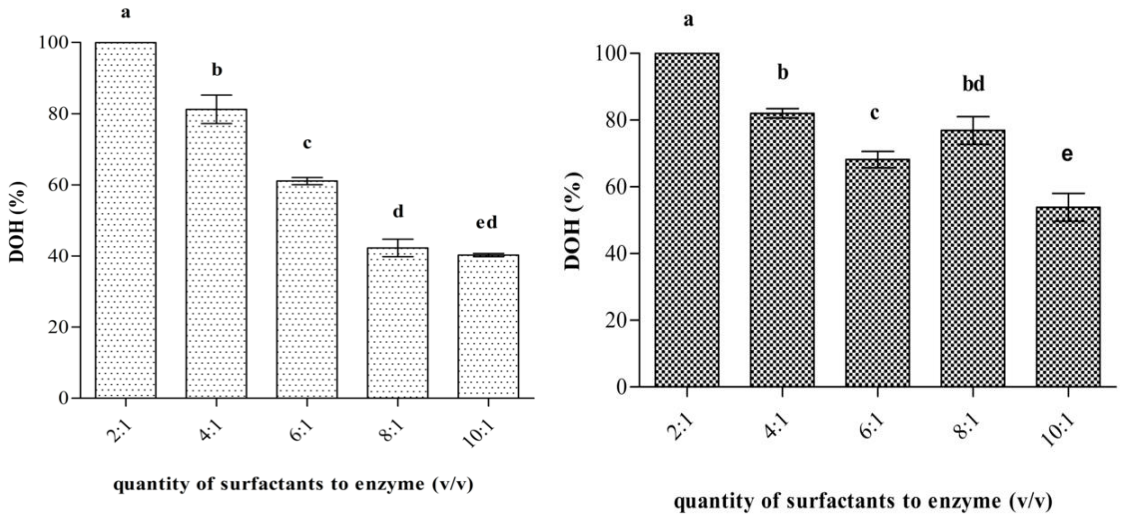


(A) **(B)**

Figure 5.8: Effect of dilutions of mixed surfactants on DOH of CRL (A) and PCL (B). In case of CRL, the DOH corresponds to 7.47% was taken as 100% and 10.92% for PCL was taken as 100%.

5.3.4.1.4 Effect of quantity of surfactants in enzyme solution

It is observed that 2:1 (v/v) of mixed surfactants to lipase solution resulted in the highest DOH in both CRL and PCL (Figure 5.9 (A) and Figure 5.9 (B)). Beyond this ratio, the DOH tends to decrease due to the inhibitory effects of surfactants by impairing the lipase adsorption at liquid- water interfaces. This usually happens when surfactants are used at concentrations above their critical micelle concentration (CMC). At this stage, the surfactants compete with lipase for adsorption at the interface and lower the interfacial surface tension by saturating the interface with increased concentration of surfactant monomers and thus preventing the lipase to reach the interface making it inactive (Delorme et al. 2011).

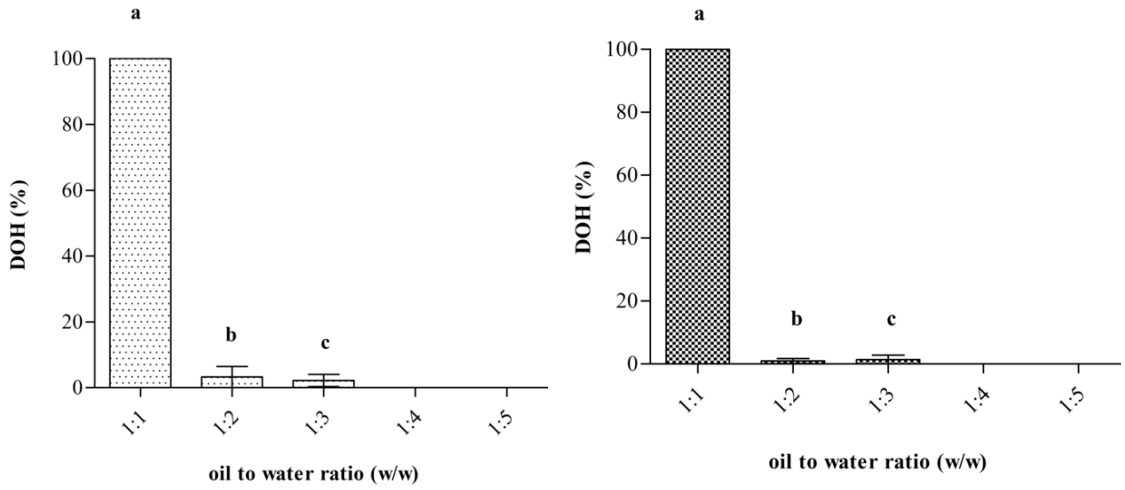


(A) **(B)**

Figure 5.9: Effect of quantity of surfactants in CRL (A) and PCL (B). In case of CRL, the DOH corresponds to 7.61% was taken as 100% and 10.97% for PCL was taken as 100%.

5.3.4.1.5 Effect of oil to water ratio on DOH of CRL and PCL

CRL and PCL catalyse the cleavage of the ester bonds of triglycerides with the consumption of water molecules. Hence it becomes important to study the amount of water required for hydrolysis as it increases the capability of the enzyme for hydrolysis at the interphase in a biphasic system. From the Figures 5.10 (A) and 5.10 (B), it is clearly seen that the DOH was highest at the oil to water ratio of 1:1 (w/w). The ratios above 1:1 (w/w) showed lesser activities because higher quantity of water leads to a thicker water layer around the CRL and PCL which increases the flexibility of the lipases, causing denaturation (Aditi et al. 2014). Han and Rhee (1986) stated similar reaction conditions for the hydrolysis of olive oil catalyzed by CRL enzyme in the range of pH 6.5–7.1, temperature 30-35°C and 0.72–9.78 (v/v) oil to water ratio.

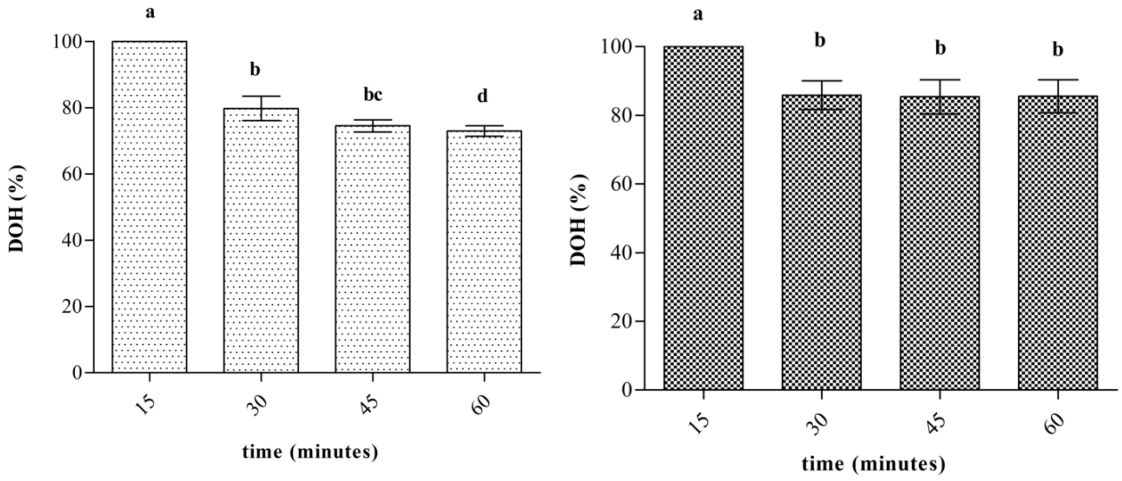


(A) **(B)**

Figure 5.10: Effect of oil to water ratio on DOH of CRL (A) and PCL (B). In case of CRL, the DOH corresponds to 7.63% was taken as 100% and 11% for PCL was taken as 100%.

5.3.4.1.6 Effect of time of hydrolysis of CRL and PCL

Maintaining the conditions as mentioned above for all the parameters, the time course study was performed under the following conditions: a mixture of 1g of oil, 1 g of water and 10 mg/mL of CRL and 4 mg/mL of PCL incubated at 30°C/40°C with a constant stirring at 300 rpm. Figure 5.11 (A) and figure 5.11 (B) shows the time courses of the two enzyme forms at the optimal conditions. In both the cases the refined Indian sardine oil was hydrolyzed rapidly for the first 15 min and then the DOH almost remained consistent. This could mean that both CRL and PCL have attained equilibrium in terms of the conversion rates (Halldorsson et al. 2004). The time taken by the enzymes to reach a maximum DOH (7.68% /11.03%) is 15 min. Although the time taken by both the enzymes is the same, the increase in DOH was much higher by PCL than CRL. A further increase in time up to 1 hour did not lead to any improvement in the product formation. A further progress in hydrolysis did not proceed linearly and inclined to slow down with time which may be due to the decreasing oil concentration, the increase in product concentration or the inactivation of the enzymes (Gardossi et al. 2010).



(A) **(B)**

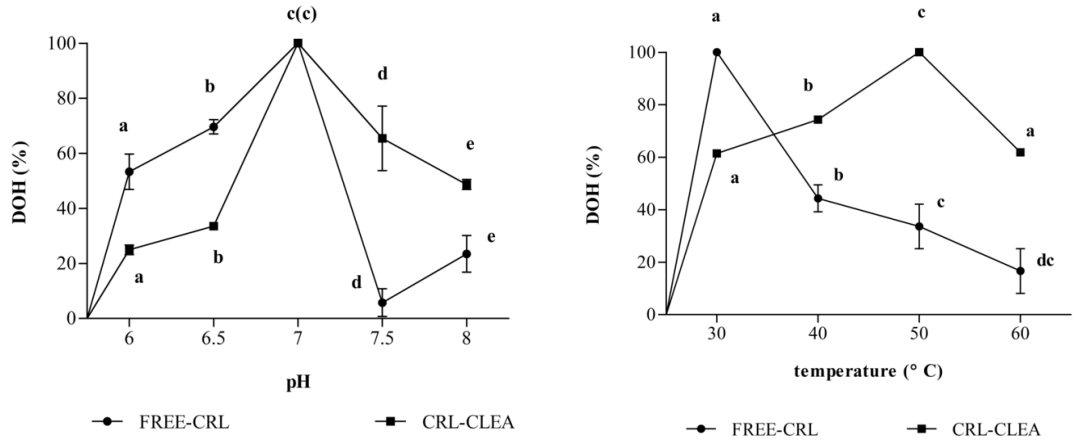
Figure 5.11: Effect of duration of hydrolysis on DOH by CRL (A) and PCL (B). In case of CRL, the DOH corresponds to 7.68% was taken as 100% and 11.03% for PCL was taken as 100%.

5.3.4.2 Hydrolysis of oil using free-CRL and CRL-CLEA

5.3.4.2.1 Effect of pH and temperature on hydrolytic activity of the free-CRL and CRL-CLEA

The results of the effect of pH on hydrolysis of oil using free-CRL and CRL-CLEA are shown in the Figure 5.12(A). The optimum pH for both free-CRL and CRL-CLEA was found to be 7, which is conflicting with the published reports (Talekar et al. 2012a, 2013b) which in general state that immobilization by cross-linking leads to the shifting of pH to a higher range due to the intense cross-linking of the amino groups of surface amino acid residues on the enzyme. However, in the present case, the optimum pH remained the same in spite of the cross-linking with glutaraldehyde. Perhaps this could be due to the presence of co-aggregates, BSA and PEI. It is known that shift in optimum pH is the result of the change in acidic and basic amino acid side chain ionization in the microenvironment around the active site, which was caused by the newly formed interactions between basic residues of enzyme and glutaraldehyde during cross-linking (Aytar and Bakir 2008). It is not unreasonable to say that participation of BSA and PEI in cross-linking reaction with glutaraldehyde and their

presence might have influenced the microenvironment of the enzyme thereby negating the effect of side chain ionization due to pH.



(A) **(B)**

Figure 5.12: Influence of pH (A) and temperature (B) on DOH of free-CRL and CRL-CLEA. In case of influence of pH, the DOH corresponds to 30.94% for CRL-CLEA and 3.35% for free-CRL was taken as 100%. In case of influence of temperature, the DOH corresponds to 32.87% for CRL-CLEA and 3.35% for free-CRL was taken as 100%.

The thermal stability of the free-CRL and CRL-CLEA were assessed by incubating both the enzymes in buffer (pH 7) at different temperatures. The profiles of DOH of free-CRL and CRL-CLEA at different temperatures were represented in the Figure 5.12(B). At 40°C, the free-CRL retained only 43.1 % of activity, while CRL-CLEA retained 74.35% of its initial activity. Similarly, at 60°C, free-CRL could retain only 16.92 %, whereas CRL-CLEA retained 61.88% of activity. This data proves that cross-linking with BSA and PEI conferred good thermal stability to the enzyme. These results are in accordance with the findings of Yan et al. (2012) and Vaidya et al. (2012) which proves the efficiency of PEI as a precipitating agent.

Further, thermal deactivation kinetics of the free-CRL and CRL-CLEA was studied. The deactivation energy of the prepared CRL and free-CRL was found to be 80.97

KJ/ mol.K and 55.93 KJ/ mol.K, respectively indicating 1.45 fold increase in deactivation energy after the modification of CRL. From this it can be inferred that, more number of stabilizing linkages are formed between the enzyme molecules due to which increased amounts of energy must be put into the system to disrupt these linkages. These results are in agreement with the findings of Vaidya et al. (2012), who experimented with L-aminoacylase.

5.3.4.2.2 Effect of enzyme load on DOH

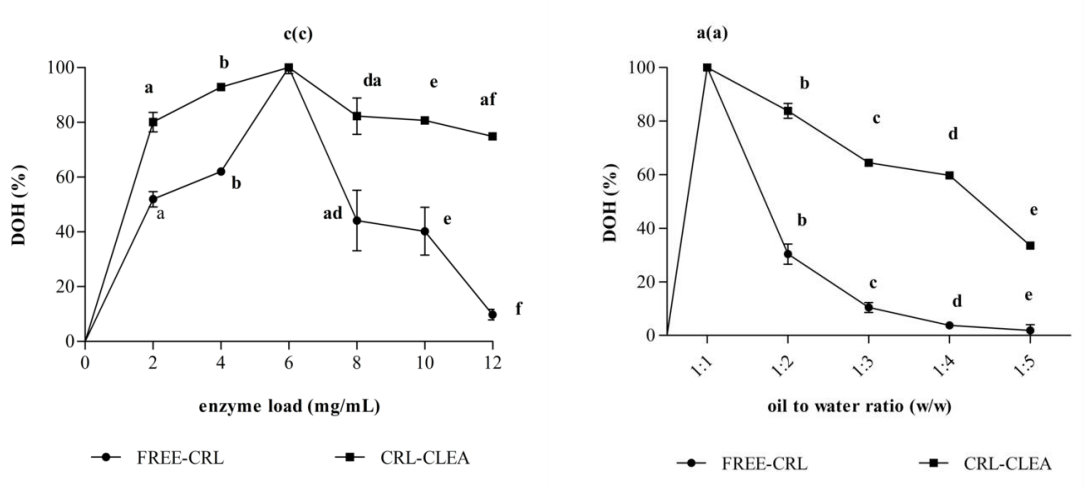
A reaction mixture containing 1 g of refined sardine oil, 1 g of water and various concentrations of free-CRL/ CRL-CLEA, was stirred at (30°C/50°C), respectively for 15 min at pH 7.0, to examine the effect of enzyme load on the hydrolysis.

Highest DOH was achieved when 6 mg/mL of the free-CRL (200U) and CRL-CLEA (1000U) was added (Figure 5.13 (A)). It was observed that further addition of lipase did not increase the DOH. It could be reasoned out that beyond 6 mg/mL, the oil-lipase solution formed under these conditions gets saturated with lipase along with the formation of monolayer and accumulated intermediates (Pongket et al. 2015). As can be seen from the Figure 5.13(A), although the reduction in the DOH was witnessed by both the forms of lipase at higher enzyme concentrations, the reduction of DOH by CRL-CLEA was not as drastic as the free-CRL. This could be explained by the fact that particles of CRL-CLEA did not agglomerate and hence facilitated the distribution of the lipase in the reaction medium (Yan et al. 2010).

5.3.4.2.3 Effect of amount of water on the hydrolysis reaction

Since lipases are dispersed in water during hydrolysis and water is utilized for hydrolysis reaction, it becomes essential to study the effect of water on hydrolysis. From the Figure 5.13(B), it is evident that the DOH was highest at the oil to water ratio of 1:1 (w/w) for both free-CRL and CRL-CLEA. Further increase in water to oil ratio, progressively decreased the DOH. This could be due to the increased micelle formation as water content increases. As it is evident from various studies, lipase positions itself at the oil-water interface to catalyze hydrolysis. With the increase in the number of micelles and micelle volume, increased numbers of enzyme molecules

are taken away from the oil-water interface due to which a drastic reduction in DOH is observed.



(A) **(B)**

Figure 5.13: Influence of enzyme load (A) and amount of water (B) on DOH of free-CRL and CRL-CLEA. In case of influence of enzyme load, the DOH which corresponds to 35.73% for CRL-CLEA and 3.4% for free-CRL was taken as 100%. In case of water quantity in the system, the DOH corresponds to 35.97% for CRL-CLEA and 3.45% for free-CRL was taken as 100%.

5.3.5 n-3 PUFA glyceride enrichment using the four enzymes

Refined Indian Sardine oil was subjected to hydrolysis for 15 min under optimal conditions (1g of oil, 1 g of water and 6 mg/mL of both free-CRL/ CRL-CLEA incubated at 30°C/50°C with a constant stirring at 300 rpm and pH 7.0 **and** 1 g of oil, 1 g of water and 10mg/mL and 4mg/mL of CRL and PCL incubated at 30°C/ 40°C with a constant stirring at 300 rpm). The resulting FFA free oil was assessed for n-3 PUFA content. Under optimal conditions, CRL which resulted in 7.63% DOH, showed a peaked concentration of EPA (19.46%) and DHA (9.5%) whereas, PCL which led to 10.73% hydrolysis degree resulted in (15.23%) 1.65 and (7.4%) 1.56 fold increase in EPA and DHA, respectively. It is evident from the above GC results that CRL enhanced the nutritionally important n-3 PUFA like EPA and DHA in the oil better than PCL in spite of PCL showing better hydrolysis rate than CRL. This observation can be explained due to the fact of CRL having fatty acid chain length

specificity which shows an increased discrimination against the long chain PUFA like C18 to C22. CRL hydrolyses the short chain fatty acids, saturated fatty acids and mono unsaturated fatty acids because of the reduced steric hindrances when linked to the glycerol backbone acids leading to the protection and enhancement of EPA and DHA (Okada and Morrissey 2007). PCL, on the other hand, hydrolyses even long chain fatty acid (Kapoor and Gupta 2012) which is why the enhancement of EPA and DHA by PCL was lesser as compared to CRL. Contrasting to these results, the DOH exhibited by PCL is higher than CRL which can be explained by the difference in the anatomy of the fatty acid binding sites of the two modified enzymes. PCL is known to have a crevice-funnel shaped substrate binding sites which accommodates bulky substrates and results in the hydrolysis of glycerides in sardine oil (Kapoor and Gupta 2012) whereas the active site of CRL is not straight, rather showing a L-shape (tunnel like conformation) due to which it presents a low activity towards LC-PUFA (Dominguez de Mario et al. 2006).

Similarly, free-CRL which gave 3.45 % DOH, showed 1.54 and 1.39-fold enhancement in EPA and DHA, respectively (18.16 %w/w EPA and 8.5 % w/w DHA). Whereas, CRL-CLEA, exhibited 35.97 % DOH, showed 3.87-fold increase in EPA content (45.75 % w/w). However, a slight decrease in DHA content (6.1 % to 5.04 % w/w) was witnessed. This shows CRL-CLEA hydrolyzed ester bonds of small amounts of DHA. These results concur with the observations made by Halldorsson et al. (2004) while studying fatty acid selectivity of various lipases.

Numerous reports are available describing the application of commercially available lipases to concentrate EPA and DHA from marine oils. They reveal that the commercially available lipases discriminate against n-3 PUFA, and that the lipases, which display any significant activity toward n-3 fatty acids usually, prefer EPA to DHA as substrate. The reason is discussed to be due to the close proximity of the carbon-carbon double bond to the carboxyl group being located one bond closer in DHA. This presumably adds strain on the active site of the lipase to accommodate DHA properly (Miller et al. 1988). Lipase fatty acid selectivity may be due to steric hindrance resulting from the chain length, whereas DHA is 2 carbons longer than EPA. However, this is not true for all lipases, e.g. the bacterial lipases from

Pseudomonas are known to display higher activity toward DHA than EPA, despite DHA being theoretically the less favourable substrate (Haraldsson and Kristinsson 1998).

In the current study, use of 200 U/ 270U of free-CRL and modified CRL resulted in the enhancement of n-3 PUFA to 26.66 % and 28.96 %, respectively from the initial 17.91 %. On the contrary, Okada and Morrisey (2007) had reported n-3 PUFA content of 63.86 % from an initial 26.86 % after 6 h of hydrolysis of Pacific Sardine oil with 250 U of free-CRL. PCL (340 U) led to a better enrichment of EPA than DHA. These results were similar to the studies of Lyberg and Aldercreutz (2008) where the highest enrichment of EPA was achieved by PCL without any significant losses of oil.

Treatment with 1000 U CRL-CLEA enhanced the n-3 PUFA content to 50.79%. From this it is evident, enzyme aggregates showed better performance compared to free-CRL. However, the extent of n-3 PUFA enrichment obtained in the current study seems to be less compared to the published reports. Yan et al. (2012) has reported improved performance of *Geotrichum* lipase after preparing CLEA with PEI. The DOH increased to 42% from the initial 12%. This result seems to substantiate well with the present study. Similar studies conducted by Yan et al. (2010) on the enrichment of n-3 PUFA using cross-linked imprinted lipase with 960 U, witnessed an enrichment of 41% from 22% n-3 PUFA in the original oil after 8 h of hydrolysis. In our study, enrichment of 50.79% (CRL-CLEA hydrolyzed oil) from 17.91% (unhydrolyzed oil) was evinced in 15 min of reaction time. The method adopted in the present work resulted in 2.84 times enhancement of n-3 PUFA and thus can be concluded that the suggested method can be used for the application of hydrolysis of sardine oil. From the discussions given above, it is evident that the differences in the DOH and the enrichment in n-3 PUFA content vary. These variations depend upon fatty acid content of the oil, which in turn depends on the sources and also the lipases or modifications of the lipases used.

The HPLC analysis of FFA-free hydrolyzed oil obtained after hydrolysis using modified CRL and PCL, free-CRL and CRL-CLEA revealed differences in the glyceride profile of oil (Figure 5.14). The hydrolyzed oil contained pronounced

quantities of monoglycerides with the drastic reduction in the diglycerides and the triglycerides (Figures 5.14 (B), 5.14(C), 5.14(D)). In comparison to free-CRL and modified CRL, modified PCL and CRL-CLEA (Figure 5.14), showed negligible content of di- and triglycerides, proving higher efficiency of these enzymes in catalyzing hydrolysis. These results bear similarities with the results of Okada and Morrissey (2007).

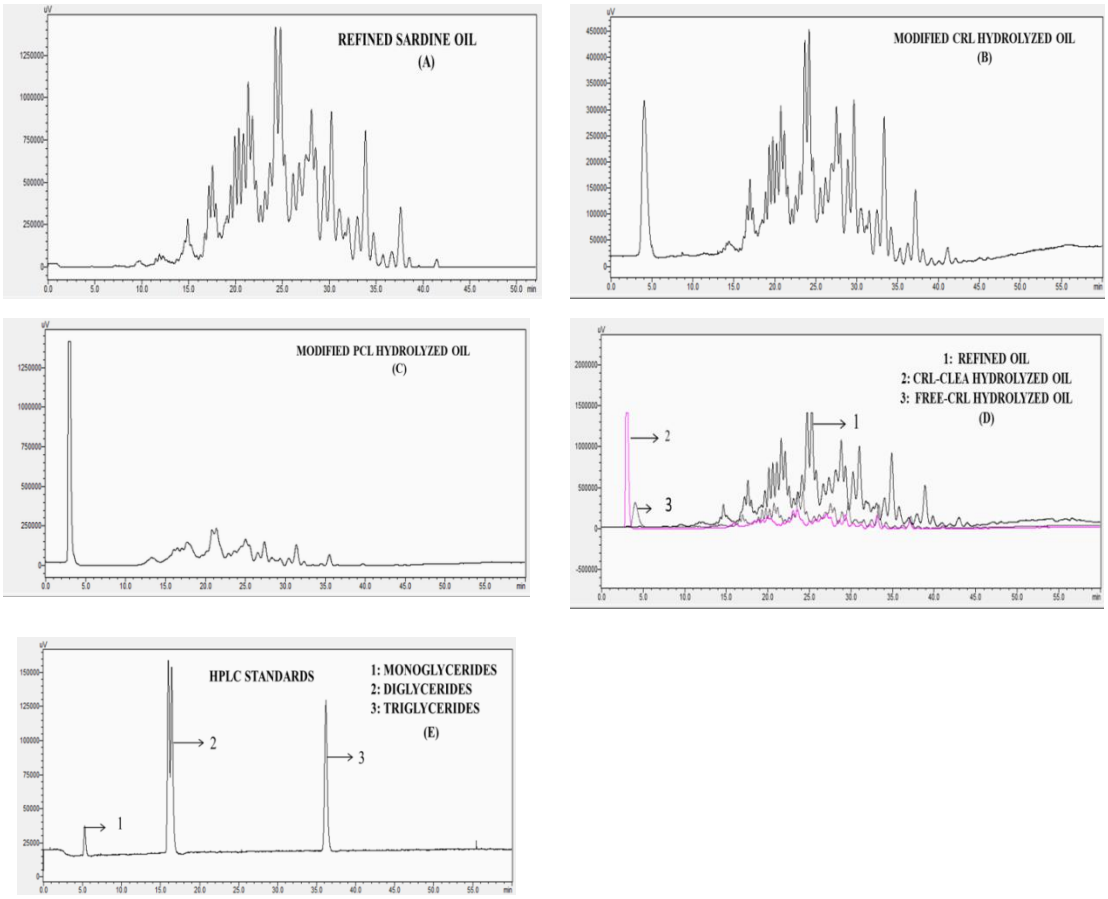


Figure 5.14: The comparison of chromatograms of (A) Refined oil, (B) Hydrolyzed oil using modified CRL, (C) Hydrolyzed oil using modified PCL, (D) comparison of hydrolyzed oils using free-CRL and CRL-CLEA with refined oil and (E) HPLC glyceride standards using ELSD. Mobile phase: acetonitrile/2-propanol (60:40,v/v) , flow rate: 0.5mL/min, Column: RP-C18.

Further profiling of the oil was performed by LC-MS with the purpose of confirming detailed lipid information on the glyceride clusters shown as peaks in the HPLC chromatograms. By extracting the mass of EPA and DHA in association with the

various fatty acids based on the GC report obtained, the lipid composition of different hydrolyzed samples were established. It was obtained from the LC-MS reports that free-CRL and modified CRL resulted in hydrolyzed oil containing large quantities of DHA and palmitic acid (PA) in the form of monoglycerides. These results concur with the findings of Aziz et al. (2015). LC-MS data also revealed the presence of DHA as diglycerides along with myristic acid (MA) and OA in the glycerol backbone. Although, EPA was not present as monoglyceride, it was in the form of diglyceride with myristic acid (EPA-MA) and palmitic acid (EPA-PA). This could be attributed to the abundance of PA and MA in the sardine oil (Table 5.1). Similarly, the hydrolyzed oil using modified PCL unveiled the presence of large quantities of EPA, DHA, LA and OA in the form of monoglycerides. It was noticed that the diglycerides of EPA-EPA, EPA-DHA and DHA-DHA was abundantly present which specifies the efficiency of enrichment of EPA and DHA by modified PCL. Likewise, LC-MS data analysis of the hydrolyzed oil using CRL-CLEA revealed the presence of large quantities of monoglycerides of EPA and PA. The following decreasing order of diglycerides was observed in the hydrolyzed oil, EPA-EPA>EPA-PA>EPA-MA>DHA-MA. This result was seen to be in agreement with the GC results which revealed a huge enhancement of EPA (45.75%) by CRL-CLEA.

5.3.6. Reusability of CRL-CLEA

After every run of hydrolysis, CRL-CLEA was separated from the reaction mixture by centrifugation, washed with distilled water twice, dried at room temperature and used again for the next cycle. As shown in the Figure 5.15, the % DOH of CRL-CLEA remained consistent in the first two cycles, after which there was a gradual reduction in the ability of CRL-CLEA to hydrolyze the oil. 49% of the reduction in DOH was observed from the first cycle to the fourth cycle. Despite the reduction in the hydrolyzing ability of the enzyme over 7 cycles, it was evinced that the EPA and DHA content showed a different pattern of reduction compared to the pattern of hydrolysis. As is clear from the figure, there was a gradual reduction of EPA and DHA from the second cycle to the third which remained almost consistent until the 5^{th} cycle after which there was a sudden reduction as opposed to the trend of DOH. This could imply that the hydrolysis by CRL-CLEA released free forms of fatty acids other

than EPA and DHA, which is the most striking result emerging from this data. It can be deduced from the graph that the reduction in % DOH of CRL-CLEA after the first five cycles may not be due to the leakage of the enzyme. The amount of EPA and DHA in the first five cycles can be a confirmation to the above inference made. SEM images of the CRL-CLEA before the first cycle (Fig. 5.5B), after the fifth cycle (Fig. 5.5C) and seventh cycle (Fig.5.5D) shows a distinct change in morphology of the CRL-CLEA. The distinct granular structure found in the fresh CRL-CLEA was getting transformed into less-distinct clusters. Perhaps, due to the continuous stirring and repeated washing with water, compaction of CLEA might have occurred (Montoro-Garcia et al. 2010, Wang et al. 2011, Talekar et al. 2013a). Thus, decrease in the performance of the enzyme (DOH) could be because of the squeezing of the enzyme molecules which reduces the accessibility of substrates like oil through the CLEA (Talekar et al. 2012b, 2013b). Interestingly, the residual DOH exhibited by free-CRL was almost nil when used for the second time. Therefore, CRL enzyme subjected to cross-linking was more stable, withstanding various conditions much better than free-CRL.

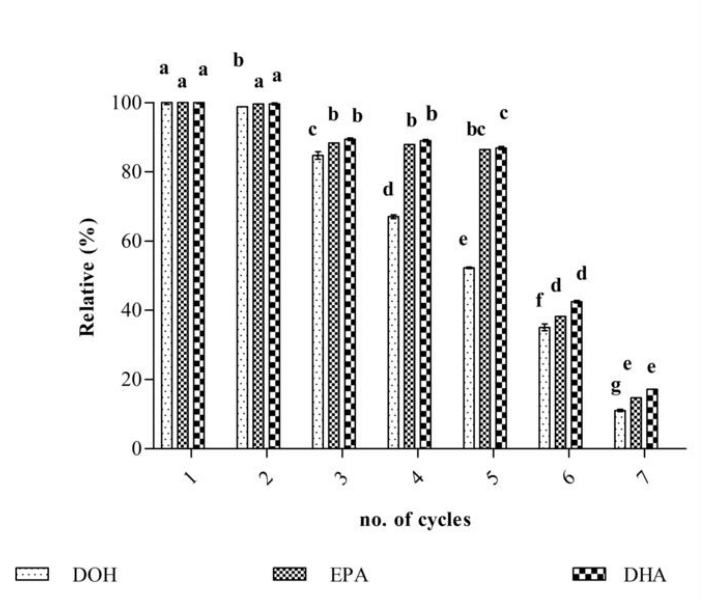


Figure 5.15: Reusability of CRL-CLEA. 32.87 % DOH, 46.75% EPA and 5.04% DHA achieved in the first run is taken as 100% and the performance of the remaining runs were compared

5.4 SUMMARY AND CONCLUSIONS

Table 5.8: Summary of hydrolysis of refined sardine oil using four different lipases

Enzyme type	Effect of enzymes on hydrolysis of Indian Sardine oil		
	DOH (%)	EPA (%)	DHA (%)
Refined unhydrolyzed oil	-	11.81	6.1
Modified CRL	7.68	19.46	9.5
Modified PCL	11.03	15.23	7.4
Free-CRL	3.45	18.16	8.5
CRL-CLEA	35.97	45.75	5.04

- A hydrolysis process was designed by first modifying CRL and PCL in the presence of solvents and surfactants. Upon optimization of various factors, CRL/ PCL in solvent-surfactant system at pH 7.0, temperature 30°C/ 40°C, and oil to water ratio of 1:1 (w/w), surfactant dilution of 2%, surfactant to lipase ratio of 2:1 (v/v) and time 15 min offered maximum DOH with the enhancement in EPA and DHA as seen in Table 5.8.
- CRL was the most superior lipase in concentrating the EPA and DHA contents in the oil while PCL showed a higher DOH as compared to CRL.
- This investigation mainly revealed that the selectivity of both the lipases is primarily fatty acid selectivity rather than a positional selectivity.
- Bioimprinting with oleic acid in the presence of Tween 60 and ethanol, and immobilization with co-aggregates and cross-linking agent was found to have doughty catalytic ability of CRL, even in aqueous environment.
- Characterization of prepared CRL-CLEA revealed type 2 CLEAs are course grained structures with fewer cavities when visualized under Scanning Electron Microscope.
- Under optimal conditions, CRL-CLEA showed 10.4 times higher DOH and 2.83 fold increase in n-3 PUFA content as against free-CRL.
- The free-CRL was not reusable, whereas CRL-CLEA could be reused up to 5 runs without substantial reduction in its performance.

- These studies demonstrate the use of modified enzymes for the efficient hydrolysis of the Sardine oil by a strategy of sequential optimization of production process, by first modifying the enzymes in the presence of solvents and surfactants/ bioimprinting and cross-linking, followed by the optimization of the parameters for the application in enrichment of EPA and DHA in the oil.
- The above declared results offer satisfactory platform to reinforce the candidature of the enzymes for the use in the enhancement of EPA and DHA in food industrial applications.

CHAPTER 6

SUMMARY AND CONCLUSIONS

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The last few years have seen a noticeable increase in the consumption of n-3 PUFA (EPA and DHA) as nutritional supplements and food products. Therefore, the search for new n-3 PUFA natural sources and design of competitive processes for the production of n-3 PUFA enriched lipid extracts have become the areas of research worth delving into. Also, there exists a demand for the development and improvement of different approaches to further concentrate the oil with n-3 PUFA (EPA and DHA) with characteristics suitable for consumption. A logical selection of various stages is extremely essential to maintain a good quality of oil. In this context, the current research undertaken is mainly aimed at studying different production processes which yields lipid extracts with a high content of n-3 PUFA through techniques such as refining and hydrolysis (mild conditions) using the Indian Sardine fish oil as the raw material. Further studies on the storage stability of the oil was performed to meet up with the needs of the industries and the society. Summary and significant findings of this work is presented below.

6.1 Summary

The refining process developed in this work aims at removing phospholipids, FFA, and metal ions without affecting 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).

Due to the high propensity of n-3 PUFA to oxidation, several strategies were investigated to avoid oxidation and prolong the shelf life of the oil apart from the common practice of addition of antioxidants. The refined sardine oil was placed under the influence of extrinsic and environmental factors. The basic motivation of this work was to identify the most detrimental factor/s causing hydrolytic and oxidative instability and deterioration of n-3 PUFA content in the oil during five week storage to protect the refined oil from getting rancid. The effect of various extrinsic factors (light, temperature and moisture content) and intrinsic factors (metal ions, phosphotidylcholine, phospholipase-A and oleic acid) on storage stability was explored. The hydrolytic stability was evaluated by acid value and expressed as FFA content. The oxidative stability was assessed by peroxide value and p-Anisidine value, collectively expressed as totox value (TV). The oil samples under the influence of individual factors like moisture, ferric ions, oleic acid and sunlight were found to cause highest oxidative and hydrolytic instability and highest reduction in n-3 PUFA content. Though phosphotidylcholine and phospholipase-A showed a sharp rise in FFA and TV during five week storage, relatively small reduction in n-3 PUFA content was witnessed. Another interesting observation was made from the oil samples (combination 2) containing ferric ions and oleic acid, phosphotidylcholine and phospholipase-A which exhibited n-3 PUFA protection, in spite of high oxidative and hydrolytic instability.

Besides this, common techniques involved in the concentration of n-3 PUFA are usually based on the separation process (distillation, chromatography, urea complexation etc), which are able to isolate EPA and DHA with good efficiency. Although these techniques leads to the enhancement of n-3 PUFA in good amounts, they are in the form of ethyl ester or free fatty acid, which is a disadvantage since these two forms are not easily assimilated into the body. As an alternative to these conventional processes mentioned above, this work proposed the use of different enzymes in order to obtain lipid extracts rich in n-3 PUFA.

A comparison of hydrolyzing ability of the oil was made using CRL and PCL. CRL and PCL modified with solvents (polypropylene glycol) and surfactants (tween 80 and SDS) showed a peaked DOH (7.68% and 11.03%, respectively) at 15 minutes of hydrolysis at pH of 7, temperature 30°C/ 40°C, oil to water ratio of 1:1 (w/w), surfactant dilution of 2%, surfactant to lipase ratio of 2:1 (v/v).Under these optimum conditions, the analysis of CRL hydrolyzed oil by gas chromatography contained 19.46% and 9.5% of EPA and DHA, respectively while the FFA free hydrolyzed oil by PCL was found to contain enhanced quantities of EPA (15.23%) and DHA (7.4%).

A further study on the preparation of bioimprinted and immobilized CRL was adopted for the hydrolysis of the oil which was compared with free commercial CRL on the hydrolyzing ability. The method of preparing CLEA using bioimprinted CRL in the presence of BSA, Polyethyleneimine and glutaraldehyde was optimized. Under optimal conditions, CRL-CLEA showed 10.4 times higher DOH, which resulted in 2.83-fold increase of n-3 PUFA content in FFA free hydrolyzed oil against free-CRL. It was evident from HPLC reports that CRL-CLEA showed negligible content of di- and triglycerides, proving higher efficiency in hydrolyzing ester bonds of fatty acids other than n-3 PUFA as compared to the free-CRL which was confirmed by the EPA and DHA content in the hydrolyzed oil as determined by GC. Similarly, the HPLC chromatograms proved that the hydrolysing ability of modified PCL was better than modified CRL due to the reduced content of di and triglycerides. LC-MS reports of the hydrolyzed oils of the enzymes used in the study revealed the presence of large quantities of EPA and DHA proving their ability to specifically concentrate EPA and DHA glycerides in the oil. Reusability studies showed CRL-CLEA could be reused up to 5 runs without a substantial reduction in its performance.

6.2 Significant findings

- The refining strategy adopted in this work resulted in an increase in n-3 PUFA from 16.39% to 17.91%.
- Substituting conventional methods of neutralization with solvents for deacidification proved to be advantageous in terms of selective FFA removal and less neutral oil loss.

- 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.
- Factors like moisture, ferric ions, oleic acid and sunlight were found to cause highest oxidative and hydrolytic instability and highest reduction in n-3 PUFA content.
- Phospholipids like phosphatidylcholine did not cause n-3 PUFA reduction though oxidative and hydrolytic instability in the oil was witnessed which could be because of its structure.
- Modified CRL proved to be efficient in concentrating n-3 PUFA (1.3 folds) compared to modified PCL.
- The prepared enzyme (CRL-CLEA) resulted in 2.83 fold increase in n-3 PUFA content on bioimprinting and carrier free immobilization compared to free-CRL.
- The enzyme was also found to retain its hydrolytic prowess upto 5 runs of continuous usage.

6.3 Conclusions

- The designed strategy for refining produced satisfactory results and could be explored for the refining of various other n-3 PUFA rich marine oils. 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.
- Extraction method and storage conditions play a pivotal role in the oil quality. On analysis of the effects of various factors, it can be concluded that storage of the oil at -21°C in dark coloured containers with little head space should be the method of choice for storage.
- The results from modifications of the enzymes like CRL and PCL in the presence of solvents and surfactants suggests its successful involvement in the hydrolytic activity of the enzyme and that these enzymes are responsive to these engineering strategies, involving the modification of the enzymes.

- The data obtained from the characterization studies of propitiously modified commercial free-CRL (CRL-CLEA) provide sufficient grounds to favour the application of the enzyme for food applications.

6.4 Scope for future work

- The significant variables affecting the refining, storage stability and the concentration of n-3 PUFA in the oil which was obtained by one factor at a time approach can be optimized by Response surface methodology.
- Various other methods of immobilization can be adopted for immobilizing the lipase for hydrolysis of oil.
- Bioimprinting of the enzyme can be performed using different template molecules which could serve as a tool to enhance the hydrolytic ability of the enzyme and hence the concentration of n-3 PUFA in the oil.

APPENDICES

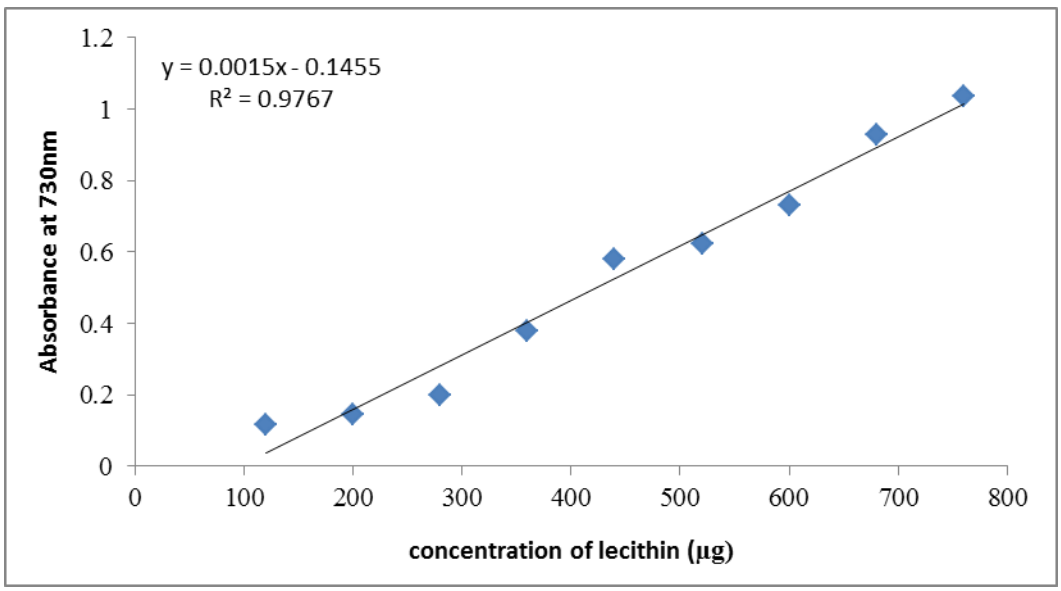
Appendix I

Sardines

Scientific Name	Sardinella longiceps
Phylum	Chordata
Class	Actinopterygii
Order	Clupeiformes
Family	Clupeidae
Environment	Deep waters (Benthopelagic)
Depth range (m)	20-200
Capture Zone	Coastal areas of Mangaluru

Appendix II

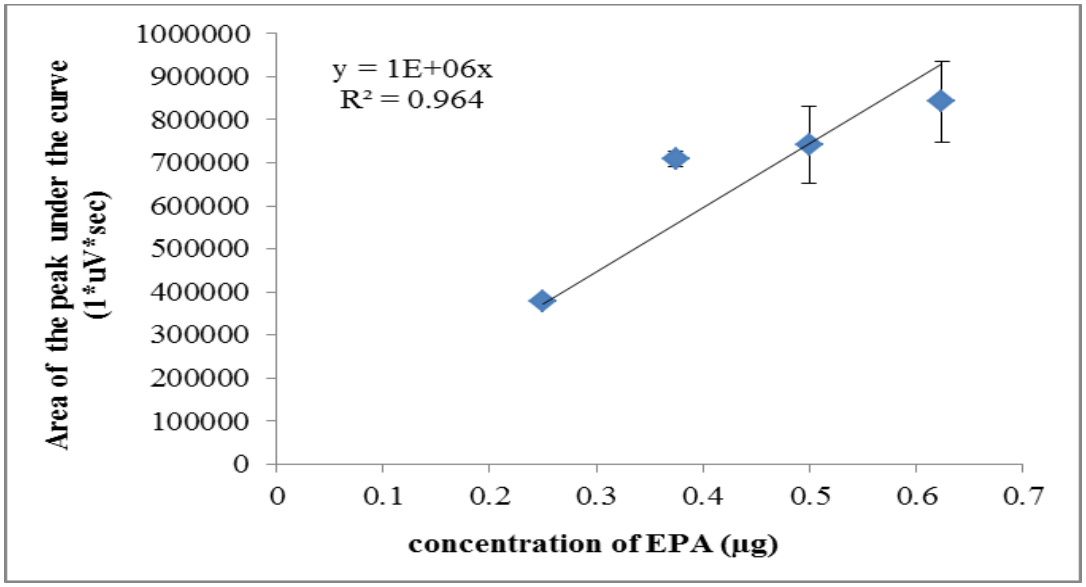
Phospholipid (lecithin) Standard Curve for Phospholipid estimation in the oil samples



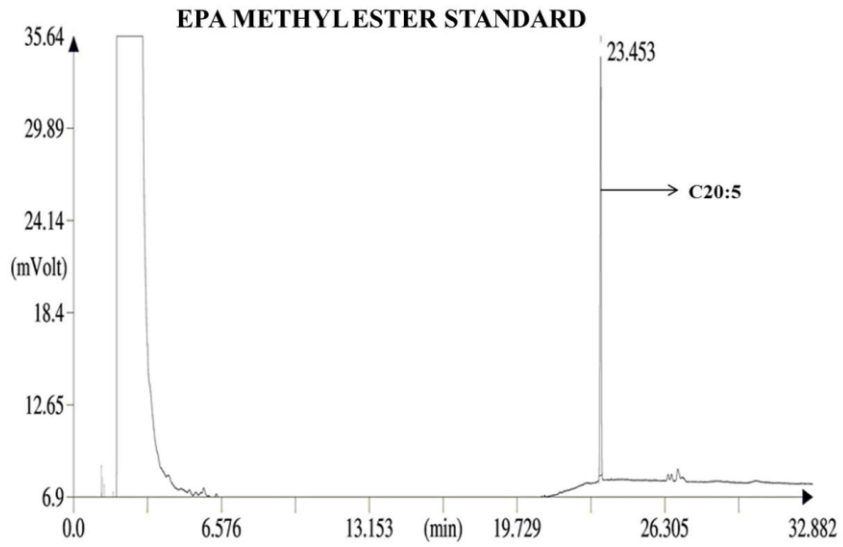
Appendix III

Trace 3330 GC Ultra (Thermoelectron Corporation) with a flame ionization detector (FID) equipped with a split/splitless injector and DB-5 column (30m x 0.25 mm x 0.2µm)

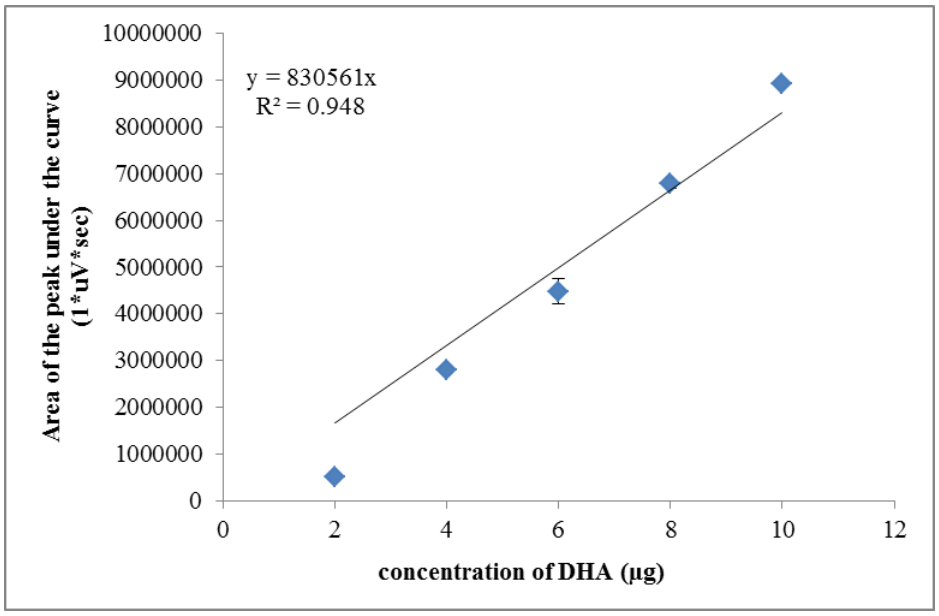
EPA Methyl Ester Standard Curve for Gas Chromatography



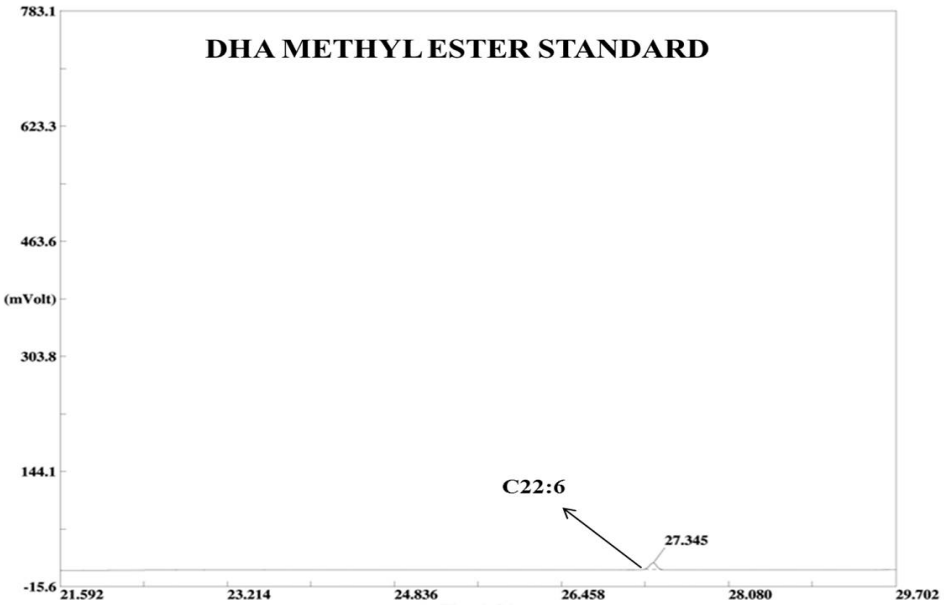
EPA Methyl Ester Standard Chromatogram



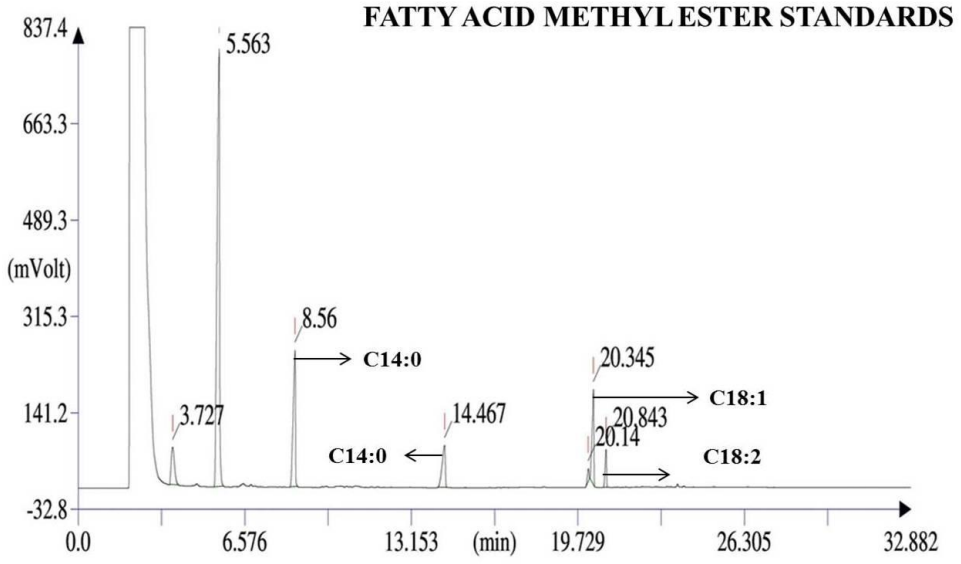
DHA Methyl Ester Standard Curve for Gas Chromatography



DHA Methyl Ester Standard Chromatogram



FAME Methyl Ester Standard Chromatogram



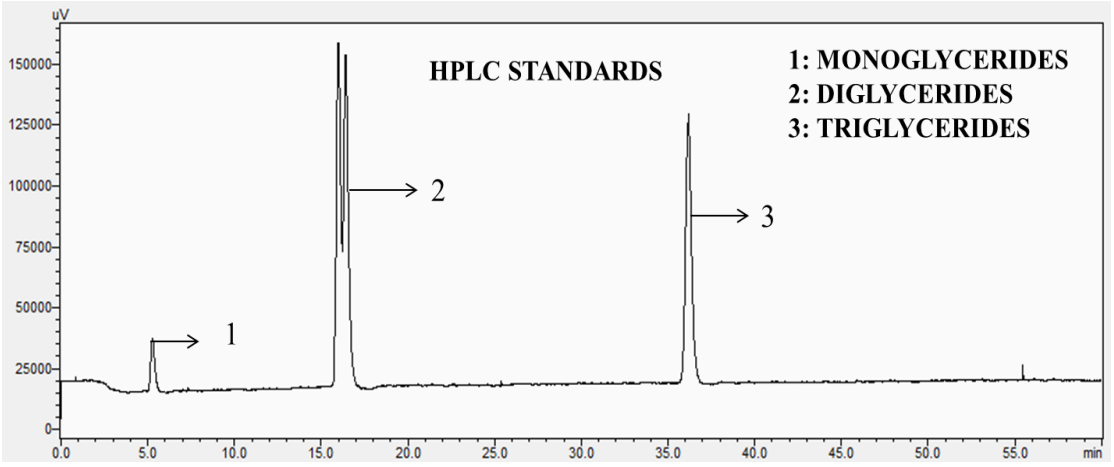
Appendix IV

Batch system of membrane unit with Polytetratfluoroethylene (PTFE) membrane (10 cm dia, Axivia, India).

Appendix V

HPLC (LC-20AD, Shimadzu Co, Kyoto, Japan) with RPC18 column using ELSD (Gilson).

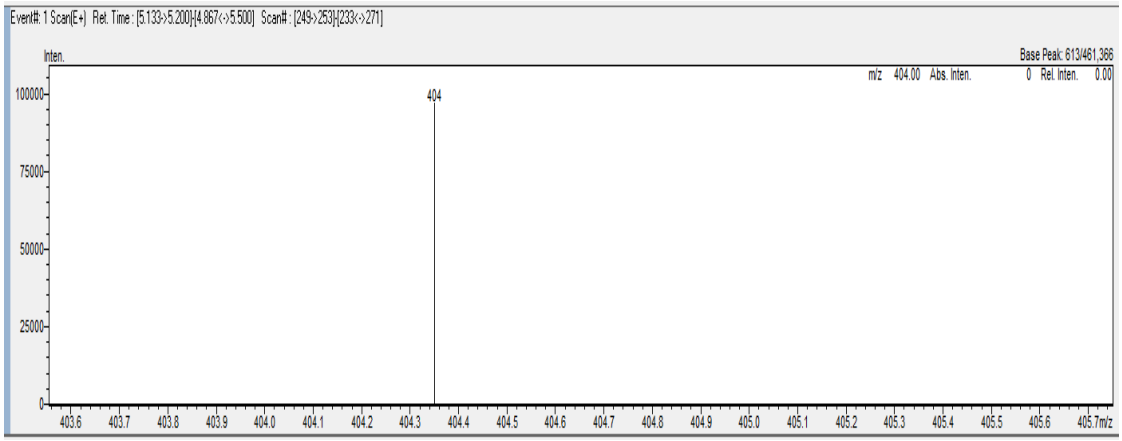
HPLC Standard Chromatograms of Glyceride sample



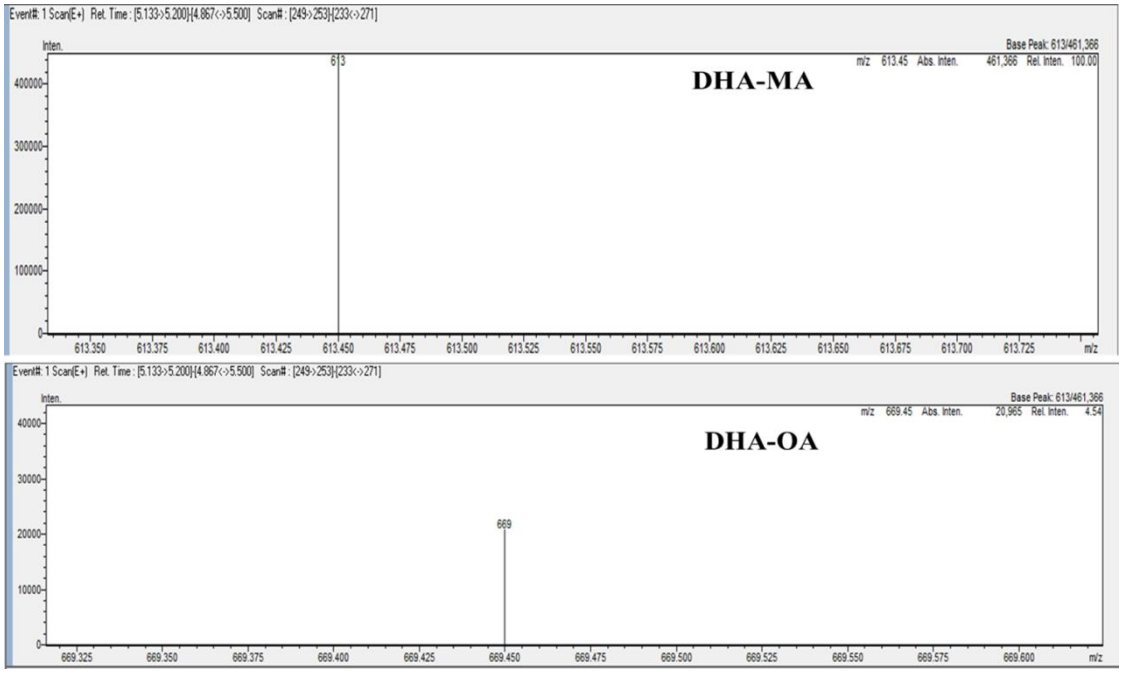
Appendix VI

LC-MS (Shimadzu Co, Japan) with a pump (LC-20AD), UV/Vis detector (SPD-20 A), column oven (CTO-10 AS) and MS fitted with an electron spray ionization source (ESI).

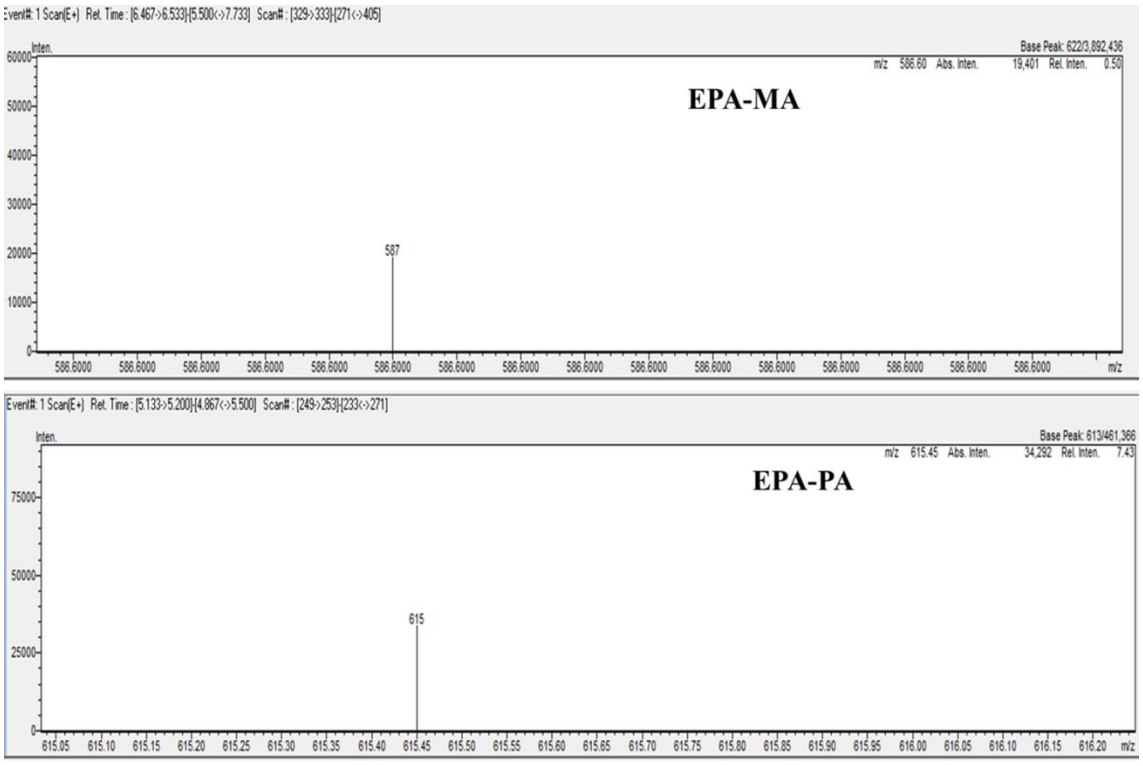
LC-MS Spectra of Monoglyceride of DHA in CRL (free and modified) hydrolyzed oil.



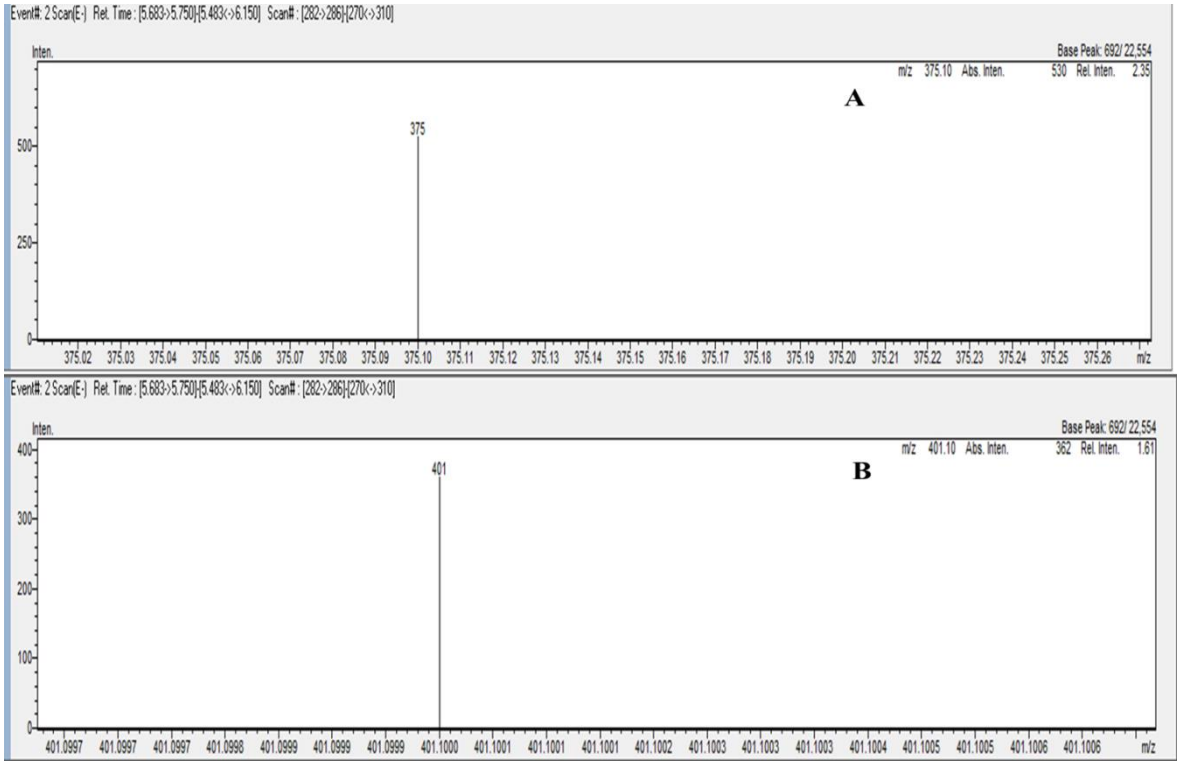
LC-MS Spectra of Diglyceride of DHA-MA and DHA-OA in CRL (free and modified) hydrolyzed oil.



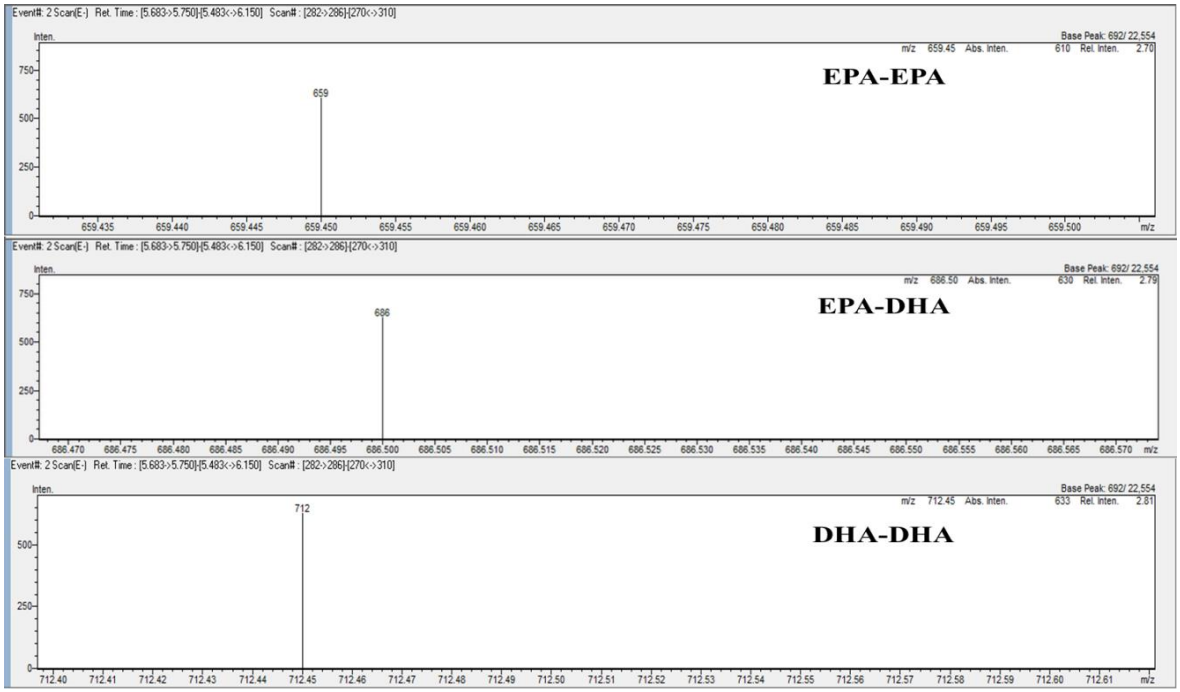
LC-MS Spectra of Diglyceride of EPA-MA and EPA-PA in CRL (free and modified) hydrolyzed oil.



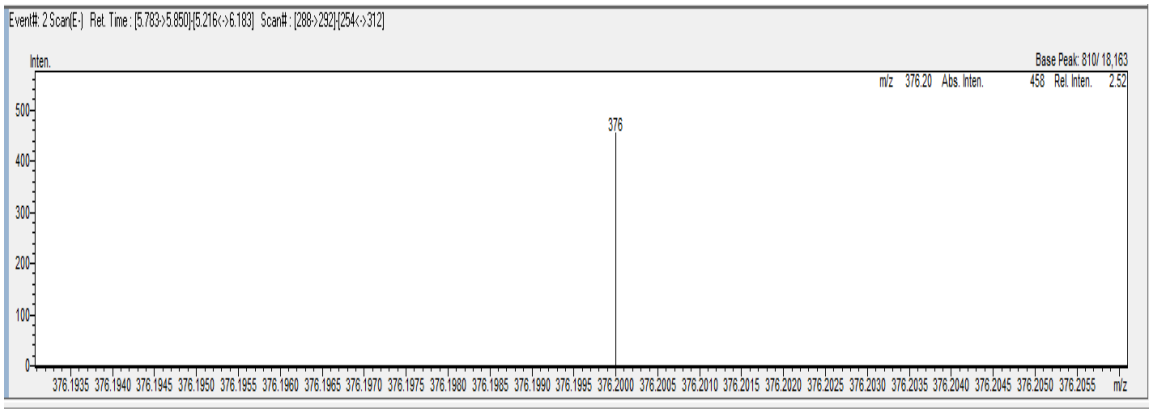
LC-MS Spectra of Monoglyceride of EPA (A) and DHA (B) in PCL (modified) hydrolyzed oil.



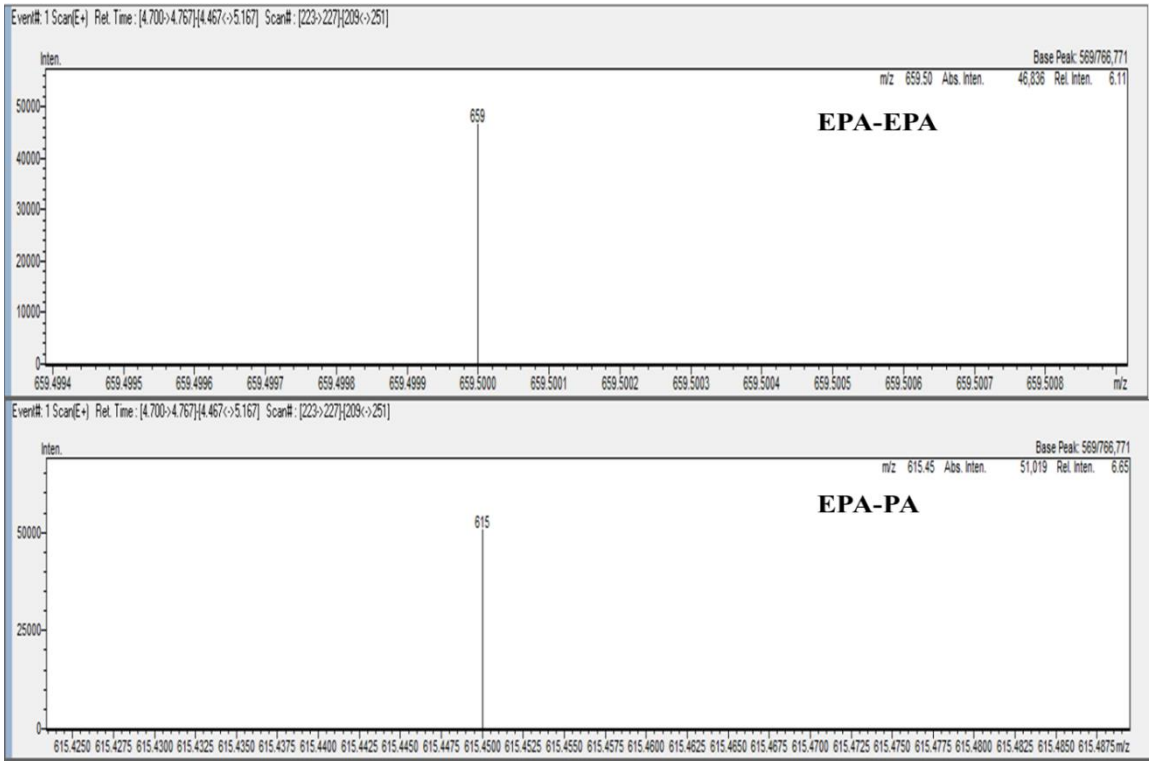
LC-MS Spectra of Diglyceride of EPA-EPA, EPA-DHA and DHA-DHA in PCL (modified) hydrolyzed oil.

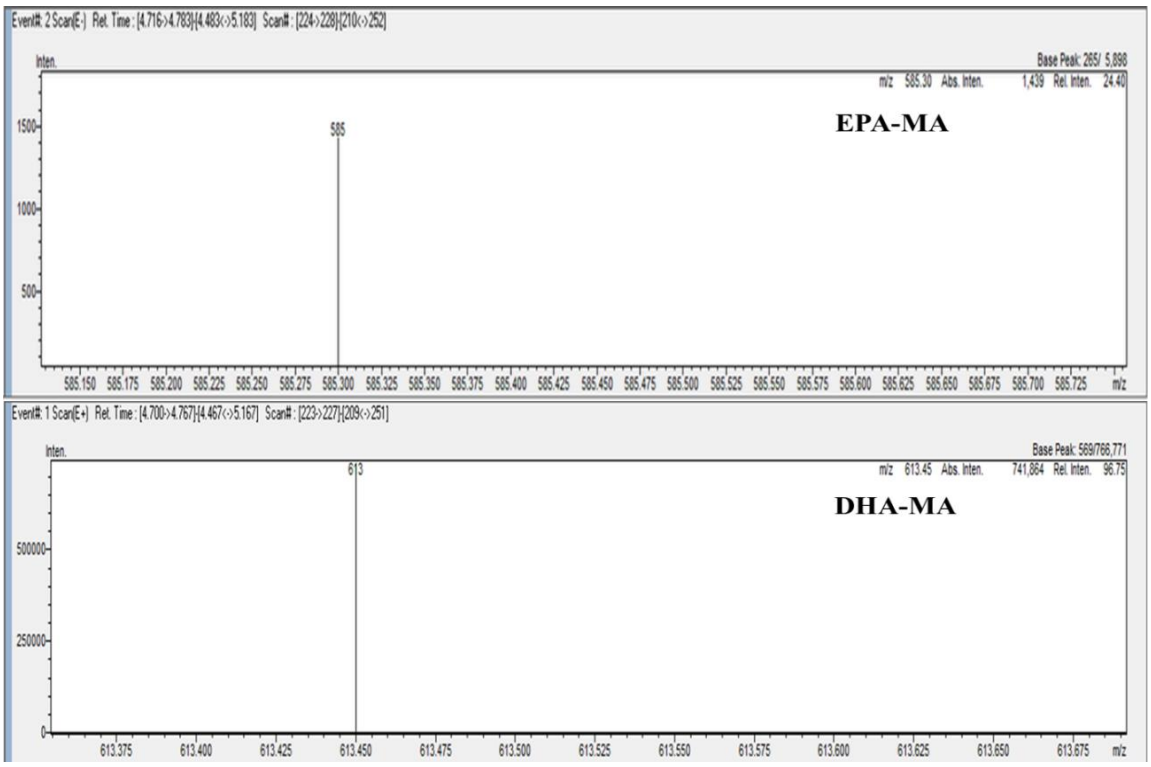


LC-MS Spectra of Monoglyceride of EPA in CRL-CLEA hydrolyzed oil.



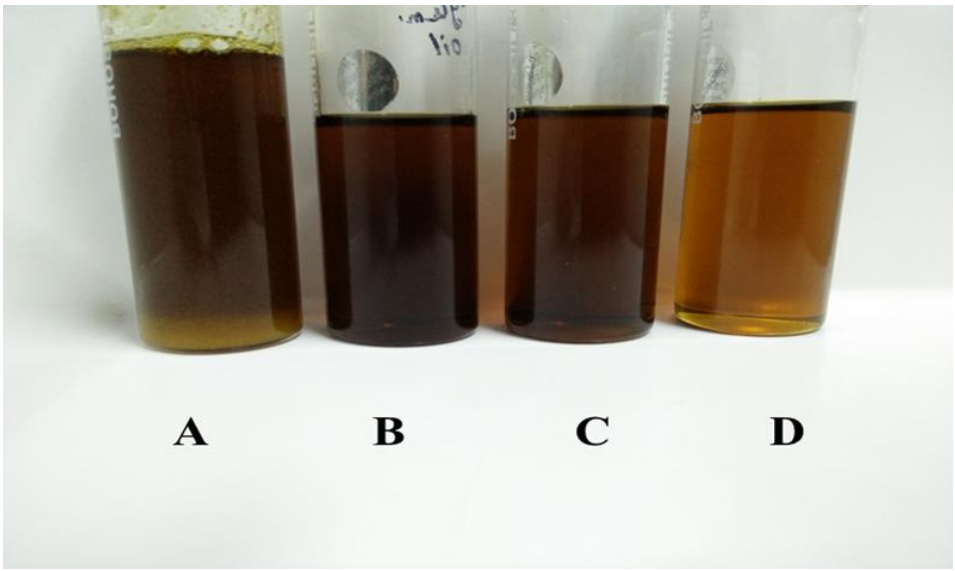
LC-MS Spectra of Diglyceride of EPA-EPA, EPA-PA, EPA-MA and DHA-MA in CRL-CLEA hydrolyzed oil.





Appendix VII

An image of the oils during various stages from refining to n-3 PUFA concentration



Crude sardine oil (A), Degummed oil (B), Bleached oil (C), Hydrolyzed oil (D).

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LIST OF PUBLICATIONS BASED ON THE CURRENT WORK

JOURNAL PUBLICATIONS

1. Vaisali, C., **Charanyaa, S**., Belur, P.D. and Regupathi, I. (2015). "Refining of edible oils: a critical appraisal of current and future technologies". *Int. J. Food Sci. Technol*., 50, 13-23.
2. **Charanyaa, S**., Belur, P.D., Regupathi, I. (2017). "A New Strategy to Refine Crude Indian Sardine Oil." *J. Oleo. Sci*., 66(5), 425-434.
3. **Sampath, C**., Belur, P.D and Regupathi, I. (2018). "Enhancement of n-3 polyunsaturated fatty acid glycerides in Sardine oil by a bioimprinted cross-linked Candida rugosa lipase". *Enzyme Microb. Technol*., 110, 20-29.

MANUSCRIPTS UNDER REVIEW

1. **Charanyaa, S**., Belur, P.D and Regupathi, I. (2017). "Effect of intrinsic and extrinsic factors on the deterioration of Indian Sardine oil". *Journal of Food Engineering.*

BOOK CHAPTERS

1. Belur, P.D., Regupathi I., **Charanyaa, S** and Vaisali, C. (2017). "Refining Technologies for Edible oil. In: Chemat, S. (eds) Edible oil extraction, processing and applications". CRC press, Boca Raton, pp 97-126.
2. **Sampath, C**., Anita, N., Prasanna, B.D and Regupathi, I. (2016). "Enzymatic Concentration of n-3 Polyunsaturated Fatty Acids from Indian Sardine Oil" In: Prasanna, B.D., Gummadi, S.N., Vadlani, P.V. (eds) "Biotechnology and Biochemical Enginnering". Springer, Singapore, pp 137-143.

PATENTS

1. Belur, P.D., Regupathi, I., **Charanyaa, S** and Vaisali, C. "A process for improving n-3 polyunsaturated fatty acid content in sardine oil" filed in Indian patent office (2016). Application no. 201641007984.

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1. **Charanyaa, S**., Belur, P.D. and Regupathi, I. (2017). "Optimization of Lipase Mediated Enrichment of n-3 PUFA Glycerides in Indian Sardine Oil.". *Int. Eng. Symp..*, Kumamoto University, Kumamoto, Japan published in International Journal of Earth Science and Engineering, 10 (4), 863-867.
2. **Charanyaa, S**., Vaisali, C., Belur, P.D. and Regupathi, I. (2016). "Screening of polymeric membranes for membrane assisted deacidification of sardine oil". *Technoscape,* VIT, India published in Resource- Efficient technologies Technologies, 2, S119-S123.
3. **Charanyaa, S**., Belur, P.D. and Regupathi, I. (2015). "Enzymatic Concentration of n-3 Polyunsaturated Fatty Acids from Indian Sardine Oil." *Proc. Int. Conf. Adv. Chem. Eng.,* Surathkal, India published as a book chapter in "Biotechnology and Biochemical Enginnering". Springer, Singapore, pp 137-143.

Bio-Data

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ACADEMIC DETAILS

I. **PhD Chemical Engineering** (2012-2017)

National Institute of Technology, Karnataka, India

- Advisor: Dr. Prasanna D Belur and Dr. I. Regupathi
- Thesis: "Studies on the production of n-3 polyunsaturated fatty acid glyceride concentrate from Indian sardine oil".

II. **Master of Technology in Biotechnology** (2010-2012).

Vellore Institute of Technology, Vellore, India

- Project title: "Standardization of proteolytic enzyme assay".
- Score: **8.47 CGPA**.

JOURNAL PUBLICATIONS

1. **Sampath,C**., Belur, P.D and Regupathi, I. (2018). "Enhancement of n-3 polyunsaturated fatty acid glycerides in Sardine oil by a bioimprinted cross-linked Candida rugosa lipase". Enzyme and Microbial Technology, 110, 20-29.
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6. Nayak, S., Harshitha, M.J., Maithili., **Sampath, C**., Anilkumar, H.S., Rao, C.V. (2012). "Isolation and characterization of caffeine degrading bacteria from coffee pulp." Indian Journal of Biotechnology, 11, 86-91.

CONFERENCE PROCEEDINGS

1. **Charanyaa, S**., Belur, P.D. and Regupathi, I. (2017). "Optimization of Lipase Mediated Enrichment of n-3 PUFA Glycerides in Indian Sardine Oil.". International Engineering Symposium (IES 2017), Kumamoto University, Kumamoto, Japan.
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6. **Sampath, C**., Nayak, S., Harshitha, M.J., Maithili. (2010). "Isolation and characterization of caffeine degrading bacteria from coffee pulp." Srishti 2010, SSIT, Tumkur, Karnataka.

INVITED BOOK CHAPTER

1. Belur, P.D., Regupathi I., **Charanyaa, S**., Vaisali, C. (2017). "Refining Technologies for Edible oil. In: Chemat, S. (eds) Edible oil extraction, processing and applications". CRC press, Boca Raton, pp 97-126.

BOOK CHAPTERS

1. **Sampath, C**., Anita, N., Prasanna, B.D and Regupathi, I. (2016). "Enzymatic Concentration of n-3 Polyunsaturated Fatty Acids from Indian Sardine Oil" In: Prasanna, B.D., Gummadi, S.N., Vadlani, P.V. (eds) "Biotechnology and Biochemical Enginnering". Springer, Singapore, pp 137-143.

PATENTS

1. Belur, P.D., Regupathi, I., **Charanyaa, S** and Vaisali, C. "A process for improving n-3 polyunsaturated fatty acid content in sardine oil" filed in Indian patent office (2016). Application no. 201641007984.

RESEARCH EXPERIENCE

1. **PhD researcher in National Institute of Technology Karnataka(NITK), Surathkal (December 2012 – present)**

- Tailor made a refining strategy of the oil which was focussed on maximum removal of impurities without any loss of n-3 PUFA.
- Development of successful membrane technology (batch and continuous) for deacidification using various membranes for edible oil processing- studied the efficiency of deacidification in terms of the effect of solvents, flux, pressure and membrane polarity.
- Performed a comprehensive study of the effect of various factors on the storage stability of refined Indian Sardine Oil to improve the product quality.
- Specialized in immobilization of lipases for the hydrolysis of the oil for the enhancement of n-3 PUFA in the oil and its limitations - characterization of the immobilized lipase.
- Knowledge on non-aqueous and aqueous enzymology for the development of bio-imprinted lipase for the use in enhancement of n-3 PUFA in the oil.
- Methods were developed to enrich the omega-3 fatty acid content of the sardine oil

- Implemented all the chromatographic techniques, for the analysis of the experiments.
- Various analytical techniques were performed to characterise the physical and chemical properties of oil.
- Hands on experience and thorough knowledge of handling, data interpretation, and troubleshooting GC, HPLC, LC-MS, Scanning electron microscopy, viscometer, and densitometer.
- Supervised four M.Tech students
- Performed statistical significance of all the experiments by using several statistical tools

2. **Project Intern on Standardization of proteolytic enzyme assay, Biocon, Bangalore, Karnataka (December 2011- July 2012).**

- Comparison of the various purification techniques to study the enzyme activity.
- Studied the optimal extraction conditions of the enzyme.
- The confirmation of the activity by HPLC and GC.

TEACHING EXPERIENCE

- 7 months of teaching experience in the post of Assisstant lecturer in the Department of Chemical Engineering, National Institute of Technology, Surathkal, Karnataka.
- Volunteered as a teacher under "MAKE A DIFFRENCE" organization for subjects like Science and English.

PROJECTS AND TRAININGS

1. **Project, Department of Biotechnology, Vellore Institute of Technology, Vellore**

   Isolation and characterization of proteins of wood apple

2. **Project, Department of Biotechnology, Vellore Institute of Technology, Vellore**

   Part 1: The preparation of new cryogel matrices.

Part 2: The effect of prepared cryogel matrices on the wood apple proteins on its cell growth.

3. **Training, School of life sciences, Manipal**
   Techniques involved in the study of animal cell lines
   Immunotechnology: Trained on blood testing, ELIZA test, Western blot, PCR techniques.

LABORATORY SKILLS

- Basic chromatographic skills (HPLC, LC, HPLC-ESI-MS, HPLC-APCI-MS, GC) and other instrumental analysis like thermogravimetry (TG), SEM.
- Biochemical tests, Enzyme assays and inhibitor screening, protein extraction and purification procedures, electrophoresis techniques (SDS-PAGE, Native PAGE).
- Microbiology skills like culture preparation, pure culture isolation, inhibitory studies and microbial-biochemical tests.
- Analytical techniques for performing oxidation studies.

AREAS OF INTEREST

- Environmental Biotechnology, Downstream processing and bio-separation technology of bioproducts, Enzyme technology, Microbiology, Food biotechnology and Membrane technology.

Declaration:

I hearby declare that all the information mentioned above is true to the best of my knowledge.

CHARANYAA SAMPATH