# Development of Novel Refining Techniques and Enzymatic Synthesis of Antioxidant Esters for Improving the Oxidative Stability of Sardine Oil

**Thesis**

Submitted in partial fulfilment of the requirements of the degree of

**DOCTOR OF PHILOSOPHY**

By

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## DECLARATION

I hereby declare that the Research Thesis entitled "**Development of novel refining techniques and enzymatic synthesis of antioxidant esters for improving the oxidative stability of sardine oil"** which is being submitted to the **National Institute of Technology Karnataka, Surathkal**, in partial fulfillment 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 "**Development of novel refining techniques and enzymatic synthesis of antioxidant esters for improving the oxidative stability of sardine oil"** submitted by **Ms. Vaisali C (Register Number: 135026CH13F04)** 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

The growing market for n-3 polyunsaturated fatty acids (PUFA) has lead to the increasing amount of research in fish oil as they are excellent sources of n-3 PUFA. However, the highly unstable nature of these compounds has to be addressed. Hence, the present work deals with improving the stability of Indian sardine oil obtained from the western coastal region.

This study aimed at developing novel refining strategies for removing impurities that caused poor stability and quality. Novel technologies involving simultaneous degumming and deacidification and membrane assisted free fatty acid removal were employed in the present work to eliminate the impurities in crude sardine oil that might increase the rate of oxidation. A novel degumming method involving triethanolamine resulted in simultaneous degumming and deacidification with phospholipid reduction from 303 ppm to 37 ppm, while the free fatty acids were reduced from 3.75% to 1.21%. Further removal of impurities was done by membrane assisted deacidification followed by bleaching using activated charcoal, resulting in refined sardine oil. The n-3 PUFA content in sardine oil was retained without much loss

The best and convenient method for improving the oxidative stability of fish oils is by adding antioxidants which delays the oxidation process. In the current study, several natural antioxidants were studied for their effect in reducing the oxidation process in Indian sardine oil. Quercetin, rutin, gentisic acid and caffeic acid showed maximum effect in imparting oxidative stability. Further, the effect of antioxidants in stabilizing sardine oil in the presence of metal ions and trace water were tested. Though the effectiveness of antioxidants was not affected when sardine oil possessed trace metals, their efficiency reduced drastically with increase in trace water levels.

Considering the relatively high antioxidant activity of rutin in Indian sardine oil and the complex heterogeneous nature of the oil, rutin was enzymatically modified to

improve its solubility in oil. It was identified that rutin was more effective during oxidation of refined sardine oil, whereas rutin ester showed higher activity in sardine oil containing trace water indicating the importance of antioxidant polarity and oil heterogeneity.

**Keywords**: Indian Sardine oil, n-3 PUFA, Refining, Oxidative stability, Natural antioxidants, Hydroxycinnamic acids, Hydroxybenzoic acids, Flavonoids, Trace metals, Trace water, Esterification, *Candida antartica* lipase, Rutin fatty ester

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## ABBREVIATIONS

| | |
|---|---|
| AAS | Atomic Absorption Spectrometry |
| ALA | Alpha Linolenic Acid |
| BHA | Butylated Hydroxyl Anisole |
| BHT | Butylated Hydroxyl Toluene |
| CD | Conjugated Diene |
| DHA | Docosahexanoic Acid |
| DO | Dissolved Oxygen |
| DPPH | Diphenyl-1-picrylhydrazyl |
| EPA | Eicosapentanoic Acid |
| ESI | Electron Spray Ionisation |
| FAME | Fatty Acid Methyl Ester |
| FFA | Free Fatty Acids |
| GC | Gas Chromatography |
| HLB | Hydrophilic Lipophillic Balance |
| HP | Hydratable Phospholipid |
| HPLC | High Performance Liquid Chromatography |
| LC | Liquid Chromatography |
| MDA | Malondialdehyde |
| MS | Mass Spectrometry |
| NHP | Non-Hydratable Phospholipid |
| pAV | p-Anisidine Value |
| PES | PolyEtherSulfone |
| PVA | Polyvinyl Alcohol |
| PTFE | Poly Tetra Fluoro Ethylene |
| PUFA | Polyunsaturated Fatty Acids |
| TBARS | Thiobarbituric Acid Reactive Substances |
| TBHQ | Tertiary Butyl Hydroquinone |
| TPTZ | 1,1,3,3–tetramethoxypropane |

## NOMENCLATURE

| | |
|---|---|
| °C | Degree centigrade |
| g | grams |
| L | Litre |
| µ | micro |
| m | milli |
| M | Molar |
| meq | Milli equivalents |
| rpm | Revolutions per minute |
| Pa | Pascal |
| ppm | Parts per million |
| w/w | Percentage weight by weight |
| w/v | Percentage weight by volume |

# CHAPTER 1

# INTRODUCTION

Fatty acids are essential compounds of human metabolism. They usually have even number of carbon atoms with either single bond (saturated) or with double bonds (unsaturated) connecting them. Polyunsaturated fatty acids (PUFA) contain one or more double bonds and they are named based on its position (Figure 1.1). Recently, PUFAs are being given much importance due to their beneficial effects on human health, especially in reducing cardiovascular diseases. These PUFAs are tagged as essential as they are precursors of many metabolites, thus providing biological significance (Ziaullah et al. 2013). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the two important n-3 PUFA that helps in improving human health.

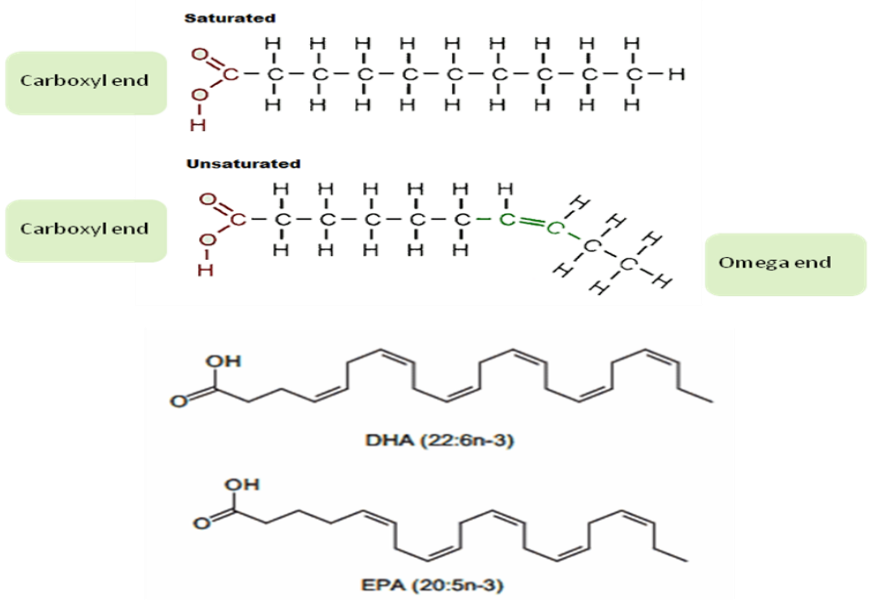



**Figure 1.1 Structure of essential fatty acids**

The numerous health benefits of n-3 PUFA has been widely explored in prevention of many diseases (Von Schacky and Harric 2007, Riediger et al. 2009, Bendsen et al. 2011). Hence, these fatty acids have to be consumed as dietary supplements. As a result, there is a growing market for edible forms of n-3 PUFA as food supplements which is mostly satisfied by the use of fish oil. Menhaden, sardine, herring, cod liver and anchovy are the major commercial sources of fish oil that are widely available (Yamaguchi et al. 2004, Vaisali et al. 2015).

Fish represents a valuable source of essential fatty acids, as they are a rich source of long chain n-3 PUFAs, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Gebauer et al. 2006). **Indian sardines** (*Sardinella longiceps*) are a major marine pelagic fisheries resource of India and is highly valuable owing to its nutritional value and industrial use. Since the major part of harvest is being used for canning and fish meal production (Tacon and Metian 2008), an impetus exists for the exploitation of its oil, which is usually the by-product of fish meal industry (Dahl and Malcata 1999). Additionally, fish oil can be effectively processed to gain a nutraceutical agent. Sardine oil is one of the richest and cheapest sources of essential fatty acids, especially EPA and DHA. The level of n-3 PUFA in sardine oil usually ranges from 20% to 50% depending upon the type of species, geographic location of fish and season (Chakraborty and Joseph 2015, Vaisali et al. 2015). These fatty acids exist in the form of glycerides in oil, which is more preferred than their free form (Bendsen et al. 2011).

In order to utilise this abundant natural resource effectively, it should meet the quality standards of the consumer. Further, care has to be taken to remove undesirable compounds from crude fish oil, which not only lowers the quality and stability of oil but also affects human health negatively on consumption (Ichinose et al. 2004). For this purpose, the raw material has to undergo a series of processing stage to obtain the desired quality. In addition to the tedious process of removing undesirable components from sardine oil, another major drawback in effectively utilising these high value products is their poor oxidative stability. Such a setback is due to the presence of high degree of unsaturated fatty acids in sardine oil (Vaisali et al. 2015). These unsaturated fatty acids undergo auto-oxidation and breakdown into numerous products that reduce oil quality and produce off-flavours. Hence, crude fish oil has to be processed and the valuable components have to be protected from oxidation process for human consumption.

### 1.1. Strategies to improve sardine oil quality and stability

The crude oil obtained from sardine fish usually contains impurities, which further speeds up the oxidation process. Additionally, the impurities in fish oil results in off-

odours, poor taste and appearance. Hence, fish oil needs to be processed for edible and technical application. Crude edible oils do not meet these criteria as they often contain large amount of undesirable compounds. These numerous impurities are removed through operations collectively known as ***refining***. Preservation of essential components and valuable substances is desired while eliminating impurities. Refining of marine oils is a tedious process, as harsh processing methods might lead to the loss of n-3 PUFA compounds. Though many industries follow conventional refining methods involving degumming, deacidification or physical refining, bleaching and deodorisation, there is still a wide scope for improving the refining technology that will ensure protection of PUFA rich glycerides. Though refining of fish oil ensures increased shelf life, it doesn't necessarily arrest oxidation. However, addition of antioxidants can be considered as a way to protect the oxidation of these n-3 PUFA.

In case of fish oils, the main barrier for their successful use is its higher susceptibility to ***oxidation*** and the poor sensorial qualities. PUFA compounds are known to oxidise faster, as the hydrogen atoms next to double bonds are less stable due to low C-H bond energy (Ruxton et al. 2007). The most popular, effective, convenient and economical method for stabilising the lipid products is by ***antioxidant addition***. The use of natural antioxidants is an interesting alternate to synthetic antioxidants for improving the oxidative stability of n-3 PUFA rich oil. Natural antioxidants like phenolic acids, flavonoids and tannins not only provide good sensorial qualities and antioxidant activity to food, but also impart beneficial properties (Lule and Xia 2005). Although oxidation and antioxidation in many edible oils have been studied, little systematic research has been performed in Indian Sardine oil. Since the importance of sardine oil is well established, there is a great scope for strategies that stabilise this particular resource for commercial use. The choice of antioxidants to stabilise fish oils is restricted to few substances, with tocopherols being the most frequently used due to the complex nature of fish oil (Yanishlieva and Marinova 2001). However, they do not provide effective protection at all conditions.

Natural phenolic compounds like flavonoids from tea and rosemary extracts, along with a wide range of plant phenolic extracts have been gaining importance due to their

safety as well as additional flavours (Brewer 2011). As the activity of antioxidants in lipids is influenced by numerous factors (Chang et al. 2003) antioxidants that are effective in one system may not be suitable for other systems. Additionally, the effect of polarity and molecular characteristics influence the antioxidant compounds to have varying effects in different food systems. Hence, it is necessary to make a comparison of different phenolics to find the best possible antioxidant for a specific system.

As PUFA rich systems are highly hydrophobic, all antioxidants are not effective due to their hydrophilic nature. One way to use the existing natural antioxidants would be to manipulate its properties to interact effectively with the bulk oil structures. These antioxidants can be designed in such a way that they are made available at micro/nano structures of oxidation in the particular food system. This could be obtained by altering the solubility properties of the antioxidant which can be done by modifying the original phenolic compounds to give an amphiphillic molecule that could be readily incorporated into oil. Such molecules can be synthesized by conjugating the carboxylic acid function of phenolics with hydrophobic compounds through chemical or enzymatic esterification. In terms of synthesis route of these lipophilic antioxidants, enzymatic synthesis is most preferred as phenolic acids are sensitive to heat and harsh treatments. Lipases are diverse group of enzymes that have the ability to reverse hydrolysis reaction to form esters in organic media under controlled conditions. Based on the nature of substrate used, two types of reaction can be performed for ester synthesis, viz. esterification and trans-esterification. The study on oxidation mechanism and improving the oxidative stability of Indian sardine oil can be considered a great advantage for Indian economy, as sardine oil is an abundant resources that is not utilised effectively due to poor technological development. Additionally the increasing interest in n-3 PUFA rich sources has increased the circumstantial demand for developing effective strategies to improve and maintain the quality standards of sardine oil.

## 1.2. Scope of the research work

- The available n-3 PUFA products in the market are usually produced by a series of chemical refining process that results in the formation of trans fats with loss of

other nutritional properties. Hence, there exists a huge opportunity for designing novel refining techniques by simultaneously retaining the inherent properties of these oils.

- The existing literature implies on the adverse effects of harsh chemical treatments for fish oil. Hence, an impetus exists for exploring novel and safer techniques involving membrane processes.
- Meagre reports on improving the oxidative stability of Indian sardine oil are available. There is a wide scope for the search of natural antioxidants for application in sardine oil, as they may lead to a more target directed antioxidant approach.
- Though many in vitro antioxidant assays are available to understand and predict the mechanism of action of a natural antioxidant, their efficiency in food systems fail to replicate the same results. Thus there exists an opportunity to conduct in vitro assays in bulk oil systems to correlate antioxidant activity and oxidation mechanism.
- There is a great difference between antioxidant polarity and the polarity of sardine oil. Hence, a prospect for altering the solubility properties of the antioxidants to interact better with sardine oil exists.
- The available literatures on the lipophilisation of antioxidants are restricted to cinnamic acid derivatives and few other natural antioxidants. Hence, there is a large scope for exploring new antioxidant derivatives for their application in improving the oxidative stability of sardine oil.
- The existing reports on this direct esterification of antioxidants with lipids have not discussed all the reaction parameters in detail. This further increases the scope of exploring the process parameters that affect the esterification reaction in detail.

### 1.3. Objectives

In line with the above observations, the research work on Sardine oil was taken up with the following objectives,

1. Characterisation of Indian Sardine oil and development of novel refining strategies for processing crude sardine oil
2. Comparison and identification of best natural antioxidants for improving the oxidative stability, and establish structure-activity relationship for antioxidants in sardine oil.
3. Study on the effectiveness of chosen antioxidants in the presence of major impurities
4. Enzymatic synthesis of novel esters of antioxidants and to study the effect of reaction parameters on ester synthesis
5. Efficiency of synthesized antioxidant esters in improving oxidative stability of sardine oil.

### 1.4. Thesis Organisation

The stated objectives have been addressed in this doctoral work and they are reported in the following chapters.

**CHAPTER 1: Introduction**

This chapter proposes the basis of the work, discusses the background and the significance of the proposed work. The chapter further highlights the importance of n-3 PUFA rich sardine oil and its potential as a neutraceutical and other food applications. The scope and motivation for the current work has been stated and the formulated objectives have been presented.

**CHAPTER 2: Review of Literature**

This chapter provides a detailed and comprehensive review of the literature pertaining to the research work. The beneficial applications of n-3 PUFA has been stated briefly followed by the importance of Sardine oil. The properties of crude oil and the need to stabilise the sardine oil by various stages of refining has been discussed. Further, concise information on the oxidative stability of such marine oils has been provided along with the strategies to improve the oxidative stability. The various antioxidants

and their mechanism of action have been briefly discussed and the relevant published research works on the application of antioxidants in oil has been summarised.

**CHAPTER 3: Characterisation and Refining of Indian Sardine oil**

This chapter presents the characteristics of crude sardine oil sourced locally. Experimental results pertaining to the development of refining process are presented here.

**CHAPTER 4: Comparison of natural antioxidants for improving oxidative stability of refined sardine oil**

This chapter discusses the results on the screening of different natural antioxidants for improving oxidative stability of sardine oil. The results on the structure-activity relationship of antioxidants in sardine oil are discussed. This chapter further presents the results on the effectiveness of the chosen antioxidants in the presence of major impurities while improving oxidative stability, with a brief description on the mechanism of oxidation.

**CHAPTER 5: Synthesis of antioxidant ester for improving the oxidative stability of sardine oil**

This chapter presents the results of enzymatic synthesis of lipophilic antioxidant esters using immobilised *Candida antartica* lipase along with the optimum conditions required for maximum conversion of native antioxidant to its corresponding ester. The results on the application of lipophilic antioxidant ester for improving the oxidative stability of sardine oil are discussed and compared with the efficiency of native antioxidant.

# CHAPTER 2

# REVIEW OF LITERATURE

## 2.1. HEALTH BENEFITS OF n-3 PUFA

Since the early studies in Greenland Eskimos in 1980, increased dietary intake of fatty fish has been consistently associated with the prevention of cardiovascular diseases (He et al. 2004). Hence, interest in the benefits of n-3 PUFA on human health has led to the increasing amount of literature over the last few years. The effect of dietary consumption of EPA and DHA on human health has been presented in many literatures, all of which suggests the prevention of coronary heart disease, myocardial infarction and strokes (Pike and Jackson 2010). n-3 PUFA are known to reduce the production of inflammatory eicosanoids, cytokines and the expression of adhesion molecules (Calder 2006) which found its application in the treatment of inflammatory diseases like eczema, psoriasis, inflammatory bowel disease and rheumatoid arthritis (Cleland et al. 2003, Chen and Yeh 2003). Additionally, EPA and DHA have been reported to induce breast cancer cell apoptosis and have anticancer effects (Wu et al. 2005). These compounds act by getting incorporated into the cell membranes of many cell populations and consequently influence the disease process by decreasing the platelet aggregation and thrombosis, and also reduce the accumulation of oxidation products in liver (Gogus and Smith 2010). A brief depiction of some of the well-established beneficial effects of n-3 PUFAs are shown in Figure 2.1.

The nutritional guidelines for maternal diets have always signified the importance of protein and caloric requirements. However, recently the requirements of fatty acids have also been deemed important (Swanson et al. 2012). Evidence on the effect of long chain n-3 polyunsaturated fatty acid in the treatment and prevention of mental illness are also available (Song and Zhao 2007). The importance of n-3 PUFA on the neurological development and mental health, including cognitive function could be of much importance in the near future. As EPA and DHA play a vital role as a component in brain and nervous tissue and also for the development of these organs, it could be given as a supplement for pregnant woman or as infant nutritional supplements. These compounds are essential for proper fetal development and healthy aging. Though there are numerous studies supporting these claims, there are no detailed dietary intervention studies to convince the authorities (Pike and Jackson

2010). In a recent study it has been established that long chain n-3 PUFAs supplementation to elderly female patients has reduced depressive symptoms, improved phospholipid fatty acid profile resulting in overall improvement in health (Rondanelli et al. 2011). Based on the availability of vast literature on the advantages of n-3 PUFA consumption, a huge market demand for PUFA based food products can be expected in the near future.

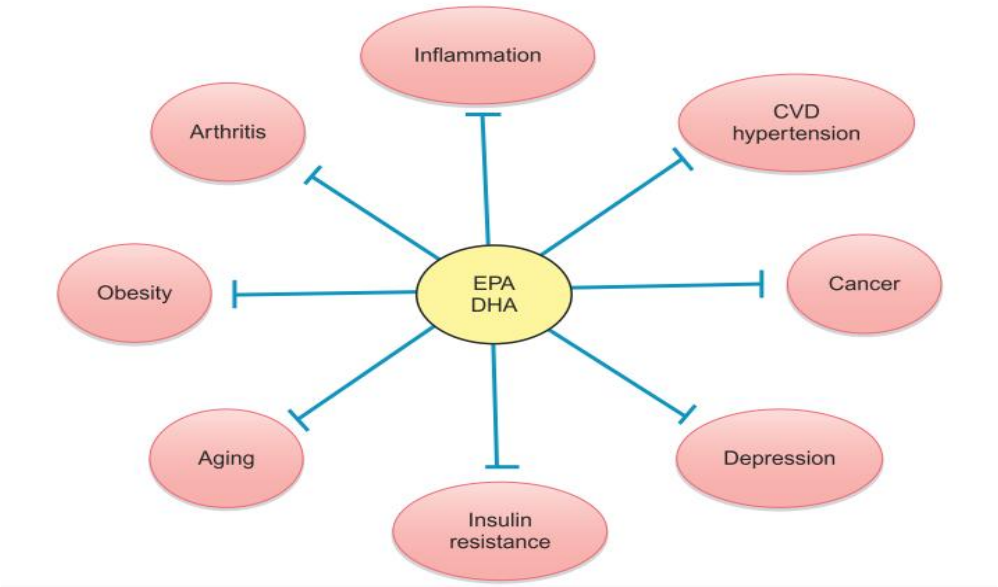



**Figure 2.1 Scientifically validated benefits of EPA and DHA (Source: Siriwardhana et al. 2012)**

Many reviews were presented on the importance of consumption of polyunsaturated fatty acids (Ruxton et.al. 2007, Gogus and Smith 2010, Rubio-Rodriguez et al. 2010, Massaro et al. 2010) based on which, works related to incorporation of polyunsaturated fatty acids in food systems were developed (Wang et al. 2012, Okada and Morrissey 2007, Frankel et al. 2002, Febrianto and Yang 2001). The *in vivo* derivatisation of n-3 PUFA from other fatty acids is shown in Figure 2.2. As can be seen from the figure, the consumption of vegetable and seed oil ensures the presence of these precursor fatty acids (α-linolenic acid and linoleic acid) in human body. Consequentially, these fatty acids become essential nutrients. Technically, EPA and DHA are non-essential fatty acids as the human body is capable of producing these

fatty acids from alpha lenolenic acid (Siriwardhana et al. 2012). However, the conversion in humans is not efficient enough to meet the demand required for beneficial effects. Few studies suggested 2-10% conversion of alpha lenolenic acid to EPA and DHA, while several others found lesser conversion (Swanson et al. 2012). Hence, consumption of marine oils rich in these n-3 PUFA (Figure 2.2) can serve as an alternate for gaining wide beneficial effects from these compounds.

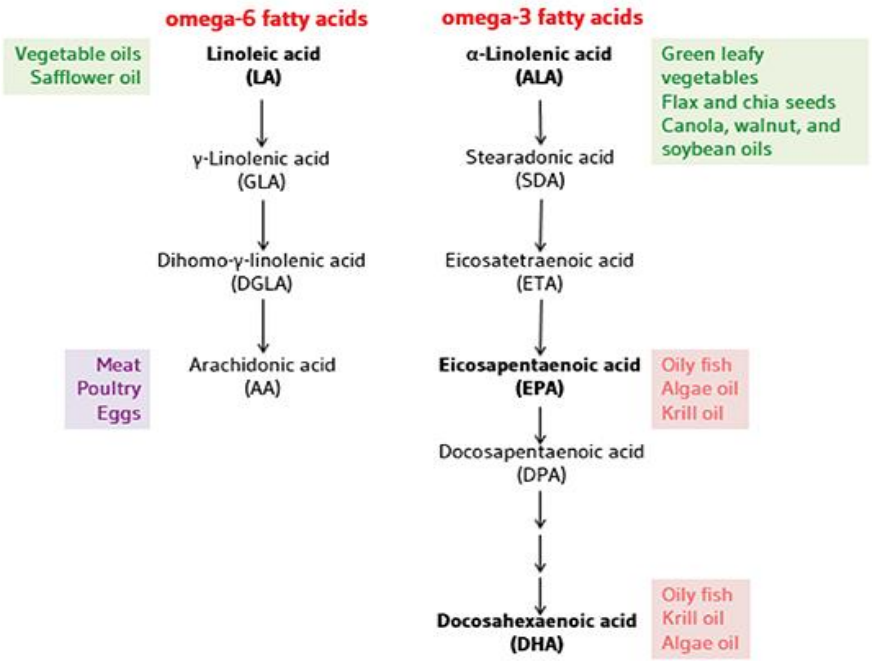



**Figure 2.2 Synthesis of n-3 polyunsaturated fatty acids (Source http://lpi.oregonstate.edu/mic/other-nutrients/essential-fatty-acids)**

### 2.1.1. Recommended consumption of n-3 PUFA

The typical recommended consumption of EPA and DHA by the World Health and North Atlantic Treaty Organisation is 0.3-0.5 g/day of both EPA and DHA. Though the average intake of n-3 PUFA in United States is 1.6 g/day, only 0.1-0.2 g/day is from EPA and DHA (Kris-Etherton et al. 2000). While the average intake of n-3 PUFA in Japan is 1-2 g/day, Canada recommends a consumption of 1.2-1.6 g/day (Arab-Tehrany et al. 2012). The current trend for fast food consumption has increased the intake of saturated fat that has very low quantities of essential omega-3 fatty acids.

This low intake of EPA and DHA is presumed to be associated with the increase in heart diseases. Hence, the American Heart Association has recommended consumption of fatty fish meals at least two times a week for a healthy life. Based on the availability of vast literature on the advantages of n-3 PUFA consumption, a huge market demand for PUFA based food products can be expected in the near future.

## 2.2. EDIBLE SOURCES OF n-3 PUFA

With the increasing knowledge on the benefits of n-3 PUFA, search for good sources of these components that could be effectively utilised as food supplements are constantly in demand. Plant derived foods like nuts, oil, seeds are good sources of alpha lenolenic acid form which EPA and DHA are derived (Figure 2.2). However, the primary and well known sources of n-3 PUFA are fatty fish and fish oils with anchovy, salmon, sardines, sturgeon, herring, mackerel, tuna and trout being the popular sources of fish oil. The three significant features in fish oil make it unique in comparison to other commercially available vegetable oil are:

(1) High unsaturation and number of carbon atoms in fatty acids,

(2) High concentration of long chain n-3 PUFAs,

(3) Variable composition of glycerides and fatty acids (Arab-Tehrany et al. 2012).

Due to the various health benefits and recommendation by several world health agencies, fish oil has become a major dietary supplement. In addition to fish oil, marine algal species are also reported to be good source of EPA and DHA. However, the concentrations in these species are very less in comparison to fish sources (Siriwardhana et al. 2012). This has led to a drastic increase in the world fish production with 160 million metric tons at present. A major part of this is from pelagic fisheries caught for fish meal and fish oil production (Bockish 2010). Bockisch 2010 has critically reviewed the developments in fish oil industry over past decade and its progress to becoming a precious and good dietary ingredient.

The versatile use of fish body oils has not yet assumed commercial significance in India due to the uncertain quality of oil. However, much importance is being given these days to ascertain and improve the quality of edible oils by various technologies (Bhosle and Subramanian 2005, Saravanan et al. 2006, Rao et al. 2013, Greyt 2012, Dijkstra 2013). Since India is the second largest aquaculture producer in 2010 (Ghaly et al. 2013), the effective use of our aquatic resources can be done by utilising the available knowledge to gain a nutritionally significant oil of good quality.

The Indian subcontinent harbours a rich fish biodiversity. On testing the EPA and DHA content along with the fatty acid profile of 39 types of fish species in India, it was identified that *Sardinella longiceps* (Indian Oil Sardines) is one of the richest source of n-3 PUFA (Mohanty et al. 2016). According to National Fish Processing Development Board (NFPDB) fish processing in India is mainly done as an export business for sun dried fish and fish meal. According to Department of Animal Husbandry, Dairying and Fisheries, sardine fish production in India has increased from 21805 tonnes in year 2007 to 82924 tonnes in the year 2012. A recent case study on the production and marketing of fish meal in India indicated that oil sardines are the major fish used in majority of the fish meal plants with a variable operating capacity of 20 to 350 tonnes per day (Ponnusamy et al. 2012).

Table 2.1 depicts the data collected from various fish industries in the southern part of India, reflecting the production and marketing of fish meal. The main by-product of fish meal production is crude fish oil, which in turn is exported to mostly south-east Asia. These exported products are processed and then re-exported to meet the standards of the world market. Hence, there is a great economic potential for the proper processing and utilisation of fish products in India. The sustainability of this fish meal industry rests on the proper utilisation of all by-products and hence research efforts for conversion of fish oil to edible oil are a necessary task to be taken up by these industries. Additionally, these raw materials are in crude form with high n-3 PUFA content and are easily susceptible to oxidation. Hence, research efforts focusing on improving and maintaining the end product quality are needed.

**Table 2.1 Comparative analysis of production parameters of fish meal plants in three states in India**

| Particular | Karnataka | Kerala | Tamil Nadu |
|---|---|---|---|
| Year of establishment | 1968, 1991, 2001 to 2007 | 2006 | 2003 to 2008 |
| Installed production capacity | 20 to 350 tonnes per day | 80 to 160 tonnes per day | 60 tonnes per day |
| (a) Raw material | Oil Sardine constitute 95-97% followed by threadfin breams, carangids, flatfish, lesser sardine, silverbellies | Oil Sardine is the predominant fish | Oil Sardine is the only species |
| (b) Sources of availability | From Kochi to Mangalore-Malpe coast, Goa and upto Ratnagiri in Maharashtra | From entire Kerala coast | 70% from Tamil Nadu and 30% from Kerala |
| (c) Period of availability | Maximum availability of oil sardine from September to January and Lesser availability during February to May | September to December | October to February |
| (d) Procurement | Rs. 600 to 850/t | Rs. 650 to 700/t | Rs. 530 to 650/t |

Data adapted from Ponnusamy et al. 2012

## 2.3. COMPOSITION OF FISH OIL

It is mandatory to know the type and composition of the oil before designing any strategies to improve its quality. A new species as a source of marine oil has to be investigated completely for its chemical and agronomic characteristics before its application in oil production. In case of vegetable oil, changes in the seed oil

composition due to the factors like days to flower, days to seed yield and plant height during different seasons. Also the extraction procedure affects the components of oil to a greater extent. Usually, boiling and expression methods are used for the production of fish oil.

Composition of oil greatly affects its quality, type of refining treatment along with shelf life of the product. In case of fish oil, higher PUFA content is generally preferred and considered as a high quality product. Further, the composition differs greatly based on the species, strain, environmental conditions and also the method of oil extraction followed. Depending on these factors, fish oils may contain 100-300 g/kg of n-3 PUFA. Due to the low temperature acclimatisation and transfer to sea water, higher content of n-3 PUFA can be noted in marine fishes (Kolakowska et al. 2006). In Table 2.2 fatty acid composition of four common fish species are shown. All the species are found to possess good amounts of n-3 PUFA. However, this composition is not consistent with every fish landings as there is a possibility of seasonal effect and size which affects the fatty acids profile.

**Table 2.2 Fatty acid composition (% w/w) of some of the major fish oils available in market**

| Fatty acids | Sardine oil | Anchovy oil | Menhaden oil | Salmon Oil |
|---|---|---|---|---|
| 14:0 | 6.70 | 10.6 | 8.60 | 2.40 |
| 16:0 | 19.0 | 16.1 | 21.2 | 12.4 |
| 16:1 | 8.80 | 11.4 | 10.6 | 5.50 |
| 18:0 | 3.70 | 2.80 | 3.3 | 5.10 |
| 18:1 | 17.1 | 10.2 | 13.2 | 18.2 |
| 18:2 n-6 | 1.10 | 1.00 | 1.40 | 2.60 |
| 18:3 n-3 | - | 0.40 | 1.30 | 0.50 |
| 18:4 n-3 | 2.60 | 1.50 | 4.10 | 0.30 |
| 20:1 | 2.50 | 0.50 | 1.20 | 7.60 |

| Fatty acids | Sardine oil | Anchovy oil | Menhaden oil | Salmon Oil |
|---|---|---|---|---|
| 20:4 n-6 | 1.60 | 1.70 | 1.90 | 1.00 |
| 20:5 n-3* | 19.0 | 24.6 | 13.4 | 9.00 |
| 22:1 | 1.00 | 1.00 | - | 4.50 |
| 22:5 | 1.90 | 1.90 | 2.00 | 0.30 |
| 22:6 n-3* | 11.0 | 9.80 | 12.4 | 20.6 |

*indicates two major essential n-3 Polyunsaturated fatty acids, EPA and DHA. (Adapted from Moffat et.al. 1993 and Aursand et.al. 1994)

The fatty acids in fish oil are usually found as glyceride derivatives as they are more stable. Hence, the fatty acid composition is usually contributed by mono-, di- and tri-glyceride, phospholipids and free fatty acids (FFA). Fish oil consists of 95% acylglycerols, 1% phospholipids and unsaponifiable matter like cholesterol, vitamins, hydrocarbons and fatty alcohols. The highly significant polyunsaturated fatty acids are present in the form of acylglycerols. Since acylglycerol form of n-3 PUFA are easily digestible than their corresponding methyl esters, many works were published to concentrate n-3 PUFA in their glyceride form (Rubio-Rodríguez et al. 2010). It can be seen from Table 2.3 that the acylglycerols (mono-, di-, triglycerides) contribute to the major portion of fats and oils with high nutritional significance (Sun et al. 2002). The non-fatty components of oil contribute to the unsaponifiable matter. The unsaponifiable matter constitutes up to 1–10% of vegetable oil and in some fish liver oil it is found in higher quantities (Channon, 1928).

**Table 2.3 General composition of edible oils and their overall effect on oil quality**

| Type of components in oil | Character | Quantity | | Effect on oil quality |
|---|---|---|---|---|
| | | Crude | Refined | |
| Acylglycerols | Desirable | 90% | >99% | Improve the nutritional value |
| Tocopherols, squalene, sterols | Desirable | 200-800 ppm | 50-300 ppm | Improve oxidative stability |

| Type of components in oil | Character | Quantity | | Effect on oil quality |
|---|---|---|---|---|
| | | Crude | Refined | |
| Phospholipids | Undesirable | 100-500 ppm | <10 ppm | Settling at bottom during storage |
| Free fatty acids | Undesirable | 5-20% | <1% | Act as pro-oxidant leading to oxidation of acylglycerols |
| Metal ions and metal complexes | Undesirable | 2-15 mg/kg | <1 mg/kg | Harmful for consumption and act as pro-oxidant |
| Oxidized products | Undesirable | 2-6 meq/kg | <1 meq/kg | Rancidity and harmful |
| Moisture | Undesirable | 1-3% | <1% | Act as pro-oxidant |

Adapted from Vaisali et al. (2015)

The unsaponifiable portion of oil is made up of many components, such as tocopherols, phytosterols, polyphenols and carotenoids in case of vegetable oil and compounds, such as squalene, tocopherols, fatty alcohols and sterols in fish oil (Channon, 1928; Ghazani and Marangoni, 2013). The presence of some of these compounds is beneficial while some compounds result in low quality product. Hence, processing of marine oil should ensure the removal of some compounds while retaining the nutritional important components.

The major difference between fish oil and vegetable oil that has significant effect on the choice of refining techniques is the fatty acid profile. Since vegetable oil contains lower percentage of long chain fatty acids than fish oils, it is easier to refine and maintain the stability of vegetable oil for longer times in comparison to fish oil. Thus, this bottleneck provides a wide scope for researching different technologies for refining and stabilising PUFA rich oil.

## 2.4. REFINING OF FISH OIL

Any developments in commercial food production or pharmaceutical formulations should undergo critical evaluation of the Food and Drug Administration (FAO). The

new products should meet the quality standards set by the organisation before commercialisation. This is usually achieved by adapting refining techniques in case of oil based products. The evolution of refining has progressed from emphasise on one independent operation in the early days towards a current integrated approach to produce value added products. Even before refining crude oil, there are several critical steps that have to be carried out like storage, preparation, oil extraction and then followed by refining processes.

All fish oil industries aim at effective separation of oil from fat-free dry matter. The most important aspect to be considered is the ample and continuous supply of raw materials. The fish landings are screened based on the size, freshness and texture. Usually, analysing the catch for dry matter, protein and fat will give a brief idea on the product to be expected. Based on these aspects the fish landings are separated and stored until processing stage. The first stage of fish processing is cooking, for coagulation of proteins. This results in the liberation of bound water and oil, while pressing of the coagulate yields a press cake containing 60-80% of the dry matter. Oil and the aqueous phase are then separated by centrifugation.

Such extraction process results in crude oil containing variety of lipids forms such as phospholipids, sterols and acylglycerols. In addition to this, there is a possibility of reaction of lipase or lipoxygenase enzyme on triglyceride resulting in the formation minor components. In order to obtain oil with good appearance and flavour, higher shelf life and uniform quality, removal of impurities and detrimental compounds from crude oil becomes imperative. Preservation of essential components and valuable substances is desired while eliminating impurities. However, this criterion becomes tedious due to the diverse nature of oil with respect to composition. The crude fish oil obtained by this manner predominantly contains triacylglycerols along with minor impurity components that naturally occur in fish species like free fatty acid (FFA), phospholipids, metal ions, sterols and volatile matter. Refining process is carried out to reduce the concentration of these minor components. Phospholipids and FFA are found in higher amounts in fish oil when compared to vegetable oil. The presence of these compounds in oil reduces the quality and hence has to be removed by effective

refining strategy (Dijkstra, 2009). Fish oils also 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.

Millions of tons of crude fish oil are produced annually and some of them have high levels of FFA due to poor storage and transportation. The biggest challenge every fish oil industry faces is to refine high FFA crude oil economically (Wang et al. 2012). In perspective of refining industry, it is aimed to convert crude oil to edible form by removing objectionable impurities with minimal loss of desirable compounds in a cost effective manner. The different refining processes available in all the stages are shown in Figure 2.3. All the stages mentioned in Figure 2.3 are crucial for fish oil refining as the effectiveness of each process not only affects the oil quality but also the efficiency of proceeding stages. The technologies used in industries for refining, fall under two broad classifications: ***chemical refining*** and ***physical refining***. The primary difference between the two methods lies on the technique employed in the removal of free fatty acids i.e. either chemical addition or by physical removal through distillation. However, other steps are common to both the processes.

In case of chemical refining, the impurities are removed by using certain chemicals. Several stages including degumming, deacidification, bleaching and deodorisation are involved in this process. These stages are designed to target different impurities one at a time, thereby resulting in a high quality product.

Crude oils usually possess a number of desirable and undesirable compounds. Acylglycerols, tocopherols, carotenoids, phytosterols and polyphenols are some of the essential components of oil (Table 2.3). Whereas, phospholipids, free fatty acids (FFA), metal ions, oxidation products and other volatiles, reduce the oil quality (Vaisali et al. 2015). As bland and odourless oil with high oxidative stability is generally preferred, efforts to remove odoriferous compounds along with pigments, gums and metal traces efficiently are made by the refining industries (Farhoosh et al. 2009, Lamas et al. 2016).

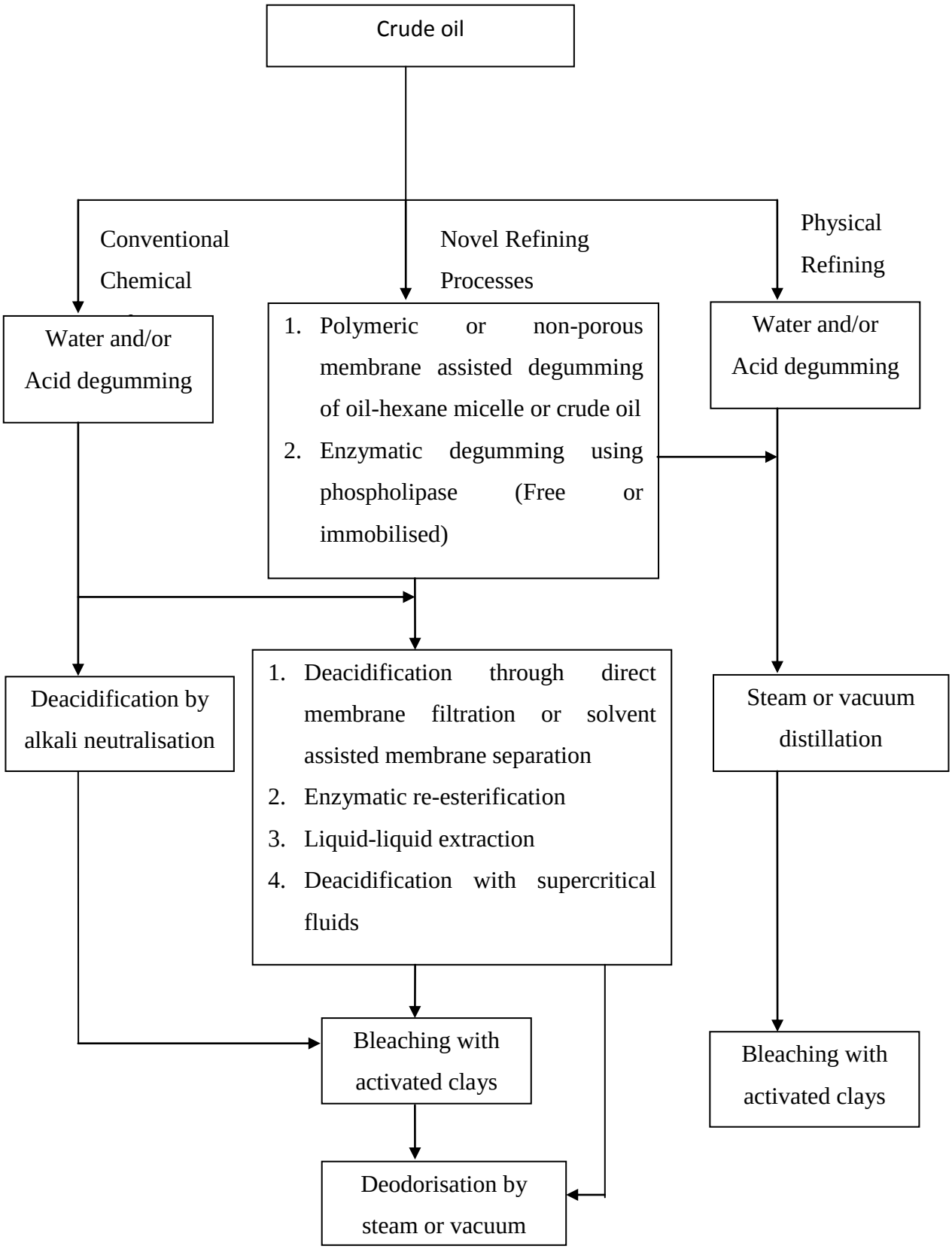



**Figure 2.3 Flow chart representing the different routes and stages in edible oil refining (Adapted from Belur et al. 2017)**

Unlike chemical refining process, physical refining utilises steam to strip the FFA, impurities and unsaponifiable matters from the oil under vacuum. However, a high quality raw material is expected for the process to show acceptable results. Hence, oils are subjected to steam refining only after the initial pre-treatment. 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).

### 2.4.1. Degumming

Oils that are usually produced by traditional mechanical pressing and solvent extraction contain gum like substances. These gums were initially identified to be phospholipids which absorb some amount of water present in the extraction process to become hydratable phospholipids (HPs). In contrast to HPs, some phospholipids fail to absorb water and exist as salts complexes. Such phospholipids are termed as non hydratable phospholipids (NHPs). Due to their emulsifying properties, they form clusters with other valuable components in oil. Separation of these clusters by sedimentation/ centrifugation or during subsequent refining stages will results in loss of quality (Jiang et al. 2015). Phospholipids can decompose and cause dark colouration during heating. As it has been suggested that the ideal phospholipid content in oil should be less than 5mg/kg for any further refining stages (Yang et al. 2008), degumming techniques were employed to reduce the phospholipid levels to meet quality standards.

#### 2.4.1.1. Conventional degumming

Water degumming is the most conventional method of phospholipid removal, where 1 to 3% of water is added to the crude oil at higher temperatures after which the phospholipids are removed by centrifugation (Lamas et al. 2016). These compounds are surface active, containing both hydrophobic tails contributed by fatty acid and hydrophilic head of phosphate group. As a result, they tend to form reverse micelles, monolayers and bilayers on addition of water (Li et al. 2016). Majority of marine oils and some vegetable oil like soybean generally contain high phospholipid content. For

such oils, 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 phospholipid levels even after degumming was found to be due to the presence of NHPs, that exist as salts of phosphatidic acid and phosphatidylethonlamine (Ghazani and Marangoni 2013).

In order to remove NHPs that are not targeted in water degumming, acid degumming was used. Some of the acids that are commonly used for this application are phosphoric acid, citric acid, lactic 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, Li et al. 2016). These NHPs lose their non-hydratability during association with monovalent ions from the acids and results in reverse micelle formation, due to which addition of small quantities of water enhances the process efficiency. In a particular 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). Though literatures record considerable oil loss in acid degumming, this method is continued to be widely used in industries as the process 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 non-hydratable phospholipids more hydratable by binding calcium and magnesium ions before adding water.

In addition to water and acid degumming, several other conventional techniques are followed in many industries for degumming. A dry degumming process is usually employed for oils with low phospholipid content (Johnson 2008). In this case the oil is treated with an acid to agglomerate the phospholipids, which is later removed during bleaching. Similarly, super degumming process is also followed in industries by using a strong solution of citric acid against warm oil to precondition the gums.

This conditioning results in liquid phospholipid crystals that can be easily removed by centrifugation (Johnson 2008).

The efficiency of these conventional degumming processes greatly depends upon the amount and type of phospholipids (Jiang et al. 2015). Since, majority of the conventional degumming processes provides oil with a phospholipid content of 15-80 mg/kg (Yang et al. 2008), several other emerging technologies have been tested for the same purpose.

#### 2.4.1.2. Non-conventional degumming processes

Predominantly, two approaches involving membrane technology and enzyme technology are being followed for degumming of oil. The former exploits the use of semi permeable membranes to separate the phospholipids from oil, based on the difference in the properties of phospholipids and triglycerides. The latter used phospholipid specific hydrolases to decompose phospholipids. Different types of membrane processes like microfiltration (MF) and ultrafiltration (UF) have been explored for oil degumming in literature. The separation process is highly dependent on membrane material, size and shape of oil components, interaction between solute and the membrane surface (Manjula and Subramanian 2006). With regards to UF, the separation efficiency is based on the molecular size exclusion, whereas interaction between the solute and the membrane material is the primary reason for separation in non-porous membranes (Coutinho et al. 2009). Membrane degumming has been performed in literatures by two following two approaches: 1. Degumming of crude solvent-free vegetable oils and 2. Degumming of oil-solvent micelle.

In industry perspective, crude oil handling is considered easier than handling oil-solvent micelle. As a result, many studies were carried out to evaluate the efficiency of different types of membranes for degumming crude oil. Conceptually, there is no significant difference between the molecular mass of phospholipids and glycerides for efficient membrane application in degumming process (Subramanian et al. 2004). However, because of the emulsifying properties of phospholipids, they exist in the form of micelles without any need for surfactant addition.

With this consideration, many studies were performed with direct crude oil degumming using membranes. However, it was noted that the type of membrane played a main role in degumming efficiency. Only 12% reduction in phosphorus levels were noted on soybean oil microfiltration by polyethylene membranes (Subramanian and Nakajima 1997). In contrast, 99.5% reduction was noted in soybean and rapeseed oil when a polymeric composite membrane was used (Subramanian and Nakajima 1997). One primary drawback identified with crude oil membrane degumming was the drastic flux drop at the initial stages of membrane process due to the concentration polarisation, pore blocking and cake formation (Pagliero et al. 2005, DeSouza et al. 2008).

Considering the importance of polymeric membranes, Ulsoy and team aimed at overcoming the drawbacks by altering the membrane surface properties of polyvinyldenefluoride (PVDF) membrane to degum crude oil (Ulusoy et al. 2014). This alteration was found to increase the phospholipid rejection from 58% to 75.5%. Simultaneously, the flux of both modified and original membranes was tested and found to exhibit similar profile. 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. As crude oil membrane degumming posed many challenges regarding flux, a recent study was conducted to decide if dry degumming can be employed to corn oil. According to this study, a computational fluid dynamic (CFD) model was developed to predict the thickness of concentration polarisation when corn oil was degummed using tubular ceramic membrane. Based on this model it was concluded that increasing the Schmidt (Sc) number resulted in a significant reduction in retention layer thickness (Wibisono and Widodo 2015). Since, Sc number is based on the ratio of momentum viscosity and mass diffusivity, use of solvents to reduce the viscosity of oil feed can be the best approach in membrane degumming process. Hence, alternate approaches involving phospholipid micelle in solvents was carried out.

Phospholipid tend to form micelles due to their surface active properties, when oil is diluted using a suitable solvent (usually hexane). These micelles are known to have a

molecular weight of 20KDa approximately and hence can be easily separated by using membranes (Manjula and Subramanian 2006, Ribeiro et al. 2008). However, the process greatly depends on the critical micellar concentration (CMC), and the amount of solvent required to achieve the appropriate CMC is crucial. Hence, the micelles are generally formed with 30-40% oil in hexane (Coutinho et al. 2009). Table 2.4 presents a brief list of different types of membranes that shows maximum reduction in phospholipids.

Vegetable oil micella degumming by membrane process has been performed in literature using several polymeric membranes including polyethersulfone (PES), polyamide, PVDF, polyimide, ceramic membranes and also by few inorganic membranes (Ochoa et al. 2001, Garcia et al 2006, Saravanan et al. 2006, Marenchino et al. 2006, Manjula and Subramanian 2009, Tres et al. 2010). Interestingly, the bottlenecks faced in membrane degumming tend to vary depending on the type and composition of oil used. For instance, marine oils and few vegetable oils are known to have higher wax content along with nutritional compounds that has to be conserved during refining. For this purpose, conditioning of membranes can be considered.

**Table 2.4 Different membranes used for degumming of vegetable oils (Belur et al. 2017)**

| **Type of oil** | **Membrane Used** | **Phospholipid content (mg/kg)** | | **References** |
|---|---|---|---|---|
| | | **Before Treatment** | **After Treatment** | |
| 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 |
| Neutralised (40%) Soybean oil | | 141 | 141 | |
| Neutralised (20%) Rapeseed | | 123 | 12 | |

| Type of oil | Membrane Used | Phospholipid content (mg/kg) | | References |
|---|---|---|---|---|
| | | Before Treatment | After Treatment | |
| oil | | | | |
| 25% soybean oil / hexane micelle | Polyvinylidefluoride (PVDF) | 1297 | 1.6 | Pagliero et al. 2005 |
| 25% sunflower oil / hexane micelle | | 367 | 0 | |
| 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 |
| Water degummed soybean oil/hexane micelle | | 97 | 22 | |
| 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 |

The main drawback in membrane degumming of crude oil is the poor flux, while use of solvents reduce the stability of the membranes. Currently several researches are continuing to evolve that address these challenges. With this accumulating knowledge on membrane processes, few industries have commercialised this technology. However, further studies need to be carried out to employ this application commercially to a broad variety of oil.

### 2.4.1.3. Enzymatic degumming

Phospholipases are the preferred enzymes for this application. According to the process of Lurgi's EnzyMax (Mei et al. 2013), degumming is carried initially by adjusting the pH of the system for maximum enzyme activity. The enzyme is allowed to act on the phospholipids after which the gums are removed by centrifugation. This process is advantageous as it removes both HPs and NHPs simultaneously. Based on the degumming reaction type, the phospholipases used for this application are classified as $A_1$, $A_2$, B, C and D with lipase $A_1$ being the most common. A pictorial representation of the mechanism of action of different phospholipases is shown in Figure 2.4.

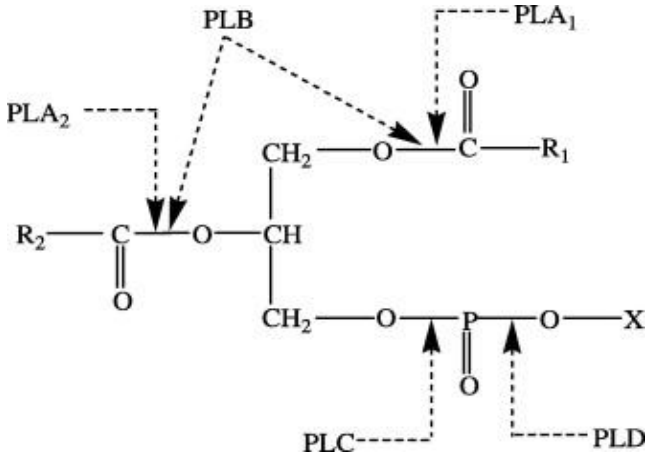



**Figure 2.4 Mechanism of action of different types of phospholipases (Adapted from Jiang et al. 2011)**

All the enzymes used for degumming are known to have inherent activity towards both phospholipids and triglycerides. In order to have the more preferable phospholipase activity, use of high temperatures is recommended (Jiang et al. 2014). However, care should be taken to have the appropriate temperature, as higher temperature might lead to instability in enzymes. The enzymatic degumming occurs at the interface between aqueous phase of enzyme and that of oil phase. Hence, an ideal strategy to gain maximum efficiency is to increase the interfacial area for maximum reaction with small time period. In order to achieve this, researchers have attempted using ultrasound technology (Jiang et al. 2014b).

Though the reports claim many advantages of the enzymatic degumming process, it is not implemented in many industries due to the high cost and inefficient separation and reuse of enzymes. Additionally, the longer reaction times are considered a major drawback at industrial levels. The choice of recombinant enzymes or immobilisation of enzymes could be considered as a possible solution for this problem (Huang et al. 2014, Qu et al. 2016, Lamas et al. 2016, Li et al. 2016). 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. However, no reports are available on the effect of degumming on marine oils and the n-3 PUFA content.

### 2.4.2. Deacidification

The main purpose of this step is to remove free fatty acids (FFA) and other impurities that are saponifiable. This stage is considered to be the most energy intensive and tedious process in edible oil refining due to its economic impact on the whole process. As FFA are highly susceptible to oxidation that further results in oil rancidity it has to be removed effectively prior to commercialisation. FFA is also known to trigger hydrolytic and oxidative deterioration (Paradiso et al. 2010). Similar to degumming, different conventional methods like physical, chemical and micelle refining have been employed in literature for this purpose. Additionally, new approaches like solvent extraction, chemical and/or enzymatic re-esterification, supercritical fluid extraction have also been explored.

#### 2.4.2.1. Alkali Neutralisation

This is one of the most conventional method and widely being used at large in industries till now. It involves the reaction between FFA and an alkaline solution like sodium hydroxide, potassium hydroxide, calcium hydroxide. This results in a saponification reaction thus separating FFA as soaps. Since, higher concentrations of alkaline solution is preferred, it results in the loss of neutral oil as well. Over the years several modifications have arisen to this traditional process for maximising the process efficiency with minimal loss of oil (De and Patel 2011, Tang et al. 2013,

Vaisali et al. 2015). Additionally, it was identified that the FFA content in the oil is directly proportional to the oil loss in this conventional process. This leads to the poor yield of refined oil especially in case of marine oils which are known for their high FFA content. Another major disadvantage of this process is the loss of nutritional compounds like tocopherols and carotenoids. 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.

#### 2.4.2.2. Physical refining

In order to overcome the excess oil loss in case of alkali neutralisation, physical refining was employed in many industries. This process makes use of steam to strip the FFA, other unsaponifiable impurities from oil under vacuum. For the process to show good efficiency, the oil has to undergo an initial pre-treatment stage. As this process is similar to deodorisation stage employed for odorous marine oil, both deacidification and deodorisation can be combined into a single step for such oils. Though this process poses many advantages as it results in a high quality product with low FFA content with minimal oil loss, use of high temperatures for longer periods may pose disadvantage to heat sensitive oils like canola oil, flaxseed oil and some marine oils that are rich in PUFA content (Bhosle and Subramanian 2005). Further, there has been recorded a considerable reduction in the tocopherols content and carotenoids in vegetable oil (Ooi et al. 1996) and also conversion of PUFA into trans fats (Ghazani and Marangoni 2013). However, 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. The oil produced is consistent with the market requirement, without appreciable loss of n-3 PUFA and minimal cis- to trans- isomerisation.

#### 2.4.2.3. Other approaches of deacidification

The difference in the solubility of FFA and glycerides in polar solvents is the basis of this particular technology. Different studies suggest that short chain alcohols are the most effective solvents for this application (Rodrigues et al. 2007). Methanol, ethanol

and acetone are some of the solvents that have been successfully used in literature for deacidification of many vegetable oils (Turkay and Civelekoglu, 1991). A majority of these reports conclude only partial removal of FFA with concomitant oil loss suggesting the requirement of an additional deacidification step. Hamm (1992) has proposed an efficient solvent extraction of FFA from fish oil by using several short chain alcohols.

The preferential selectivity of FFA by supercritical fluids is exploited in this process. The deacidification by this process at 250 bars and 353K resulted in a 97.8% reduction in FFA when $CO_2$ was used as a supercritical fluid to extract FFA from rice bran oil (Chen et al. 2008). Similarly, this application was reported to remove arsenic along with FFA during treatment of n-3 PUFA rich salmon oil (Rodriguez et al. 2012). 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. This advantage presented by the process makes it to be used specifically for removing the high quantities of FFA along with off flavours in marine oils. Table 2.5 summarises recent studies performed on deacidification of vegetable oils using various solvents. Similar to degumming, separation of FFA from glycerides based on molecular size difference is ineffective due to the minor difference in the mass. Hence, membranes that show preference towards hydrophilic FFA against the hydrophobic glycerides can be explored for deacidification.

Raman et al (1996) suggested the use of hydrophobic membranes to effectively separate FFA dissolved in solvents from triglycerides. As polar solvents fail to permeate through hydrophobic membranes, efficient separation of FFA from triglyceride can be achieved with simultaneous reduction in the solvent content in the oil (Bhanushali et al. 2001). Many studies have been conducted in literature testing the efficiency of membrane with various materials and surface properties. However, not many industries apply this process due to the high cost of membranes and also the inefficient separation of solvent form oil. A recent report proposed the use of hydrophobic polyvinylidenefluoride to remove FFA from palm oil. Additionally, the

membrane properties were modified by cross linking with polyvinyl alcohol (PVA) in the presence of glutaraldehyde to enhance the process efficiency.

**Table 2.5 Deacidification of oils by solvent extraction (Source: Belur et al. 2017)**

| Oils | Solvent 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. |
| 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 partitioning between TAG and FFA content | Mariano et al. 2011 |

### 2.4.2.4. Re-esterification

This process involves the use of catalysts to synthesize glyceride derivatives using glycerol and the FFA in oil. The catalysts used could either be chemical or enzymatic. This process is highly advantageous as it results in the increase in neutral oil. The reaction leads to liberation of mono-, di-, and triglycerides along with the generation of water. The by-product water has to be removed with utmost care as it might result in the reversible reaction (hydrolysis). In both chemical and enzymatic process, the main parameters that affect the process are the type and amount of catalyst, substrate

glycerol concentration and reaction temperature (Kombe et al. 2013). Various chemical catalysts that have been used so far includes zinc chloride, naphthalene-beta-sulphonic acid and zinc dust. In a report comprising the deacidification of rice bran oil with an initial FFA content of 37.9% using zinc dust, the FFA reduced to 1.5%, with a simultaneous increase in glyceride content from 40.32% to 80.62% (Ebewele et al. 2010). Kombe (2015) studied the FFA reduction in castor oil at low temperatures of 56°C and 99.01% reesterification efficiency was noted. Unlike chemical process, enzymatic reactions involve lower reaction temperatures which could be better suited for oils that are heat sensitive like fish oils (Bhosle and Subramanian 2005). Some of the enzymes that are used for this purpose include Mucor miehei lipase, Candida rugosa lipase, Candida antartica lipase (Bhattacharya and Bhattacharyya 1989, Sengupta and Bhattacharyya 1996, Makasci et al. 1996). However, the high cost of enzymes has become a major shortcoming in its application in industries. Hence, Laomin et al. (2012) developed a process suggesting the use of packed bed reactor with immobilised lipase to reuse the enzymes so that the process can be cost effective. However, the process is yet to draw industrial attention.

### 2.4.3. Bleaching

Bleaching is a mass transfer operation aimed at removing minor impurities like metal ions, pigments, oxidation products, redundant FFA and phospholipids that are not targeted in the earlier stages (Dijkstra 2013, Pohndrof et al. 2016, Acquah et al. 2016). The overall quality of the product is improved at this stage. This step was initially designed for vegetable oils as a batch process, with the sole purpose of reducing the pigment content in oils. However, in the current scenario, bleaching has become a critical process in edible oil industry. Bleaching is usually carried out by bringing oil in contact with bleaching materials like neutral clays, activated earth, silica gel and activated carbon by adsorption. The interaction between oil and the clays could be physical or chemical (Sabah et al. 2007, Ahmad et al. 2009, Kuuluvainen et al. 2015, Aachary et al. 2016). The operation is generally carries out under vacuum to avoid oxidation in oil.

Due to the low cost, bleaching earth is the most common material used in many edible oil industries. However, it results in 20-40% loss of oil, in addition to the tedious disposal of spent bleaching earth (Loh et al. 2013). Hence, several recent studies tested the use of new bleaching clays (Abedi et al. 2015, Aachary et al. 2016) and the use of high voltage electric field (Abedi et al. 2016). Although activated bleaching earth clays still have the highest market share, oil refiners are showing a growing interest in non-activated 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 the process is highly dependent on the acidity of the clay used in refining. While the acid activated adsorbent has the ability to act as support, filter aid, catalyst and adsorbent, neutral earths acts only as adsorbents (Silva et al. 2013). Though acid activation was found to increase the adsorption capacity of the bleaching material, it might result in a change in the crystalline structure. As a result, fish oils bleached by these materials might undergo degradation reactions including PUFA isomerisation. To overcome 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). The other factors that highly impact the outcome of this process are the operation temperature, particle size of the adsorbent and acidity.

For fish oils, bleaching is an important stage in refining, as it removes the oxidation products, that dominates other impurities. Unlike vegetable oils, using high temperature during bleaching is discouraged as it may 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).

### 2.4.4. Dewaxing

Waxes are usually monoesters of long chain fatty acids and alcohols and the esterification process which results in these products in spontaneous which is usually affected by the temperature and time. Hence, fish oil generally has higher wax content than vegetable oils and has to be removed by refining stage. The presence of wax in oil results in a cloudy end product after refining. Due to this, dewaxing is carried out prior to deodorisation stage in oil refining. Waxes are removed from oil by cooling the oils to lower temperatures (6-8 °C) slowly, over 4 h and then holding at this lower temperature for another 6 h. This cooled oil is then gradually heated to 18 °C and filtered (Pestana-Bauer et al. 2012). As some industries claim reasonable reduction in wax content during degumming stage, this step is not followed widely by many industries.

### 2.4.5. Deodorisation

Deodorisation is usually carried out as the last stage in edible oil refining, where the volatile compounds in the oil are targeted. The compounds responsible for undesirable odour and flavours like oxidation products, polycyclic aromatic hydrocarbons, dioxins are removed. Deodorisation operation is carried out at higher temperatures ranging from 170 °C to 270 °C and under higher vacuum of 3 to 8 mmHg with simultaneous sparging of steam (Johnson 2008). Deodorisation is carried out as a multistage process involving de-aeration, heating, deodorisation and cooling. Deodorization is particularly important for fish oil due to their off-flavour. However, use of high temperature to remove volatile components leads to deterioration of oil quality by cyclization of polymerisation of long chain PUFA and leading to formation of trans fats (Fournier et al. 2006, Fournier et al. 2007).

(a) Initially air is removed from oil and the holding vessel by generating the vacuum. This protects oil from further oxidation during the process.
(b) The oil is then heated to a predetermined temperature by indirect heating. This is usually achieved by using thermic fluids. Care should be taken for heat

sensitive oils like n-3 PUFA rich oils, as there is a possibility of formation of trans fats.

(c) Next a stripping agent (steam) is injected into the oil. The steam strips away the volatile matter leaving pure refined oil. Sometimes nitrogen is also used as a stripping agent. However, it is cost intensive, hence majority of the industries continue to employ steam.

(d) The steam stripped oil is then allowed to cool by passing through heat exchangers. The final temperature of the oil should be brought to 30 °C for ensuring the stability of oil. The oil is then added with antioxidants to avoid further oxidation.

The design and process conditions have to be carefully optimised to ensure minimal formation of trans fats. Care should be taken to avoid the stripping of minor essential components as well (Hamm et al 2013). Therefore, there is a huge demand for the evolution towards the milder deodorisation conditions. This can be achieved by using packed columns or dual temperature reactors. A recent study analysed the potential of an alternative process which aimed at improving the batch deodorizers by coupling them with desorption column (Laortani and Iribarren 2017). An increased efficiency in separation was noted with simultaneous reduction in the steam consumption by 16.5%. Another recent publication suggested a modified method of deodorisation involving mild temperature process for initially period, followed by a higher temperature process for another hour. This resulted in the maintenance of the carotene content in palm oil (Riyadi et al. 2016). Similarly, Schols et al. (2013) have described a three stage process involving the use of flash vessel, packed column and a cross flow system. In any deodorisation process, some amount of thermal denaturation is inevitable. Hence, there is a huge scope for development of new technologies 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.

Similarly, it is critical to design the entire refining process based on the composition of the oil by adapting techniques based on the oil quality requirement. Thus, it is

necessary to carefully analyse the merits and demerits of each process before making an effective choice of refining route. While all these refining steps greatly reduce the concentration of impurity compounds, one should understand the significance of these minor components even at lower concentrations. Thus, it is important to realise that these compounds continue to affect the quality and stability of oil by being available at low levels.

## 2.5. STABILITY OF PUFA RICH OILS

Lipids are important part of our diet as they serve as condensed source of energy, required for growth and functioning of living organisms. However, lipid based food are known to undergo hydrolytic process and auto oxidative routes which leads to its spoilage over longer storage time. In case of fish oil, hydrolytic instability is a major concern as it results in the formation of free fatty acids (FFA), which in turn affects the oxidative stability. However, removal of impurities during refining ensures the hydrolytic stability to a certain extent. Similarly, the oxidative stability of oil can be improved on removal of impurities. Nonetheless, the auto-oxidation of PUFA rich oil occurs due to many external factors regardless of refining process. The lipid oxidation is a major cause for the deterioration of lipid based foods and it also affects the integrity of biological systems. This oxidative instability in oils and fats results in the generation of compounds with off-flavours, loss of nutrients and also in the formation of potentially toxic compounds, thus making oils unsuitable for consumption.

Oxidation of edible oils is generally affected by an energy input, which could be heat, light, fatty acid profile, oxygen and minor components like mono and diglycerides, free fatty acids, pigments, phospholipids and metals. In the presence of an initiator, PUFA can undergo oxidation and form alkyl radicals. These alkyl radicals further get oxidised rapidly to form peroxy radicals, which themselves act as initiators for further oxidation process. Based on the type of catalysts it can be

(a) autooxidation,
(b) photooxidation,
(c) thermal oxidation and

(d) enzymatic oxidation.

Except autooxidation, other types of oxidation can be controlled by managing the storage conditions and refining of oil. Autooxidation is the most common process, as it cannot be controlled and occurs as a spontaneous reaction with atmospheric oxygen via a series of chain reaction. Additionally, higher temperatures further accelerate this process.

As it has been suggested to increase the dietary intake of n-3 PUFA, effective methods to incorporate these compounds into food systems have to be addressed. The main barrier for the use of fish oils in food is due to the low oxidative stability which is attributed to high degree of unsaturation (Jacobsen et al. 1999). They develop undesirable flavour and toxic products during storage, which makes it a challenge for food industry to use them (Sun et al. 2011). This problem is exacerbated when unsaturated oils are considered for domestic use like frying oils. The prolonged heating leads to the extensive oxidation, forming compounds harmful to human health.

The literature on the stability of long chain n-3 PUFA is controversial, which is attributed to the wide fatty acid and triglyceride composition (Frankel et al. 2002). It has been proved that the auto oxidation of PUFA in free form increases linearly with the number of doubly allylic methylene groups present. Though fish oil is mainly composed of PUFA containing triglycerides which ensure better stability than the free form, factors like PUFA position and other fatty acids in triglyceride molecule affect the oxidative stability (Wijesundera 2008). This is probably due to the interaction of acyl chains within the same triglyceride molecule and possible steric hindrance. Oxidative susceptibility also depends upon the position of the double bond in the PUFA rich system. Thus, in a colloidal or lipid emulsion system with fatty acids that are oriented so that hydrocarbon chain is located in the hydrophobic interior will be more stable when the double bonds are buried deeper or closer to the methyl end of fatty acids (McClements and Decker 2000). Apart from these factors, external catalysts like light, heat, enzymes, metal ions etc. also contribute to the oxidation process in fish oils. Any progress in the development of foods with high fat content

depends on improving the oxidative stability, which in turn relies on the thorough understanding of the oxidation mechanisms in different food systems (McClements and Decker 2000).

### 2.5.1. Lipid Oxidation mechanism

Lipid oxidation in a homogenous system of bulk oil has been investigated by many researchers over the past years (Frankel 1998, McClememnts and Decker 2000, Colakoglu 2007). PUFA in the form of glycerides and phospholipids do no directly contribute to the off-flavours or aroma in marine oils. However, these fatty acids will decompose upon oxidation resulting in the formation of small volatile compounds which are usually associated with the off-aromas. The mechanism of lipid oxidation depends on the type of reactive species present, which in turn depends on the physicochemical environment (McClements and Decker 2000). Theoretically lipid oxidation occurs through a series of chain reaction comprising of free radicals. This reaction occurs between double bonds of the unsaturated fat and oxygen spontaneously. PUFA are chemically reactive, having low stability to heat, light, atmospheric oxygen and impurities, posing oxidation problems during storage (Chaiyasit et al. 2007). The overall mechanism of lipid oxidation involves following steps:

(1) Initiation – free radical formation
(2) Propagation – free radical chain reaction
(3) Termination – formation of volatile non-radical products

A simple set of equations can be used to summarise these steps: While equation 2.1 represents the initiation of oxidation, (2.2) and (2.3) represents the propagation. And the rest represents termination stage

$$In + LH \rightarrow InH + L^{\circ} \qquad (2.1)$$

$$L^{\circ} + O_2 \rightarrow LOO^{\circ} \qquad (2.2)$$

$$LOO^{\circ} + LH \rightarrow LOOH + L^{\circ} \qquad (2.3)$$

$$LOO° + LOO° \rightarrow LOOL + O_2 \quad (2.4)$$

$$L° + LOO° \rightarrow LOOL \quad (2.5)$$

$$L° + L° \rightarrow LL \quad (2.6)$$

Generally, a fatty acid radical or the alkyl radical (L°) is formed in the initiation stage, when a hydrogen is abstracted from the fatty acid by influence of an initiator (In°) (Eq. 2.1). After the formation of this alkyl radical, it has the ability to delocalise over the site of unsaturation. This results in the shifting of double bonds, and formation of conjugated dienes in case of PUFA. As mentioned previously, the degree of unsaturation is directly proportional to the ease of formation radicals in bulk oils. The initiator can be a variety of components. However, the most significant of all is oxygen, which can be in the form of singlet or triplet oxygen. Because non-radical and electrophilic oxygen can directly react with the double bonds of food components without the formation of free radicals (Rawls and Van Santen 1970). The singlet oxygen oxidation is more significant as the rate is high and is easily increased by the environmental catalysts like heat and light.

Propagation step involves the addition of an oxygen atom to the alkyl radical and results in the formation of a peroxy radical (LOO°) (Eq. 2.2). This peroxy radical is known to have higher energy than that of alkyl radical, with the ability to abstract hydrogen from another unsaturated fatty acid (Chaiyasit et al. 2007). Lipid hydroperoxides (LOOH) are usually formed by this process (Eq. 2.3). This process is a chain reaction and occurs spontaneously. With the increase in unsaturated fatty acids and complexity of oil, this process proceeds exponentially. The attack of new lipids by peroxide radicals takes place several thousand times during propagation reaction until hydrogen source is depleted or the chain is interrupted by antioxidants (Shahidi and Zhong 2010). The scheme in Figure 2.5 represents the oxidation of FFA. However, oil also contains mono, di and triacylglycerols. Oxidation of triglycerides with unsaturated fatty acids results in the formation of mono hydroperoxides located on the sn-1,3 and 2-positions of the glycerol moiety (Frankel 2005). Further oxidation results in bis and tris hydroperoxides. These products are also capable of participating

in auto-oxidation like FFA. As mentioned earlier the low C-H bond energy makes the hydrogen atoms at double bond site less stable, hence they are easily attacked and replaced by free radicals during initiation stage. The interaction between two free radicals results in termination process. The radicals can either be peroxy radicals or alkyl radicals (Eq. 2.4 – 2.6).

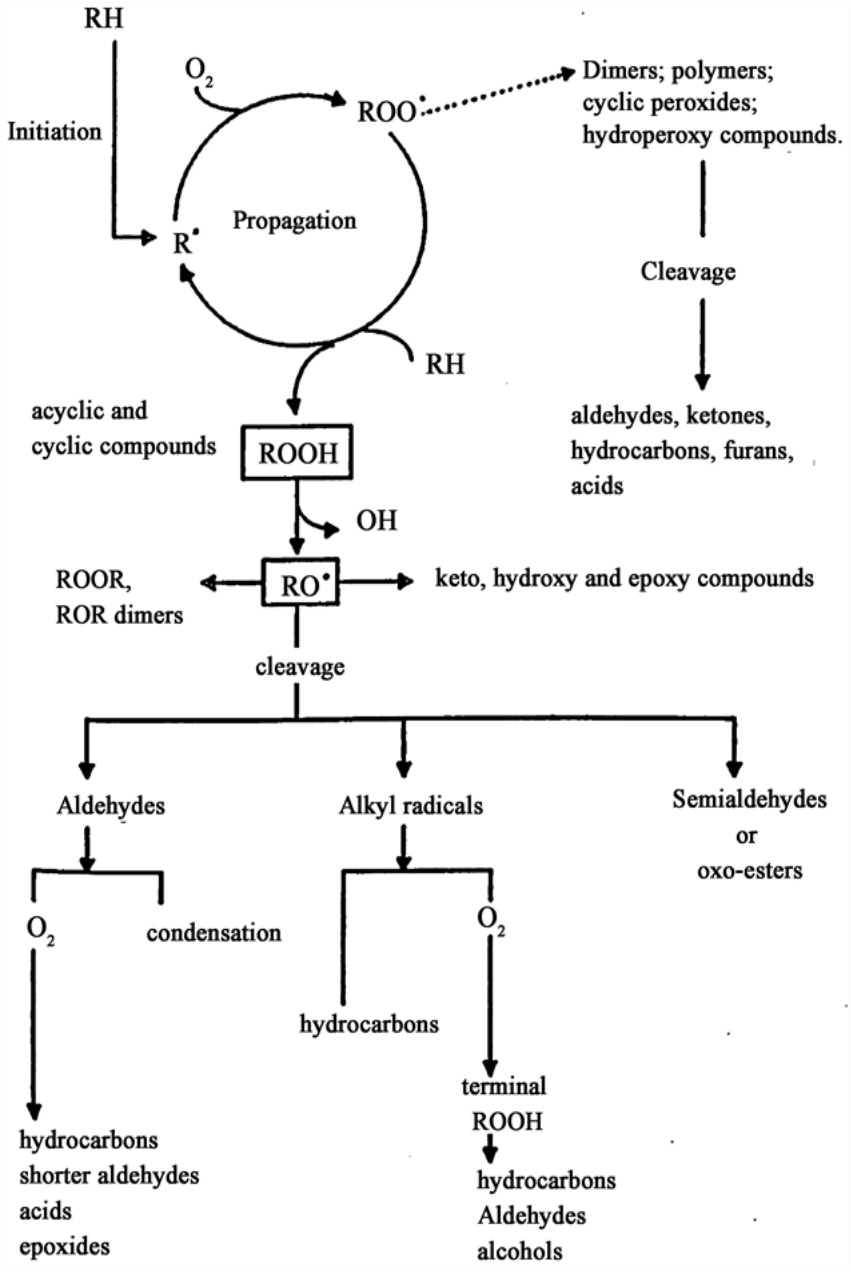



**Figure 2.5 Schematic representation of lipid oxidation (Adapted from Gad and Sayd 2015)**

### 2.5.2. Products of lipid oxidation

Oxidation products resulting in rancidity are volatile in nature and they result directly from the decomposition of lipid hydroperoxides (LOOH). The decomposition pathway of this lipid hydroperoxides by homolytic cleavage between the two oxygen atoms has been described by Min and Boff (2002). This cleavage yields either a hydroxyl radical (OH°) and alkoxyl radical (LO°) or a peroxyl radical (LOO°). The alkoxyl radical has lower activation energy than the other two products and hence can spontaneously enter into different pathways as shown in Figure 2.5. These radicals can also attack unsaturated regions or a covalent bond next to the alkoxyl radical. This reaction is called $\beta$-scission reaction, and it is critical for the quality of oils as they result in volatile products. Unless, further decomposition of $\beta$-scission reaction products occurs, it doesn't necessarily result in the formation of volatile compounds (Chaiyasit et al. 2007). Cleavage of fatty acid by alkoxyl radicals results in another set of radicals that interacts with a variety of compounds forming secondary oxidation products. These compounds are usually aldehydes, ketones, alcohols, furans, hydrocarbons and acids.

### 2.5.3. Quantification of lipid oxidation products

Evaluating the status of lipid oxidation in bulk oils is a challenging task, owing to the complexity of the process. Therefore, using one parameter as reference to the extent of oxidation might lead to falsifying data and hence combination of different methods is always preferred. The products of initial oxidation in oil are peroxides, especially hydroperoxides. These are primary oxidation products which undergo further oxidation to form secondary products. Despite being the intermediate of further oxidation these compounds are relatively stable and can be used as an indication towards the extent of oxidation, provided that extensive oxidation has not yet occurred.

Generally, lipid oxidation is measured by measuring the primary and secondary oxidation products in oil. As discussed previously, lipid hydroperoxides are well known indicators of primary oxidation and it can be measured by different methods

including volumetric methods, UV-VIS spectroscopic methods, ferrous oxidation and iodine oxidation methods and few chromatographic methods as well. In addition, primary oxidation can also be measured by measuring the conjugated dienes formed due to electron delocalisation of PUFA. This measurement is usually done by UV-VIS spectroscopy. In case of secondary oxidation product measurement, malondialdehyde (MDA) is one molecule frequently used as an indicator for secondary oxidation. This is measured by spectroscopic measurements. In addition to these, other chromatographic methods can also be applied to analyse the secondary oxidation products of oil oxidation.

### 2.5.4. Factors affecting lipid oxidation in bulk oil

#### 2.5.4.1. Fatty acid composition

Bulk oil or any lipid based food systems contains a variety of fatty acids with different physical and chemical properties. The factors that greatly influence the extent of lipid oxidation include fatty acid composition, oil refining method, type of oxygen and its concentration, free fatty acid, mono- and diglyceride, moisture, temperature, degree of unsaturation, surface area of oxidation, trace metals and other prooxidant and antioxidant compounds (Frankel 2001). In case of fatty acid profile, the presence of PUFA compounds greatly influence the oxidation. For instance, the number of double bonds along with their position and geometry also affects oxidation in PUFA rich oil (Nawar 1996). Thus, oils with higher degree of unsaturation oxidise at a faster rate than saturated oils or oil with less degree of unsaturation (Choe and Min 2006). In addition to these factors, fatty acids with cis isomers oxidise faster than the trans fats (Nawar 1996). Similarly, unsaturated FFA tends to show lower oxidative stability than their corresponding methyl ester or glyceride form (Miyashita and Takagu 1986).

In addition to the fatty acid composition the presence of various lipid forms also influences the rate of oxidation. For instance, fatty acids or PUFA can exist in free form, as glyceride or as a phospholipid. Though triglyceride rich oil is generally preferred, many fatty acids exist as monoglyceride and diglyceride. These minor mono and diglycerides along with phospholipids and FFA contribute to the surface

active compounds in bulk oil. These surface active compounds tend to form micro or nano emulsions or self-assemble to form other physical structures in bulk oil.

#### 2.5.4.2. Surface area

The next important factor that has significant influence on lipid oxidation is the surface area of lipids which is directly exposed to oxygen or other prooxidants. The rate of lipid oxidation was found to be directly proportional to the surface area in bulk oils that is exposed (Nawar 1996). This is contrast to the oxidation in emulsions, as the oxidation is mainly governed by the area between oil and water.

#### 2.5.4.3. Oil extraction and refining methods

Seed and marine oils show diverse composition due to the recent advances in the different extraction methods of oil. The composition of the oil is known to vary with the type of extraction method used. The saturated and unsaturated lipid profile in oils shows high variation due to the geographical origin of the seeds or type of marine organism, variable species and extraction techniques (Oliveira et al. 2012, Vaisali et al. 2015). With the advent of solvent extraction of many seed oils in the 20$^{th}$ century, processing of the extracted oil became a necessary criterion. The extraction methods were improvised to gain maximum oil yield. Although many replacements for solvent extraction of edible oil have been proposed, majority of the industries continue to use hexane for the extraction due to high oil recovery.

About 50% of the world's vegetable oil is produced by pre-pressing followed by solvent extraction (Subramanian et al. 1998). One such example is soybean oil which contributes to a major part of edible oil industry and is extracted using hexane. The oil-solvent micelle is passed through a series of evaporator and vacuum stripper to remove the solvent and produce crude oil. However, because of the high temperature there is a possibility of formation of trans fats in saturated vegetable oils or the degradation of omega-3 fatty acids as in case of fish oils (Fournier et al. 2006). Similarly, the stability of pressed oil was found to be higher than supercritical carbon dioxide extracted walnut oil (Crowe and White 2003). However, recent report with

minor modification in supercritical $CO_2$ extraction method of camelina seed oil resulted in highly stable oil (Belayneh et al. 2017).

In addition to oil extraction procedures, the refining methods employed to improve the quality of oil has a major impact on the oxidative stability. Few reports suggest higher stability of crude oil than refined oil on storage (Jung et al 1989). This was concluded to be partly due to the removal of antioxidant tocopherols during processing. However, several others claim higher stability of refined oil based on the removal of prooxidant compounds. Thus, a thorough analysis and understanding of the chemical and physical parameters of oil is necessary.

#### 2.5.4.4. Oxygen

In case of oxygen, when present at low concentrations, the rate of oxidation is approximately proportional to the concentration. If abundant levels of oxygen are present the rate is independent (McClements and Decker 2000). Some reports suggest that lipid oxidation is not spontaneous because ground state atmospheric oxygen is in triplet form and double bonds of fatty acid are in singlet form. Hence, this requires the assistance of minor components for the initiation of oxidation (Schafer 2005). Similar to any chemical reaction, temperature greatly affects the oxidation process. Thus, it was identified that increase in temperature resulted in an increase in oxidation rate. However, in case of bulk oils, reports are existent which claim the limited solubility of oxygen at higher temperatures, resulting in slow oxidation process. Though oxygen is a main cause behind the progress of oxidation in bulk oil, it cannot spontaneously react with double bonds of PUFA due to the difference in spin states. While atmospheric oxygen is in triplet state, the double bond in fatty acids is in singlet state, making it impossible to react directly due to restriction by quantum mechanics (Schaich 2005). Thus, the presence of minor components in bulk oil assists in the conversion of singlet oxygen to triplet state. For instance, presence of trace metal like iron reacts with oxygen to form alkyl radicals which further cause oxidation in bulk oil.

This minor component can exist in oil in two different ways, viz. dissolved oxygen (DO) and non dissolved oxygen (Garcia-Torres et al. 2009). While DO can be found

dispersed in the bulk oil, non dissolved oxygen is usually the oxygen concentration in the headspace of the container. As the solubility of oxygen is 10 times higher than the solubility of water in oil, the rate of oxygen dispersion is directly proportional to the rate of lipid oxidation (Montgomery et al. 1964). The headspace oxygen content in fish oil is highly temperature dependent as the solubility depends on the partial pressure of headspace gas.

#### 2.5.4.5. Trace metals

The presence of transition metals in bulk oils is inevitable as they are transferred from animals, plants, soils, dust and metallic equipments used in processing and storage. In case of soybean oil 13.2 ppb of copper and 2.80 ppm of iron was found (Sleeter 1981). Transition metals aid in the formation of free radicals by hydrogen abstraction and hydroperoxides decomposition, which accelerate lipid oxidation reactions, therefore, decreasing the quality of foods (Nawar 1996). These metals also result in formation of reactive oxygen species along with hydroxyl radical and hydrogen peroxide. Reports suggest that presence of copper results in a 50 times faster decomposition of peroxides than ferrous iron. While ferric iron decomposes 100 times faster than ferrous ion (Benjelloun et al. 1991).

#### 2.5.4.6. Water

Similarly, trace water is another major variable that has to be considered while studying the influence of minor components on oxidation of bulk oil. Despite being present in lower concentration in oil, water has impact on the oxidation rate in bulk oil. This could be due to the ability of water to act as solvents for hydrophilic antioxidants, prooxidants as well as trace metals. Since the polarity of water and bulk oil is different it is expected that they spontaneously separate into two phases. Hence, many researchers overlook the possibility of water as a minor component in bulk oil. Due to the presence of many surface active compounds like FFA, phospholipid, mono- and di glycerides water get emulsified in bulk oil (Chaiyasit et al. 2007). Thus trace water content in freshly opened commercial vegetable oils ranges between 200-2000 ppm. However, as the time progresses, the water content in bulk oil changes due

to the absorption or evaporation of water on contact with atmosphere. Water in bulk refined oil is generally derived in two possible ways. The water that exists in the fish and the water that is used during refining processes get dispersed into oil. Though centrifugation and vacuum drying ensures the removal of major parts of this water, the presences of surface active compounds results in the emulsification along with water making the presence of trace water inevitable in bulk oil.

The influence of the above mentioned factors on the oxidative stability of oils have been widely studied. However, literature regarding the presence of water in oils and their effect on stability is meagre as bulk oils were considered as homogenous system. In addition, moisture is also found to increase the oxidation rate leading to formation of volatiles (Kim et al. 2014). Hence, it is crucial to consider the effect of moisture on stability of oils, as moisture gets dispersed into oils from the headspace during storage and transportation.

#### 2.5.4.7. Phospholipids, free fatty acids, mono and diglycerides

Bulk oil is mainly composed of triglycerides along with minor compounds like FFA, monoglyceride, diglyceride and phospholipids. Though refining processes greatly reduces the concentration of these compounds, they exist in refined oil in minor quantities. For instance, crude soybean oil was found to contain 0.7% FFA which reduced to 0.02% after refining. These minor levels of FFA are capable of acting as prooxidants (Frega et al. 1999). In addition to their prooxidant behaviour FFA are also more susceptible to autoxidation than esterified fatty acids. Similar to FFA, monoglyceride and diglyceride have the ability to act as prooxidant (Choe and Min 2006). These compounds have both hydrophilic and hydrophobic group in the same molecule, making them surface active. These compounds decrease the surface tension of oil, as a result of which the oxygen diffusivity increases, ultimately resulting in increased oxidation rates.

Unlike FFA and minor glycerides which have prooxidant activity, phospholipids can act either as stabilizers or as prooxidants. The mechanism of antioxidative properties of these compounds are yet to be explored in detail, but the presence of nitrogen in

several phospholipids plays a major role in the antioxidative property (King et al. 1992). However, in refined soybean oil, phospholipids were found to act as prooxidants due to their minor concentration.

### 2.5.5. Impact of association colloids on lipid oxidation in bulk oil

One of the often overlooked issues in the oxidation of bulk oil is the presence and the properties exhibited by minor components in oil. Minor components like FFA, mono and diglycerides, phospholipids, sterols and polar compounds are surface active. These compounds tend to self assemble. Because of their variable physical properties, they have the ability to aggregate and form physical structures commonly termed as association colloids (Xenakis et al. 2010, Chaiyasit et al. 2007). Many studies identified that these surface active compounds from various physical structures like reverse micelles, lamellar structure, micro-emulsion and cylindrical aggregates in non-polar solvents and water. When free fatty acids and their corresponding methyl esters were dissolved in organic solvent in the presence of phospholipids, reverse micelle formation was noted (Ichikawa et al 2000). Similarly, Chen et al. (2010) noted the formation of lamellar structures in hazelnut oil when monoglycerides were added. From these studies it can be concluded that amphiphillic molecules in oil shows the ability to form physical structures.

Many researches on oil-in-water emulsions have indicated that the properties of these oil-water interfaces play a major role in the lipid oxidation. Since the presence of association colloids mimic the oxidation in oil-in-water emulsions to a certain extent, the complications involved in oxidation of such emulsion becomes exacerbated in bulk oil containing these physical structures. Thus, the oxidation in bulk oil containing association colloids depends on a variety of factors including substrate or reactant along with antioxidant presence, location of substrate and its positional orientation, water levels, temperature, mobility or concentration of substrates or antioxidants in the inner core region of the colloidal structures (Chaiyasit et al. 2007). This is due to the ability of these structures to solubilise antioxidant or prooxidants effectively. Hence, there is a huge possibility that the properties of association colloids to influence oxidation process in bulk oils by acting as micro or nano reactors

(Waraho et al. 2011). It was identified that addition of phospholipids enhanced antioxidant activity of tocopherol in oil containing trace water (Koga and Terao 1995). Additionally, it was also noted that the antioxidant potential of tocopherols in bulk oil increased when it was conjugated with a polar group. This was again concluded to be due to the association of tocopherols with the physical structures with better efficiency. Thus it becomes difficult to anticipate the efficiency of any antioxidant in bulk oil system.

### 2.5.6. Adverse effects of oxidation products on human health

Many reports are available which signify the adverse effects of oxidised products. For instance, hydroperoxides derived from linoleic acid are toxic to *Saccharomyces cerevisiae* at a very minor concentration of 0.2mM (Evans et al. 1998). Feeding of rats with oxidised palm oil resulted in reproductive toxicity and organotoxicity (Ebong et al. 1999). It has been reported that high intake of oxidised fish oil resulted in infertility in female rats and abnormal spermatozoa in male rats (Zidkova et al. 2004). Similarly consumption of rancid sardine oil increased the spontaneous development of liver tumor in mice (Ichinose et al. 2004). These results suggest the risk involved in the consumption of oxidised oil and hence has to be avoided. Most of the lipid researches focus on the primary question: how to retard lipid oxidation in food oils? A lot of attention has been drawn on identifying effective ways to increase the shelf life of n-3 PUFA rich oils.

## 2.6. ANTIOXIDANTS FOR IMPROVING OXIDATIVE STABILITY

The best strategy to protect foods from deterioration by oxidative stress is to use antioxidants. Antioxidants are molecules that prevent or delay the oxidation in lipids when present in low concentrations (Halliwell and Gutteridge 2007). Though the main purpose of antioxidant addition is to minimise rancidity, a nutritionally beneficial compound is also generally preferred while choosing an antioxidant. Addition of antioxidants to oil can minimise rancidity and prevent formation of oxidation products. Attention has to be given to the cost, toxicity, effectiveness at minimal concentration, colour, flavour before choosing an antioxidant (Reische et al. 1999).

With respect to their mechanism, antioxidants are compounds that donate hydrogen atoms and get converted to antioxidant radicals.

Many factors have to be considered for understanding antioxidant activity as the same antioxidant might show both antioxidant and prooxidant activity with different conditions in lipid environment (Huang et al. 1994). Based on their mechanism of action, antioxidants can be broadly classified as primary and secondary antioxidants. While the former retard oxidation by terminating or interfering with chain reaction or *β*-scission reaction by forming stable free radical (Figure 2.6), the latter prevents oxidation by several other mechanisms. When peroxy radicals ($LOO^{\bullet}$) are formed, it leads to formation of further radicals. These radicals are removed by primary antioxidants by donating hydrogen atom. As a result, antioxidant radicals are formed. The antioxidant radical is more stable compared to other radicals. Since primary antioxidants show higher preference to peroxy radicals, they should be added to oil prior to auto-oxidation to gain maximum efficiency (Reische et al. 1999).

Several mechanisms by which antioxidants delay auto-oxidation are
(1) Radical scavenging
(2) Chelating metal ions
(3) Quenching singlet oxygen
(4) Breaking of auto-oxidative chain reaction (primary antioxidants) and
(5) Reducing localised oxygen concentrations.

The effectiveness of any antioxidant depends on the chemical characteristics (like solubility and chemical potency) and physical location (like emulsions and micelle) in a food system (Wanatabe et al. 2010). According to Brewer (2011), antioxidants that interrupt chain reaction are the most effective. They usually contain aromatic or phenolic rings and they donate $H^{+}$ to the free radicals formed thus becoming radicals themselves. These radical intermediates are stabilized by resonance delocalization of the electron within the aromatic ring and forming quinone structures (Brewer 2011). However, phenolic compounds themselves lack positions suitable for molecular oxygen attacks. Primary antioxidants are usually synthetic compounds like butylate hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT) and tertiary butyl

hydroquinone (TBHQ). Hence, these compounds are usually added to oil immediately upon oil extraction or processing. However, due their suspected role as carcinogenic promoters (Sullivan et al. 2005), these synthetic antioxidants are not preferred any more. Though some literature claim that metal chelation is more efficient method of improving the oxidative stability of n-3 PUFA rich food (Frankel et al. 2002), use of chemical chelators are not generally preferred. Hence, demand for safe and nutritious oil with good oxidative stability using natural antioxidants is still pertaining.

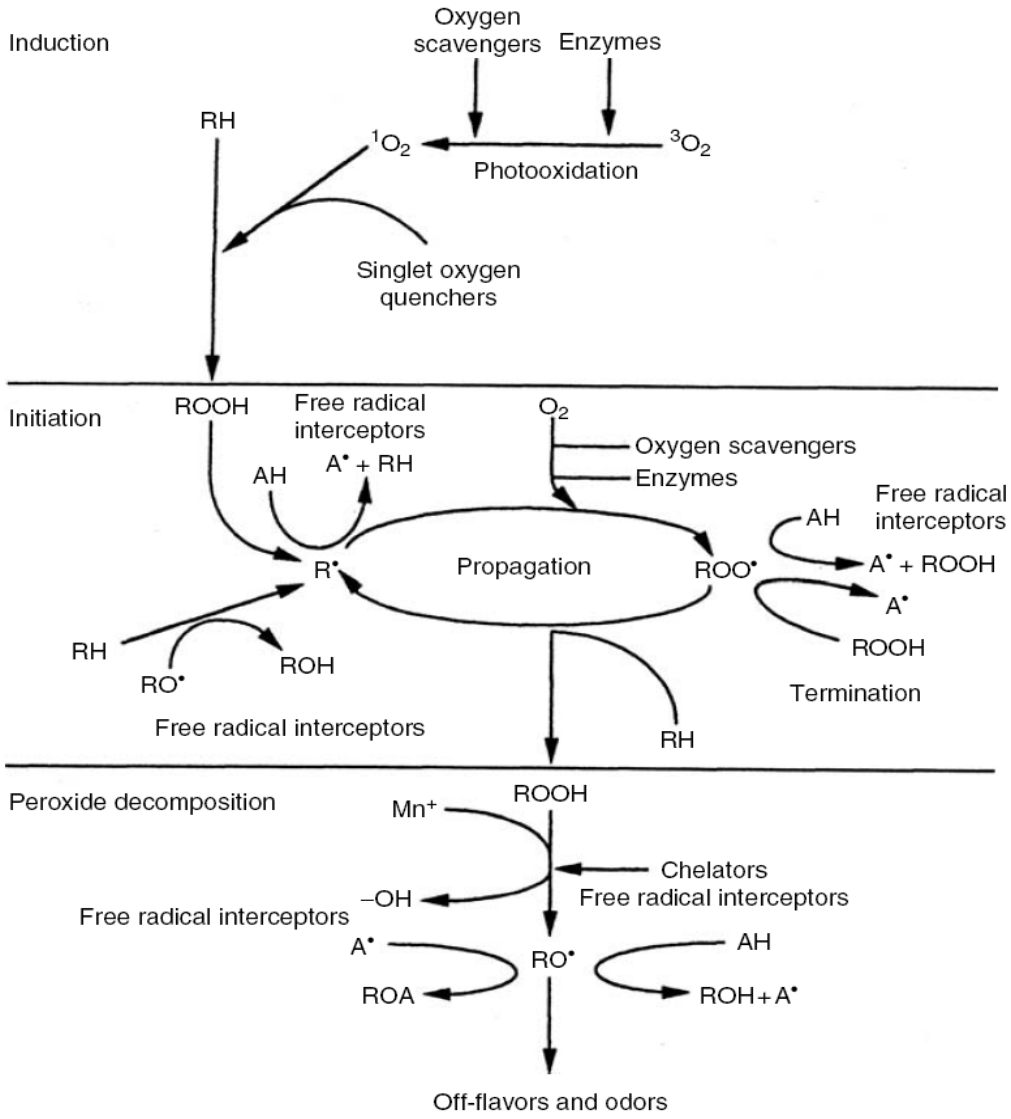



**Figure 2.6 Overview of lipid oxidation and the interaction of antioxidants (Reische et al. 1999)**

### 2.6.1. Natural phenolic antioxidants

Despite the ease of using synthetic antioxidants, increasing concerns on their role in cancer have made then loose market value. Hence, a trend towards the application of natural antioxidants has become apparent in the past years. Variety of natural antioxidants belonging to different classes are available as choice of antioxidants. Table 2.6 provides a brief list of some of the natural antioxidants in our diet.

**Table 2.6 Antioxidants from natural sources (Source: Shahidi and Zhong 2010)**

| **Antioxidant Class** | **Few examples** | **Source** |
|---|---|---|
| Tocopherols | α-, β-, γ-, δ-tocopherols | Seeds, grains, nuts, vegetable oils |
| Tocotrienols | α-, β-, γ-, δ-tocotrienols | Palm oil, rice bran oil |
| Ascorbic acid | Ascorbic acid, ascorbate | Fruits, vegetables |
| Carotenoids | β-carotene, lycopene, astaxanthin, fucoxanthin | Carrots, tomato, fish, marine algae |
| Phenolics/ polyphenolics | Ferulic acid, gentisic acid, quercetin, resveratrol, cyaniding | Fruits, vegetables, nuts, cereals |

Though tocopherols and tocotrienols are fat soluble antioxidants and can be used to scavenge lipid peroxides in fish oil, there are also evidences where they are said to have antagonistic effect with other inherent antioxidants in oil (Hradkova et al. 2013). Since, fish oil do not possess tocopherols naturally, this compound has to be added to oil like any other antioxidant. Hence, exploration of other natural antioxidants can provide beneficial alternative to tocopherols. Of all naturally occurring antioxidants, phenolic compounds are found extensively in nature in vegetables and fruits. In addition to antioxidant properties they are also known for their nutritional properties (Brewer 2011). Phenolic compounds in the form of derivatives of hydroxycinnamic acids, hydroxybenzoic acids and flavonoids are found abundantly in plants. As shown in Figure 2.7, these derivatives differ in patterns of hydroxylations and

methoxylations of their aromatic rings (Lule and Xia 2005). Phenolic acids also serve as precursors for the biosynthesis of flavonoids. The hydroxyl groups in phenolic acids determine the biological function of these compounds as they have the ability to readily donate electron or hydrogen atom (Figueroa-Epsinoza and Villeneuve 2005).

Compounds with hydroxyl groups associated with phenols are often the most effective free radical scavengers. In case of flavonoids, the C2=C3, C3 hydroxy group and 3',4'dihydroxy on the benzene ring are the important structural features responsible for biological activity (Liu et al. 2014). Some of the common phenolic compounds are presented in Figure 2.7. All these phenolic compounds not only provide antioxidant property but also anti-inflammatory, anti-viral, and anti-tumoral activities (Lule and Xia 2005). All these positive attributes have increased the development of phenolic compounds based food systems or pharmaceutical formulations. Unfortunately, such developments are seriously limited due to the poor solubility of these compounds in apolar media like oils (Gayot et al. 2003). As the polarity of the environment greatly determines the antioxidant activity of phenolic compounds (Reddy et al. 2010), it is critical to gain knowledge on the physical structures of the lipid system to be developed.

### 2.6.2. Interfacial phenomenon and polar paradox theory

To understand the effectiveness of antioxidants in a food system a hypothesis was devised called 'Polar Paradox Theory'. According to which, antioxidants that are highly hydrophilic with high hydrophilic-lipophilic balance (HLB) act well on bulk oil, while antioxidants that are non polar with low HLB are effective in polar emulsions (Shahidi and Zhong 2011). This theory was further substantiated by Frankel et al. (1994), based on the interfacial phenomenon. Accordingly, it was proposed that hydrophilic antioxidants show more affinity towards air-oil interface, which is considered as the place of oxidation in bulk oil, thus providing better protection in bulk oil. This theory on interfacial phenomenon was tested and confirmed in oil-in-water emulsions due to existence of easier methods of analysis of such emulsions (Chaiyasit et al. 2007).

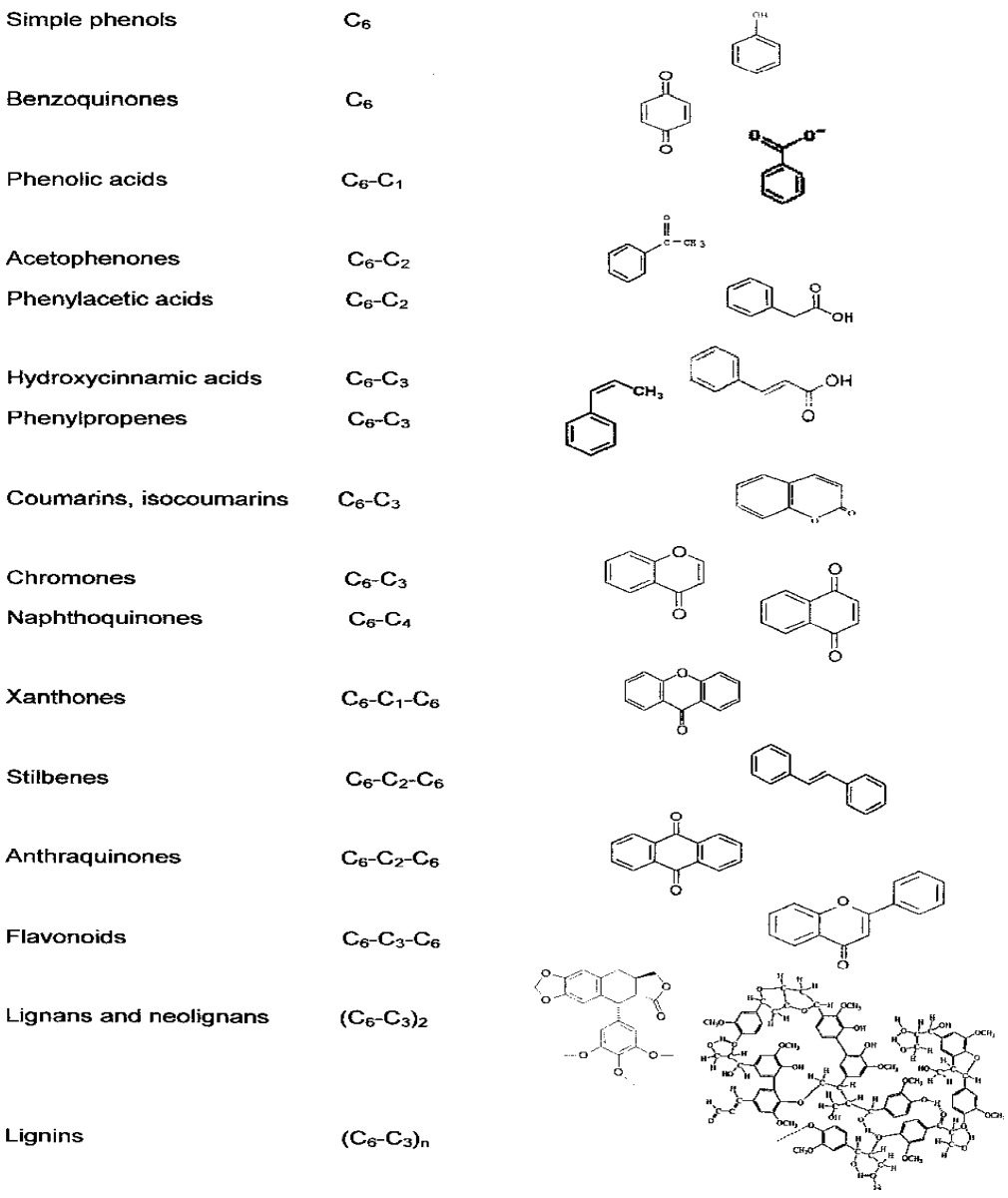



**Figure 2.7 Basic structures of some common phenolic compounds available in nature**

Early studies on the antioxidant activity on bulk oil considered oxidation in a homogenous medium. However, bulk oil consists of mixture of colloidal structures due to the presence of components used in refining stages and also due to trace impurities. As shown in Figure 2.8 polar antioxidants, instead of being oriented in oil-air interface, as previously believed (Figure 2.8A), are in fact preferentially located at the interface in colloids (Figure 2.8B). However there still exist certain discrepancies

to this theory as some of the study on effect antioxidant polarity on bulk oil oxidation has been variable.

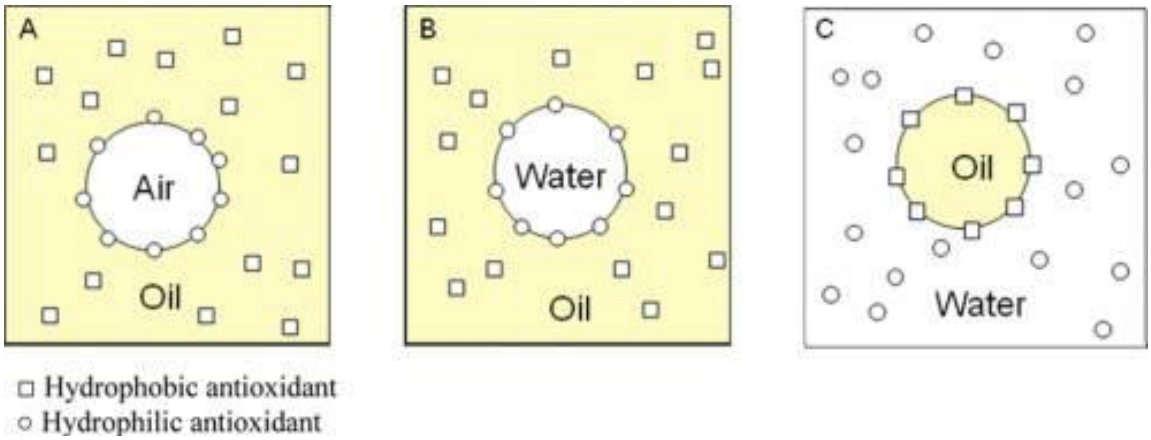



**Fig. 2.8 Distribution of antioxidants in bulk oil (A and B) and oil-in water emulsion (C) based on polar paradox theory (adapted from Shahidi and Zhong 2011)**

## 2.7. RATIONALE BEHIND SYNTHESIS OF NOVEL ANTIOXIDANT ESTERS

The choice of antioxidants with most effectiveness in food system is often determined by many properties of antioxidant like, type of substrate, physical location of antioxidant in oil and impact of other components in the surrounding environment (Katsoura et al. 2006). However, one major drawback in utilising these antioxidant compounds effectively is that most of the natural antioxidants are hydrophilic compounds. Therefore, this limits their application in hydrophobic medial due to poor solubility. Developing methods to change the characteristics of hydrophilic natural antioxidants to increase their solubility in hydrophobic medium can adopted. Hence, it becomes an effective strategy to improve their solubility properties by incorporating slight modifications into the structure of antioxidant. This can be achieved by manipulating the hydrophilicity of natural antioxidants by conjugating a hydrophobic group with the phenolic compounds either by chemical or enzymatic methods (Shahidi and Zhong 2010). As a result, the new molecules will posses both

hydrophobic and hydrophilic groups, accumulating the oil-water interface due to their amphiphillic nature.

Previously, majority of any amphiphillic molecules are prepared by chemical methods. However, these methods suffer many disadvantages like use of high temperature, non-regioselective, loss of biological activity of compounds, generation of by-products and low yield (Xiao-na et al. 2012). Moreover, phenolic compounds are heat sensitive resulting in a dark colour product on subjection to high temperature. Compared to chemical methods, enzymatic methods involve milder reactions conditions and more regioselective in reaction. Usually lipase enzymes are employed for this application.

### 2.7.1. Lipases for antioxidant lipophilisation

Several enzymes such as proteases, acyl transferases and lipase have been tested for the antioxidants modification. However, with increase in the number of commercial enzymes a wide range of biocatalysts has been tested.

Lipases are an important group of enzymes that find immense applications in pharmaceutical, dairy, and food industries (Joseph et al. 2008). Though they are water soluble, they act on lipids at the interface. They catalyse the hydrolysis of tri-, di-, and monoacylglycerols. They also catalyze the esterification and trans-esterification reactions (Figure 2.9) in addition to the hydrolytic activity (Joseph et al. 2008).

In the past few years importance of lipase mediated esterification and transesterification reaction in food industries have motivated research on *Candida antartica* (Coulan et al. 1995), *Candida cylindraceae* (Gray et al. 1990), *Rhizomucor meihei* (Krmelj et al. 1995). Among these lipases and in particular lipase from *Candida antartica* (CAL-B) has achieved higher significance. This enzyme has been widely used effectively for esterification reactions (Compton et al. 2000, Karboune et al. 2005). This immobilised enzyme is achieved by adsorption on macroporous acrylic resin. The use of immobilised enzyme provides several advantages by facilitating product recovery, resistance against denaturants (Plou et al. 2002).

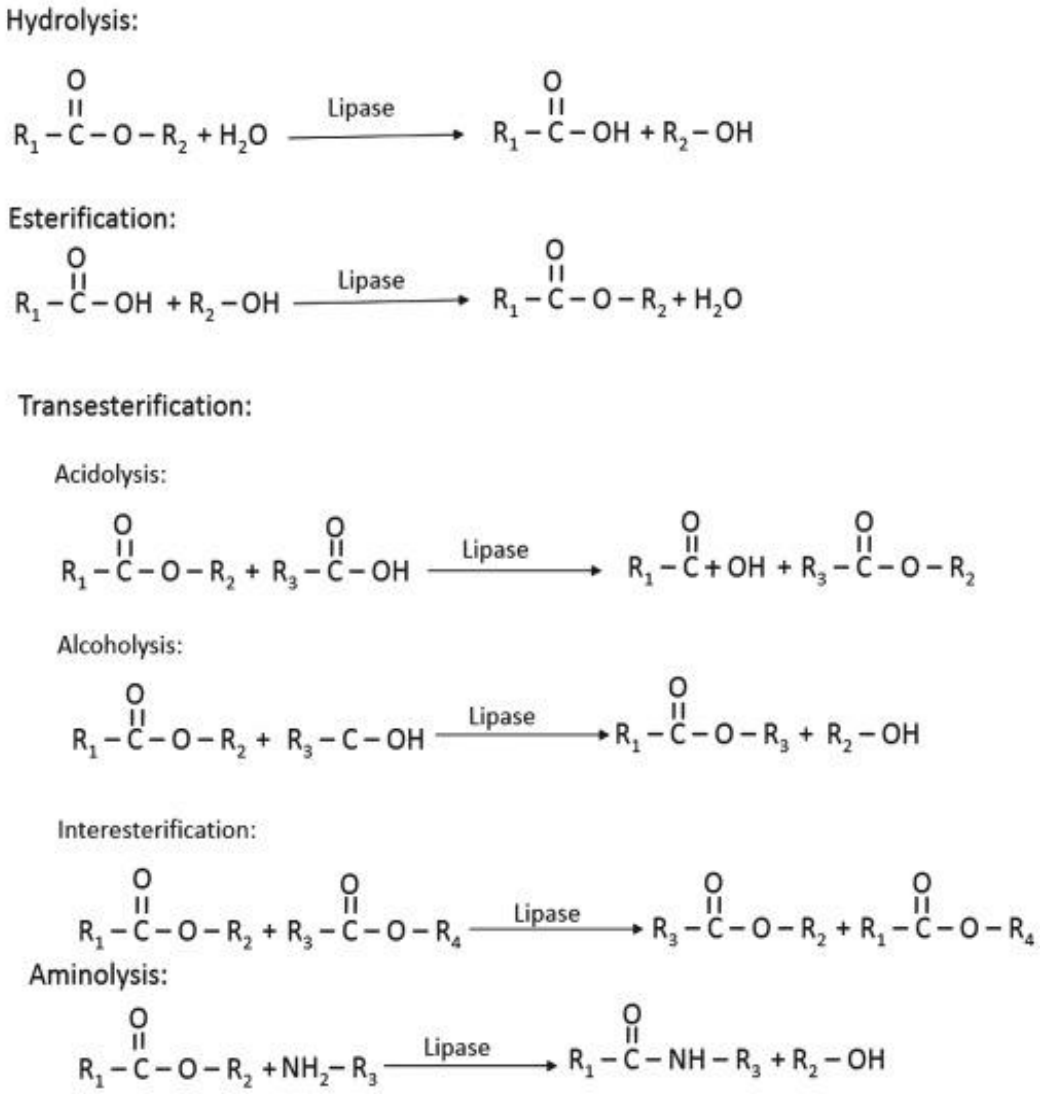



**Figure 2.9 Different reactions catalysed by lipase enzyme (Adapted from Bora et al. 2013)**

Theoretically, all hydroxyl groups can participate in the reaction and can give different products with various degrees of esterification (Gayot et al. 2003). It is also critical to avoid complete acylation of all hydroxyl groups so as to retain the antioxidants property. Hence enzymatic esterification poses better regioselective advantage over chemical esterification which gives many intermediates and products. Most of the available literature on this enzymatic esterification is focused on synthesizing lipophilic phenolic compounds using simple phenolics and acyl donors (Table 2.7).

**Table 2.7 Different Phenolic compounds used for esterification and their reaction conditions**

| **Substrates** | | **Solvent** | **Enzyme source** | **Conditions** | **Reference** |
|---|---|---|---|---|---|
| **Phenolic compound** | **Acyl donor** | | | | |
| Rutin, Esculin | Lauric acid, myristic acid, palmitic acid, stearic acid | Tert-amyl-alcohol | *Candida antartica* | Equimolar phenolic/acyl donor ratio, 60°C, 10g/l enzyme, 50-150 g/l molecular sieves | Ardhaoui et. al. 2004 |
| Cinnamic acid | Triolein | Hexane 2-butanone binary solvent | *Candida antartica* | 1:2. 1:3 1:4.5 phenolic to acyl donor ratio, 20 mg enzyme, 45°C, 150 rpm | Karboune et. al. 2005 |
| Ferulic acid, DHCA | Trilinolein | Hexane 2-butanone binary solvent | *Candida antartica* | 1:1. 1:2. 1:4 phenolic to acyl donor ratio, 25 mg enzyme, 55°C, 150 rpm,14 days | Sabally et. al. 2006 |
| Ferulic acid | Glycerol and oleic acid | Ionic liquids | *Candida antartica* | 80 °C, continuous stirring, 0.21 g/l enzyme | Sun et. al. 2009 |
| Rutin | Decanoic acid, dodecanoic acid, octadecanoic acid | 2-methyl-2-butanol. | *Candida antartica* | 1: 5 molar ratio rutin: fatty acid, 60°C, 250 rpm, 168 hours, 10 g/l enzyme, 100g/l, 4 Å molecular sieves | Viskupicova et. al. 2010 |

| Substrates | | Solvent | Enzyme source | Conditions | Reference |
|---|---|---|---|---|---|
| **Phenolic compound** | **Acyl donor** | | | | |
| Isoquercetin | Ethyl esters of fatty acid | 2-methyl-2-butanol. | *Candida antartica* | 1:10 molar ratio of isoquercetin to acyl donor, 65°C, 150 rpm under vacuum (700mbar) | Salem et. al. 2010 |
| DHCA | Octanol, Tricaprylin | Tert-butanol | *Candida antartica* | 1:1, 1:3 molar ratio of phenolic to acyl donor, 60°C, 300 rpm, 10% enzyme, 10% 3 Å molecular sieves. | Feddern et. al. 2011 |
| Phlodzin, isoquercetin | Stearic acid | Acetone | *Candida antartica* | 0.5 g phenolic, 1.6 g fatty acid, 45 °C, 12-24 hours | Ziaullah et. al. 2013 |
| Coumaric acid | Methanol, ethanol, propanol | Nil | *Bacillus lichenifo rmis* | 1:1 molar ratio, 55 °C, 8 hours, 1% enzyme, 2% 3 Å molecular sieves | Sharma et al. 2014 |
| Vanillyl alcohol | Docosahexae noic acid ethyl ester | | *Candida antartica* | Fed batch process, 0.5 g vanillyl alcohol, 10ml of DHA-EE, 37°C, 500mbar pressure, 250 rpm | Roby et al. 2015 |

The existing reports show higher significance of reaction conditions on esterification. While some studies focused on the regioselectivity of acylation (Kontogianni et al. 2001, Kontogianni et al. 2003, Chebil et al. 2007a), several others aimed at understanding the nature of substrates, type of lipase origin and operating conditions

including water activity of the system on esterification (Ardhaoui et al. 2004, Duan et al. 2006, Chebil et al. 2007a). However, data related to such enzymatic reactions are very incomplete and contradictory, making them interesting topic of research. Some of the recent works on antioxidants modification has been listed in Table 2.7.

### 2.7.2. Application of amphiphillic phenolic esters in bulk oils

Since enzymatic modification of antioxidants is a relatively recent idea, there is very little literature on their applications in food system. In order to test the effectiveness of the amphiphillic phenolic esters, many in vitro studies have been carried out. Research on the application of lipophilised antioxidants is mostly dedicated to *in-vitro* antioxidant assays, model systems and very few vegetable oils. As these systems fail to duplicate the complex oxidation mechanisms found in fish oils during storage, the utility of *in-vitro* antioxidant assays is limited.

Moreover, reports are rather scarce on the effectiveness of lipophilised antioxidants in marine oils. Aladedunye et al. (2015) and Chen et al. (2016) studies the effectiveness of lipophilic antioxidants in edible oil and identified that lipophilised compounds performed with better efficiency in improving the oxidative stability of bulk oils. Additionally, Sorenson et al. (2011), tested the effectiveness of the synthesised esters in fish oil enriched milk and concluded that these compounds were effective in preventing oxidation in variable food systems. However, no reports are available on the application of these lipophilic antioxidants in Indian sardine oil, making this work a novel and interesting.

# CHAPTER 3

# REFINING OF INDIAN SARDINE OIL

This chapter deals with the refining and characterisation of crude Indian Sardine oil. Increase in demand for n-3 PUFA rich oil has lead to the need for production of good quality product that meets consumer demands. Refining is a necessary process that ensures the quality of the edible oils for human consumption. The removal of undesirable components while retaining the essential portion of oil is critical. Though refining procedures are habitually carried out in any edible vegetable oil industry for commercial applications, the same process cannot be replicated for fish oil. Fish oils differ from other vegetable oils based on the triglycerides composition. Fish oils have variety of fatty acids, higher proportion of long chain fatty acids, considerable PUFA and also omega- 3 fatty acids. Similarly, vegetable oils have compounds that improve the oxidative stability, whereas fish oils do not posses compounds with inherent antioxidant properties. On the other hand, the presence of high quantities of unsaturated fatty acids in fish oil reduces the oxidative stability, demanding refining processes that do not involve long processing periods. Hence, the traditional refining route and technologies applied for vegetable oils might not work effectively for fish oils. Exploration of different routes and techniques of refining stages could provide with an effective choice of refining strategy.

Hence, this chapter deals with identification of the best refining route based on the oil characteristics. In order to achieve this, conventional methods like chemical refining along with several novel approaches to refining like membrane processes were studied in this chapter. After analysing the efficiency and advantages of the methods tested, a final refining route was derived for refining of Indian Sardine oil.

## 3.1. MATERIALS AND METHODS

Crude Indian Sardine oil was procured from a local seafood industry (Mukka seafood industry). The physico-chemical characteristics of crude oil were determined. The oil was stored at -20°C until further use. Ammonium molybdate, phenolphthalein indicator in methanol, potassium hydroxide, potassium iodide, iso-octane, sodium thiosulphate, wijis solution, chloroform acetic acid were of analytical grade and were purchased from Merck, India. Membranes were purchased from Axivia India. All

reagents and solvents were of analytical grade and purchased from Merck India. All chemicals and solvents were used without further purification.

### 3.1.1. Sardine Oil Characterisation

#### 3.1.1.1. Phospholipid estimation

The assay for direct measurement of phospholipid phosphorus was performed according to a method described by Hundreiser et.al (1985), with slight modifications. A chromogenic solution was prepared prior to experiments in a series of steps as follows;

- Solution I: 16 g ammonium molybdate in 120 mL deionised water.
- Solution II: Filtered mixture of 10 mL concentrated HCl, 10 mL mercury and 80 mL solution I was prepared and mixed for 30 minutes to obtain solution II. The remaining solution I was diluted with 200 mL concentrated sulphuric acid.
- Solution III: Combined mixture of solution I diluted with sulphuric acid and solution II.

The chromogenic mixture was prepared by combining 25 mL of solution III with 45 mL of methanol and 5 mL chloroform and 20 mL distilled water. This solution was then stored at 4°C for further assay uses.

In order to analyse the phospholipid content in oil, the following steps were carried out.

- The oil samples were weighed and taken in glass tubes. The oil sample were allowed to dissolve in chloroform, after which the solvent was evaporated by heating in water bath
- The residue was then mixed with 0.4 mL chloroform and 0.1 mL of chromogenic reagent.
- The mixture was mixed thoroughly by vortex and placed in boiling water bath for 1.25 minutes.
- The samples were allowed to cool to room temperature and 4 mL of chloroform was added.

- The mixture was vortexed and the absorbance of chloroform portion was read at 730 nm using LabIndia double beam UV-Vis spectrophotometer, against a blank containing no oil.
- A standard graph was plotted with increasing concentration of soy lecithin as phospholipid standard (Appendix I).

#### 3.1.1.2. Acid value estimation

The free fatty acid reduction in oil was analysed using standard AOCS (2009) methods.

- Known mass of sample (1 g) was taken in Erlenmeyer flask.
- 25 mL of isopropyl alcohol was added to the flask and the oil sample was dissolved well by continuous mixing.
- Few drops of phenolphthalein indicator dissolved in isopropyl alcohol was added to the oil solution.
- Titrate the mixture, while shaking with 0.1 M NaOH solution to the first pink colour which persists for 30 seconds.

The acid value was calculated as follows,

$$Acid\ value = \frac{(S - B) * N * 56.1}{m} \qquad (3.1)$$

where, S is sample titre value, B is blank titre value, N is normality of KOH solution, m is the mass of sample taken for analysis in grams. In order to express the acid value in terms of free fatty acids (FFA) as percent of oleic acid, the acid value was divided by 1.99.

#### 3.1.1.3. Iodine value estimation

The iodine value is the measure of unsaturation of fats and oils. This was determined by using standard AOCS (2009) methods.

- Label one 250 ml flask as Blank and the other as Sample

- 0.3 g of the sample was weighed into the Sample flask
- 20 mL of chloroform was added into each flask and dissolve the sample well by mixing
- 25 mL of iodine monochloride (Wijs) solution was mixed into both flask and disperse well. The flasks were placed in dark for 1 hour
- 20 mL of potassium iodide solution was added to the flasks and titrated against 0.1N Sodium thiosulfate solution to achieve a straw yellow colour.
- 1 mL of starch solution was added to the flask. The resultant solution was dark purple in colour
- The mixture was titrated against sodium thiosulfate solution to get a clear solution
- The volume of thiosulfate solution was recorded

The iodine value was calculated as follows,

$$Iodine\ value = \frac{(S - B) * N * 12.69}{m} \qquad (3.2)$$

where, S is sample titre value, B is blank titre value, N is normality of sodium thiosulphate solution, m is the mass of sample taken for analysis in grams.

#### 3.1.1.4. Peroxide value estimation

The lipid hydroperoxide level in oil was measured based on the standard AOCS (2009) method.

- A known mass of sample was weighed into flask and an appropriate blank flask was maintained without sample addition
- 25 mL of 3:2 acetic acid: isooctane solution was added to the flasks and the sample was dissolved well by mixing
- 0.5 mL of freshly prepared saturated potassium iodide solution was added to the flasks and mixed.
- The flasks were kept one minute with intermediate shaking for every 30 seconds.

- 30 mL of distilled water was added to the flasks and mixed well to stop the reaction
- 1 mL of 0.1% starch solution was added and the resulting solution was titrated against 0.1N sodium thiosulfate solution until the blue grey colour disappeared.

The peroxide value in terms of milli equivalents of peroxide per 1000 gram of oil was calculated as follows;

$$Peroxide\ value = \frac{(sample\ titre - blank\ titre) * Molarity\ of\ thiosulfate * 1000}{mass\ of\ test\ in\ g} \quad (3.3)$$

### 3.1.1.5. Determination of fatty acid composition

The fatty acid composition of sardine oil was measured by analysing the Fatty Acid Methyl Esters (FAME) using gas liquid chromatography (GC). The FAMEs were prepared according to the conventional method described by Ichihara and Fukubayashi (2010) with minor modifications. This method involves the saponification of fatty acids followed by their methylation to form respective esters. Oil samples were taken in a screw capped glass tube and was dissolved in diethyl ether. The lipid solution was then mixed with 2 mL of 0.5M methanolic NaOH solution. The mixture was allowed to hydrolyse for 1 hr at 70 °C under reflux. The hydrolysed mixture was then mixed with 0.6 mL 2M HCl. In order to extract the FFA resulting from the hydrolysis reaction, hexane was added to the mixture. The hexane phase was evaporated and the FFA was methylated by addition of 2.75 mL of boron trifluoride in methanol. The mixture was heated for 20 minutes. In order to extract the methylated fatty acids, solvent extraction with hexane and water (1:1 v/v) was used. The procedure was repeated three times and the entire hexane fraction was pooled and used for analysis. The fatty acid profile was compared with a standard FAME mixture (Appendix II). The fatty acid composition of the oil in term of relative weight percentage was directly obtained from the GC chromatogram.

The GC of sardine oil FAME and standard FAME mixture was analysed by Agilent Gas Liquid Chromatography with DB-5 column from Agilent Technologies. Trace 3330 GC Ultra system from Thermoelectron Corporation, USA was used. The detection of FAME was done using flame ionisation detector. The conditions of analysis were as follows: Injector temperature – 280 °C; Detector temperature – 300 °C; Column over gradient: 0-1 minute 160°C; temperature increased at 5°C/min to 185 °C; The temperature was maintained at 185 °C, for 10 minutes; The temperature was further increase at 8 °C/min to 240 °C; The temperature was further maintained at 240 °C for another 10 minutes. Along with FAME standard from Sigma-Aldrich India, EPA methyl ester and DHA methyl ester were also analysed under the same conditions. In order to quantitatively estimate the EPA and DHA content of the oil, standard graphs were plotted for EPA methyl ester (Appendix III) and DHA methyl ester (Appendix IV) respectively, by plotting the concentration of methyl esters versus area under the peak of respective compounds.

#### 3.1.1.6. Moisture Content estimation

The moisture or trace water content of sardine oil was measured by oven drying method as well by using Metrohm Karl Fischer Titrator, India. Analysis by oven drying method was carried out as follows:

- Approximately 2 g of sardine oil was weighed in a crucible ($w_1$)
- The crucible was kept in oven at 100°C for a period of 30 minutes
- The weight of the oil was measured. The drying and weight measurement was performed consecutively for three measurements until a constant weight is achieved ($w_2$).
- The percentage of moisture was estimated as follows

$$\%\ moisture = \frac{w_1 - w_2}{w_1} * 100 \qquad (3.4)$$

The moisture content in sardine oil was further confirmed using Metrohm Karl Fischer titrator and expressed as percentage or ppm.

#### 3.1.1.7. Instrumental methods of analysis of sardine oil

- The trace metal content in sardine oil was measured using atomic absorption spectrometry (AAS), GBC scientific equipment (932 plus), AU.
- The density and viscosity of sardine oil was measured using densitometer from Rudolf Research Analytics and Anton Paar digital micro-viscometer, respectively.
- The trace water content was also confirmed by analysing through Karl Fischer titration using Metrohm Karl Fischer titrator.

### 3.1.2. Refining of sardine oil

#### 3.1.2.1. Degumming

Degumming is a pre-treatment stage, which involves the removal of phospholipids from sardine oil. Conventional chemical degumming was performed similar to the method described by Zufarov et al. (2009) with minor modifications.

- Crude sardine oil was accurately measured and taken in a beaker.
- The degumming agent was added in required weight % (w/w) to the oil.
- The mixture was kept for continuous stirring for 30 minutes using magnetic stirrer.
- The oil-degumming agent mixture was then centrifuged at 10,000 rpm for 25 minutes.
- A clear and less viscous supernatant was obtained, which was analysed for the phospholipid content and used for further studies.

#### 3.1.2.2. Deacidification

The removal of free fatty acids from degummed sardine oil was performed by exploiting the application of liquid-liquid extraction using appropriate solvent by a method similar to Kale et al. (1999).

- The effect of solvent extraction on free fatty acid removal was conducted by using several solvents in different ratios.

- The degummed oil was taken in a beaker and appropriate quantity of solvent was added.
- The mixture was mixed continuously for 60 minutes and then transferred into a separating funnel
- The mixture was allowed to separate overnight. The oil part was separated, the trace solvent was allowed to evaporate and the oil was subjected to analysis.

#### 3.1.2.3. Bleaching

The degummed and deacidified oil was subjected to bleaching to remove trace impurities by the method described by Gracia-Moreno et al. (2013), with minor modifications

- Degummed and deacidified oil was subjected to bleaching under vacuum at approximately 40-70 mmHg.
- Appropriate quantities of oil was measured and taken in a conical flask. Calculated quantity of activated charcoal was added to achieve a 5% w/w concentration in oil.
- The conical flask was sealed using cork and placed on magnetic stirrer with heating mantle.
- Bleaching was performed by maintaining vacuum and at 80°C for 20 minutes
- The activated carbon was then removed from oil by centrifugation at 10000 rpm for 15 minutes.
- The clear oil supernatant was collected, analysed for its physico-chemical properties and used for further studies.

#### 3.1.2.4. Membrane process

A dead end microfiltration membrane setup with solvent resistant test cell as shown in Appendix V was used. The maximum operating pressure for this unit was 4 bars and had a feed capacity of 500 mL. The test cell was placed on a magnetic stirrer and the feed was continuously agitated using a magnetic spin bar fitted into the cell. Commercial microporous membranes with different polymeric matrix were

purchased. Microporous polyamide (0.45 μm), polyethersulfone (0.45 μm) and PTFE-polyterafluoroethylene (0.45 μm) membranes were purchased and used for the refining of sardine oil. Membranes were obtained as circular discs of 10 cm diameter or above. In order to understand the influence of solvents on the membrane properties, conditioning of membranes were performed. Membranes can be conditioned prior to their use, so that adverse effects of some solvents can be avoided. Possibility of swelling or dysfunction of membranes is high when pure solvents are used. This can be prevented by conditioning of membranes before their use. Membrane conditioning was performed similar to the method described by Rao et al. (2013). The membranes were soaked overnight in increasing concentrations of solvent over a course of period. The membrane discs were initially rinsed in water after which it was soaked in 10% solvent. The percentage of solvent used was gradually increased to a final value of 100% pure solvent. The conditioned membranes were then used in membrane application to study the change in their efficiency.

For degumming by membrane process, crude oil/hexane mixture was directly passed through the desired membranes. The membranes were placed on a polymeric support to avoid damage to membranes during the process. Permeate and retentate were then collected and used for analysis.

The degummed sardine oil along with appropriate solvent was fed into the membrane test cell for solvent assisted deacidification process. The permeate/ retentate was collected after membrane filtration and analysed for properties.

The permeate flux is the most common and important method for analysing the performance of a membrane process. The flux is calculated by the equation (3.5) using the volumetric flowrate of permeate. It is usually expressed as $L/m^2.h$ or $Kg/m^2.h$.

$$Flux = \frac{Volume\ of\ permeate\ in\ litres\ or\ weight\ in\ kg}{Membrane\ area\ (m^2) * Time\ (h)} \qquad (3.5)$$

In case of membrane process for the application of degumming and deacidification process, the efficiency of membrane was calculated based on the phospholipid or FFA content in the feed and retentate, respectively. In addition, oil loss was also calculated to analyse the efficiency of membrane process.

## 3.2. RESULTS AND DISCUSSION

### 3.2.1. Characteristics of crude sardine oil

In order to produce high quality edible oil, many refining processes like degumming, deacidification, bleaching and deodorisation are generally carried out. Crude fish oil obtained from local seafood industry was analysed for its physical and chemical characteristics so that appropriate refining strategy can be chosen. The crude sardine oil contained higher phospholipid content (303.03 µg/g) and 3.75% (w/w) of FFA as shown in Table 3.1.

**Table 3.1 Chemical properties of crude sardine oil**

| **PROPERTIES** | **CONTENT** |
|---|---|
| Phospholipid (µg/g) | 303.03 ± 5.02 |
| Acid value (mg of KOH/g of oil) | 7.46 ± 0.01 |
| % Free fatty acid (w/w) | 3.75 ± 0.01 |
| Iodine value (g of iodine consumed/100 g of oil) | 157.70 ± 2.05 |
| Peroxide value (meq of peroxide/ 1000g) | 7.35 ± 0.01 |
| % Moisture (w/w) | 0.23 ± 0.02 |
| Density ($g/cm^3$) at 30°C | 0.9181 ± 0.071 |
| Viscosity (mPa.s) at 30°C | 41.03 ± 0.02 |
| Iron (ppm) | 0.23 ± 0.25 |
| Copper (ppm) | 0.06 ± 0.1 |
| Zinc (ppm) | 0.03 ± 0.5 |
| Nickel (ppm) | 0.09 ± 0.07 |

*All experiments were done in triplicates and the results are provided as average with standard deviation

FFA value of 3.75 % denotes that the sardine oil was relatively fresh (Shahidi et al. 1997). In addition, high iodine value of 157.7 indicates the degree of unsaturation or the presence of higher amounts of unsaturated fatty acids. Generally, sardine oil exhibits iodine value around 160-200 (Bimbo 1998). However, it is subjected to change based on the freshness, geographic location and type of species. Similarly, crude sardine oil showed a peroxide value of 7.35 meq of peroxide per kg oil. Though literature suggests a peroxide value of below 5 meq/kg for standard oils (Bimbo 1998), several reports have indicated values similar to the value reported in our study (Noriega-Rodriguez et al. 2009, Suseno et al. 2015). However, refining steps has to be employed to avoid further formation of FFA or any other oxidation products. The physicochemical properties of oil purchased are also shown in Table 3.1.

### 3.2.2. Degumming

In recent times much importance has been given to degumming stage as it greatly affects further refining processes. Phospholipids can act as either antioxidants or prooxidants in oil. This depends on their concentration and presence of trace metals in oil (Choe and Min 2006). Since higher concentrations of phospholipid can lead to higher oxidation (Yoon and Min 1987), attempts were made to reduce the phospholipid content to desired levels. Further, if the degumming strategy applied is efficient enough, loss of oil in further stages can be minimised.

The phospholipid content in the procured sardine oil was high enough to hinder other refining stages and to act as pro-oxidant. Such high amounts of phospholipid could be due to the freshness of the oil and the method of oil extraction (Zufarov et al. 2009). Since, this study aimed at developing novel refining techniques, it is necessary to understand the efficiency of new method in comparison with the traditional methods. Initially, conventional degumming method by using *acid treatment* was followed to study its efficiency in sardine oil. Considering the corrosion resistant nature and practicality, organic acids were chosen for degumming application and their degumming effect was analysed. As literature suggested best results with organic acids, concentrated ortho phosphoric acid, citric acid and lactic acid were initially used for phospholipid removal from oil (Subramanian et al. 1999). It was noted that

out of the three organic acids tested, orthophosphoric acid gave mild reduction of phospholipids (12.8%), while lactic acid and citric acid gave negligible reduction (Table 3.2). Since lactic acid and citric acid failed to show effective reduction in phospholipids as efficiently as ortho phosphoric acid, it was chosen for further studies (Table 3.2). This is in contract to the results obtained by Mei et al. (2013), who noted a 58% and 27.5% degumming efficiency by citric acid and phosphoric acid respectively. The lack of similar degumming efficiency in sardine oil by these organic acids could be due to the highly complex composition of sardine oil.

**Table 3.2 Effect of degumming agents on phospholipid reduction**

| **Degumming Agent (5% w/w)** | **% Reduction in phospholipid*** | **Free fatty acid (FFA) % content in treated oil** |
|---|---|---|
| Orthophosphoric acid | 12.82 | 3.75 |
| Lactic acid | 2.64 | 3.75 |
| Citric acid | 1.09 | 3.7 |
| Monoethanolamine | 29.07 | 2.02 |
| Diethanolamine | 55.88 | 1.98 |
| Triethanolamine | 76.96 | 1.21 |

*Reduction percentage was calculated by considering crude phospholipid content as 100%

Due to the low performance of organic acids, other degumming agents were searched. Since phospholipids are emulsifying agents, addition of another emulsifier might lead to its precipitation in oil. Based on the results obtained by Zufarov et al. (2009) ethanolamines were tested for their ability as possible degumming agents for sardine oil. It was identified that all three ethanolamines performed with better efficiency than organic acids, with triethanolamine showing a maximum reduction of 76.96% (Table 3.2). Due to its surfactant properties, ethanolamines are known to have synergistic effects in complex emulsifier systems (Frauenkron et al. 2011). Hence, it resulted in effective reduction of phospholipids in oil.

Further, it was also noted that, there was a drastic reduction in FFA content in all samples treated with ethanolamines. While triethanolamine reduced the FFA content to 1.21%, mono and diethanolamine reduced the FFA content to 1.98% and 2.02% respectively, from a crude value of 3.75% FFA (Table 3.2). Similar results were obtained by Zufarov et al. (2009), where triethanolamine gave higher reduction in FFA content than mono and diethanolamines in vegetable oil. The highly basic nature of ethanolamines could be the possible reason behind the simultaneous reduction in FFA content along with phospholipid reduction. Because of the presence of basic amino group along with the hydroxyl group, ethanolamines exhibit properties of both amines and alcohols. When ethanolamines come in contact with fatty acids, salt formation takes place (Frauenkron et al. 2011), thus resulting in the precipitation of ethanolamine salts of fatty acids. Unlike the reports by Thengumpillil et al. (2004) and Zufarov et al (2009) on ethanolamine degumming of vegetable oils, triethanolamine showed better efficiency than mono or diethanolamine. This could be attributed to the type of oil and its phospholipid composition. It has been suggested that ethanomaines are efficient by acting against Ca and Mg salts of phospholipids (Zufarov et al. 2009), otherwise known as non-hydratable phospholipids (NHPs). This could indicate the presence of higher amounts of NHPs in sardine oil.

In order to make an efficient comparison between conventional acid degumming and ethanolamine as degumming agent, the concentrations of ortho phosphoric acid and triethanolamine were varied (Table 3.3). It was identified that the removal of phospholipid was proportional to the ortho phosphoric acid concentration upto a certain point after which there was no significant reduction with increase in acid concentration. Thus 3% (w/w) of ortho phosphoric acid gave a maximum reduction from 303 ppm of phospholipids in crude oil to 233 ppm in degummed oil. Similarly, the effect of triethanolamine concentration on phospholipid removal resulted in a bell shaped profile, with a maximum reduction to 37.9 ppm at 3% triethanolamine, beyond which there was a decrease in reduction.

As the main basis of phospholipid removal by ethanolamine is their emulsifiying property, increase in concentration of ethanolamines after a particular quantity failed

to show the reduction in phospholipids. In addition to phospholipid removal, the FFA content was found to reduce significantly from 3.75% to 1.21% with increase in triethanolamine concentration. At all triethanolamine concentration tested, the FFA content was less than 2% with a maximum reduction at 3-5% of triethanolamine (w/w). Since FFA are removed as salt precipitates (Frauenkron et al. 2011), increase in triethanolamine concentration resulted in higher reduction of FFA.

**Table 3.3 Effect of concentration of degumming agent on phospholipid content**

| % Degumming agent (w/w) | Concentrated orthophosphoric acid | | Triethanolamine | |
|---|---|---|---|---|
| | Phospholipid (ppm) | %Free fatty acid | Phospholipid (ppm) | %Free fatty acid |
| 1 | 241.21 ± 2.01 | 3.75 | 100.41 ± 0.33 | 1.84 |
| 2 | 234.54 ± 0.01 | 3.70 | 90.41 ± 1.21 | 1.50 |
| 3 | 233.33 ± 0.02 | 3.75 | 37.91 ± 0.31 | 1.21 |
| 4 | 252.12 ± 6.01 | 3.75 | 63.75 ± 2.64 | 1.21 |
| 5 | 264.24 ± 4.00 | 3.75 | 70.00 ± 0.03 | 1.21 |
| Crude | 303.03 ± 5.02 | 3.75 | 306.03 ± 5.02 | 3.75 |

*All experiments were done in triplicates and the results are provided as average with standard deviation

The reason for the inefficient removal of phosphorus compounds from crude sardine oil by conventional degumming procedures could be due to the presence of surface active compounds leading to inefficient interaction of degumming agents with phospholipids. Also presence of large quantities of phosphoglycolipids is also a major drawback (Thengumpillil et al. 2004). Since, there are no standard analysis methods to confirm the presence of such molecules (AOCS Lipid Library), the inefficiency of polar organic acids to remove phospholipids could explain their presence of these compounds in higher concentrations. Triethanolamine is an organic compound that has many cosmetic and pharmaceutical applications. It blends both water-soluble as

well as oil-soluble compounds. Hence, emulsifiers like triethanolamines can efficiently interact with the compounds in sardine oil and result in the effective reduction of impurities.

#### 3.2.2.1. Membrane degumming of crude sardine oil

As many recent reports on vegetable oil suggest higher degumming efficiency in membranes processes, several membranes were tested for degumming of crude sardine oil as well. Three types of microporous membranes viz. polyamide, polyethersulfone (PES) and polytetrafluoroethylene (PTFE) of pore size 0.45 µm were tested. Literature indicates membrane based degumming process was performed either with crude vegetable oil or crude oil/hexane micelle. Since the crude sardine oil obtained for the current study was highly viscous, the flux obtained with these microporous membranes could be very low and insignificant for industrial use. Hence, sardine oil/hexane micelle was used to study the efficiency of membrane process. Crude oil membrane degumming was performed similar to Subramanian and Nakajima (1997). In order to characterise the effectiveness of the process, flux studies with crude sardine oil/hexane mixture were conducted and the results are shown in Figure 3.1.

Of the three membranes tested, PTFE membrane was hydrophobic, while polyamide and PES membranes were hydrophilic in nature. It was noted that hydrophillic polyamide membrane showed very poor flux even at a higher pressure of 3 bar and PES failed to show any flux at all. As crude sardine oil contains higher concentrations of long chain fatty acids and phospholipids due to which it exhibited higher viscosity, a poor or no flux was noted in case of hydrophilic membranes. Thus, dilution with non-polar hexane failed to improve the flux in hydrophilic membranes. Interestingly, it was noted that, as the pressure increases, there was a reduction in flux of hexane-oil micelle in polyamide membrane (Figure. 3.1). This could be due to the membrane compaction resulting in a reduction in the permeability characteristics (Hussain and Al-Saleh, 2014). However, in case of hydrophobic PTFE membranes, it was noted that the flux increased with increase in pressure (Figure 3.1). This reverse trend exhibited by the membranes could be due to the difference in the surface properties of

the respective membranes. Thus novel membrane degumming techniques were found to be ineffective due to the highly viscous nature of oil.

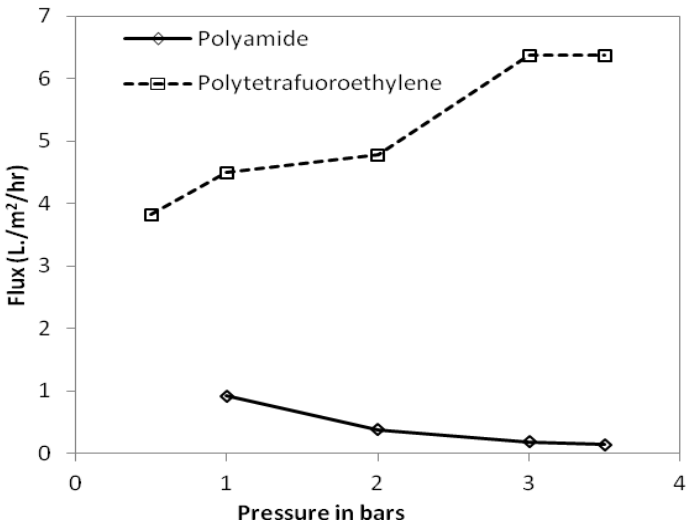



**Figure 3.1 Membrane assisted degumming of crude sardine oil/hexane (30% w/v).**

Additionally, on analysing permeate (hydrophobic membrane) or retentate (hydrophilic membrane), it was identified that there was no significant reduction in the phospholipid content. Addition of hexane to oil, results in micelle formation with phospholipids which was attributed to the amphiphillic nature of phospholipids. Degumming can be achieved by effectively separating the hexane phospholipid-micelle from the oil. In case of polyamide and PES membrane the poor flux resulted in very poor separation. Hence, polyamide and PED membranes failed to show reduction in phospholipids, as the passage of the non-polar hexane/oil micelle is restricted. However, in case of PTFE membrane, rather than selectively separating phospholipid micelles from the oil, the membrane permeated the entire mixture without any significant separation. Thus, it can be concluded that the chosen membranes cannot be applied for degumming application of crude sardine oil/hexane. Hence, degumming with triethanolamine was carried out for further studies.

### 3.2.3. Deacidification

The second stage of edible oil refining is deacidification which involves the removal of free fatty acids from the oil. As free fatty acids are more susceptible to oxidation than their esterified form and also capable of acting as prooxidants, it is necessary to reduce the FFA levels in oil (Kinsella et al. 1978, Miyashita and Takagi 1986).

#### 3.2.3.1. Solvent Extraction

As use of conventional chemical and physical refining leads to loss of neutral oil (Bhosle and Subramanian 2005), use of solvents for specifically extracting fatty acids was considered. The differential solubility of free fatty acid and triglycerides in a particular solvent is the basis of this step (Rodrigues et al. 2007). Usually, solvents like methanol, ethanol, propyl alcohol, amyl alcohols and acetone are generally used with or without dilution with water for this application.

##### 3.2.3.1.1. Effect of solvents on FFA reduction

Based on results by Rodrigues et al. (2007), short chain alcohols like methanol, ethanol, propanol and butanol were chosen along with acetone and acetonitrile for the application of solvent assisted separation of FFA from degummed sardine oil. While short chain alcohols like methyl alcohol, ethyl alcohol and propyl alcohol gave good reduction in FFA, other amyl alcohols and acetone failed to give any phase separation as they dissolved higher amounts of oil along with fatty acid. This is consistent with the results obtained by Fornasero et al. (2013). This could be due to the distribution coefficient of FFA present in our sardine oil with respect to these solvents (Batista et.al. 1999).

The results for the reduction in FFA content of sardine oil treated with various solvents are depicted in Figure 3.2. It can be noted that, as the chain length of alcohol increases there is a reduction in efficiency in FFA reduction by solvent extraction (Figure 3.2). This could be attributed to the lower solubility of oil in alcohols with shorter chain length. Thus, methanol showed the highest reduction of 81.5%, followed by ethanol and propanol at oil solvent ratio of 1:4. This is similar to the findings by

Rodrigues et al. (2007), where higher reduction in FFA with short chain alcohols like methanol and ethanol was noted. However, it was seen that acetonitrile showed that lowest efficiency of 23.3% reduction at the same ratio. Though acetonitrile is a highly polar solvent similar to the alcohols tested, it failed to show same effectiveness in reduction. This could be attributed to the lack of alcohol functional group in acetonitrile. Similar to other solvents, the reduction in FFA increased from 3.9% to 23.3% with increase in acetonitrile concentration. Though acetonitrile was not as efficient as other alcohols, the lack of equilibrium data and information on distribution coefficient of degummed sardine oil with acetonitrile restricted the opportunities to improve the efficiency of the system.

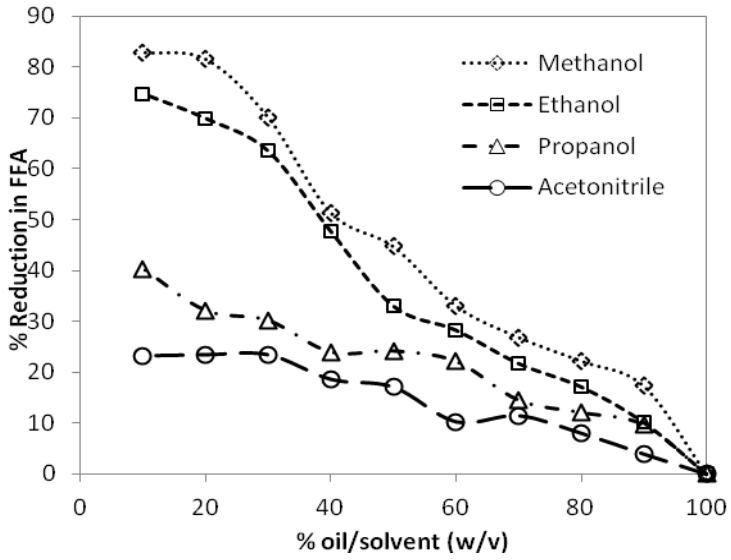



**Figure 3.2 Reduction of free fatty acid content of sardine oil using solvents**

### 3.2.3.1.2. Oil loss during deacifidication using solvents

In order to further analyse the effectiveness of solvents used, the percentage oil loss was also calculated. Since sardine oil is a rich source of n-3 PUFAs, loss of oil during any refining stage could result in the loss of n-3 PUFA content and hence, could adversely affect the quality and cost of the final product. Hence, the efficiency of the solvent extraction process with respect to oil loss was calculated for all solvents at all ratios (Figure 3.3).

It can be seen that solvents which showed higher reduction in FFA also showed higher oil loss. Methanol and ethanol showed a maximum of 23.5% and 16.7% oil loss respectively (Figure 3.3). However, acetonitrile showed lowest percentage of oil loss even when higher levels of solvent was used. This could be due to the inefficient solubilisation of many fatty acids present in sardine oil, due to which lower efficiency of FFA reduction and low oil losses were noted. It was also identified that with increase in solvent content beyond 60%, the oil loss increased exponentially. Hence, 40% oil/solvent (w/v) could be considered as an ideal ratio for deacidification using solvents. In order to avoid oil loss at higher concentrations of solvent, two-stage solvent extraction of FFA can be considered. A report by Kale et al. (1999), suggested such a two-stage solvent deacidification using methanol for rice bran oil to reduce the FFA content from 16.5% to 3.7%. However, due to the low FFA content in the degummed oil used in the current study, the use of second stage can be avoided.

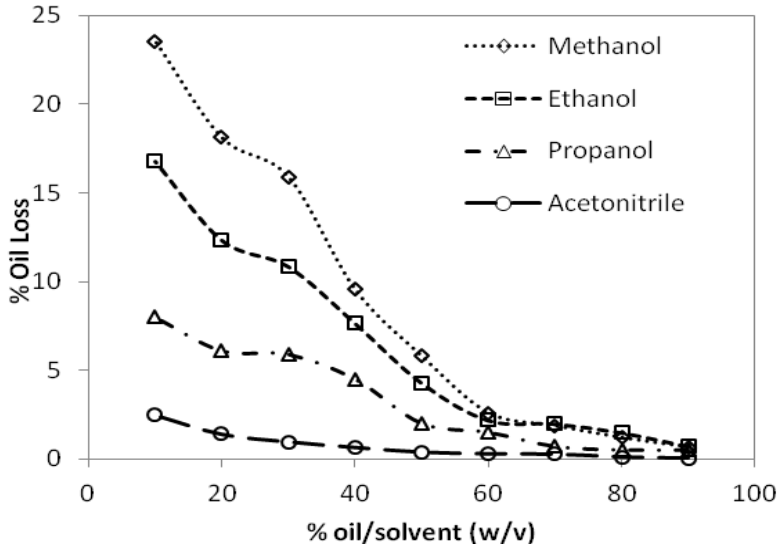



**Figure 3.3 Oil loss during deacidification using various solvents**

Since deacidification is a stage where maximum oil losses are seen at industrial level, care should be taken while designing the refining route. Thus, the present refining strategy employed becomes highly advantageous as 70% of deacidification happens in

the degumming stage itself while another 25% occurs in solvent extraction, making this refining strategy industrially significant.

#### 3.2.3.2. Solvent extraction coupled with membrane separation

Since solvent extraction was found to be effective, the possibility of extraction coupled with membrane filtration was explored. Addition of such a process stage is considered more economical as the energy requirement for the desolventisation is costlier than membrane process (Manjula and Subramanian 2008). Though membrane process for deacidification utilises the same principle of solvent extraction, it poses better advantage over traditional extraction by reduction in time of extraction, minimisation of energy requirement, continuous operation, and ease of scale up. Since sardine oil is considered as an important raw material for n-3 PUFA production, it is wise to use processing techniques that are fast, as n-3 PUFA are known to be unstable.

Initial screening for suitable membranes was done with microporous polyamide (0.45 μm), PES-polyethersulfone (0.45 μm) and PTFE- polyterafluoroethylene (0.45 μm) membranes. Since, industrial use of methanol in not preferred, ethanol was initially used in the application of membranes assisted deacidification of sardine oil. As in degumming application, the efficiency of membrane was tested by studying the permeate flux. Initially, the stability of membranes while handling oil/solvent mixture was studied by analysing the change in flux over a course of time.

##### 3.2.3.2.1. Study of permeate flux

The change in permeate flux of 40% oil/ethanol (w/v) in the three chosen membranes is shown in Figure 3.4. In case of polyamide membrane, a rapid reduction in flux was noted, while PTFE and PES membranes showed relatively lower flux. This reduction in flux over the process could be attributed to the changes in membrane structure with respect to feed (Koltuniewicz et al. 1995). As polyamide and PES are hydrophilic in nature, the passage of polar FFA-solvent mixture across the membranes might have caused swelling, resulting in lower flux (Zwijnenberg et al. 1999). However, due to

the hydrophobic nature of PTFE membranes the flux was almost constant with no significant difference.

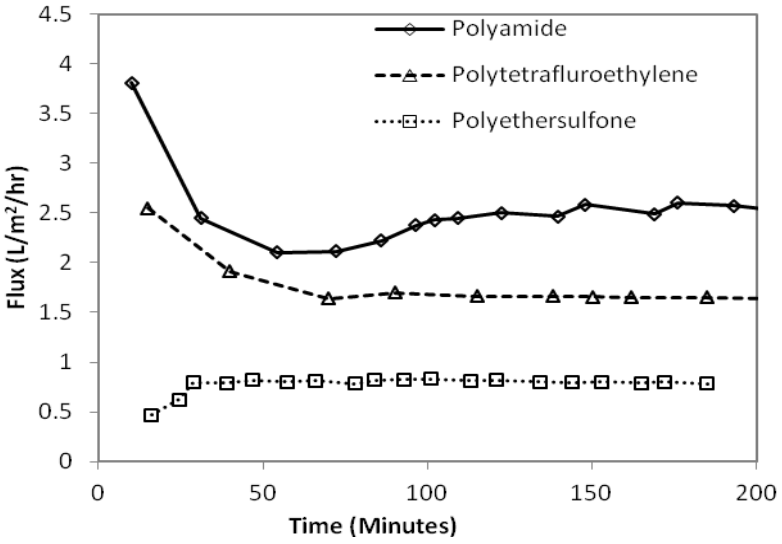



**Figure 3.4 Permeate flux of 40% sardine oil/ ethanol (w/v) mixture at 3.5 bar and 200 rpm with a filtration area of 0.00785 $m^2$**

In order to achieve an ideal membrane process, higher flux is generally preferred. This can be achieved by altering the feed pressure and study its influence on the permeate flux. This study will simultaneously provide an idea on membrane performance at higher pressures. Figure 3.5A shows the permeate flux of oil-ethanol mixture (40% w/v) at different pressure. PTFE membrane was the only hydrophobic membrane that permeated oil, while PES and polyamide were hydrophilic in nature. Both polyamide and PTFE membrane showed a steady increase in flux with increase in pressure. However, only a mild increase in flux was noted at higher pressures in case of PES membrane (Figure 3.5A). This could be attributed to the hydrophilic nature of polyamide membranes. Hence, polyamide facilitates easy permeation of ethanol/oil mixture. Additionally, a higher concentration of polar solvent in oil promotes formation of small micelles that can permeate through membrane pores.

Further, the results from the current study are in accordance to Darcy's law. With increase in trans-membrane pressure, an increase in flux was noted due to the higher

driving force (Tres et al. 2009). Thus increase in the permeate rate with increase in pressure further indicates that no compaction of membrane with this feed (Koike et al. 2002, Hussain and Al-Saleh, 2014).

The effect of solvent content on the flux was also studied and shown in Figure 3.5B. In case of all membranes, a negative effect on the permeate flux was noted when the percentage of oil increased. The highest reduction of flux was noted in polyamide membranes when the oil concentration increased (Figure 3.5B). At low oil concentration, ethanol flux is high due to the hydrophilic nature of the membrane, providing effective separation of oil and ethanol. However, the hydrophobicity of the feed increased with increase in oil content in the mixture and resulted in membrane fouling.

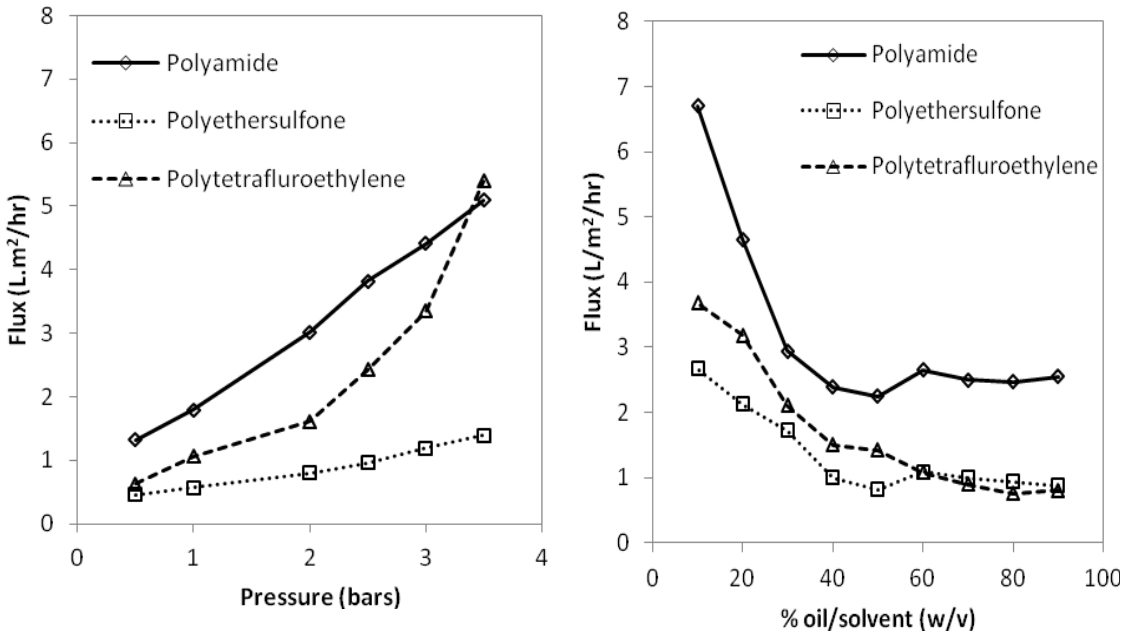



**Figure 3.5 Permeate flux of oil/ethanol mixture through different membranes (A) Effect of pressure at 40% oil/ethanol (w/v) (B) Effect of solvent percentage at 3.5 bars.**

On the other hand, PES and PTFE showed a steady decrease in the flux with increase in oil percentage in the mixture (Figure 3.5B). This reduction in flux at increasing oil

percentage could be due to the compaction of oil particles on the membrane surface causing reduction in the pore size of membrane (Tres et al. 2009). Further, increase in the percentage of oil in the mixture causes an increase in the viscosity. Viscosity affects the convective flow inversely, which is perhaps the reason for the decline in the flux across the membranes with the increase in oil concentration. A similar trend was observed by Koike et al. (2002) while studying the performance of various polymeric membranes for sunflower oil mixture with solvents like ethanol and hexane.

#### 3.2.3.2.2. Study of FFA reduction

While analysing the effects of pressure on FFA reduction (Figure 3.6), it was found that increase in pressure failed to show any significant reduction in FFA reduction. This could be due to the improper separation of oil and ethanol phases.

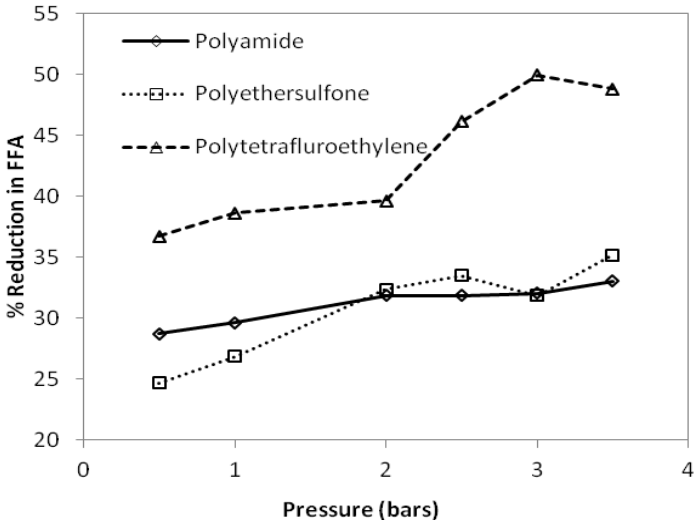



**Figure 3.6 Effect of pressure on FFA reduction during membrane processing of 40% oil/ethanol (w/v) mixture**

It was seen that oil phase also permeated along with ethanol (Visual Observation), as the selectivity of polyamide and PES membranes towards ethanol was poor. However, in case of PES membrane increase in pressure showed a moderate increase in FFA reduction from 24.6% to 33% and an increase from 36.7% to 49% in PTFE (Figure

3.6). This higher increase in reduction in PTFE membrane could be due to the hindered transport of FFA molecules than triglycerides due to the nature of membrane (Tam and Tremblay 1991). However, the percentage of oil loss has to be determined with respect to other variables in order to find the significance of this membrane assisted deacidification industrial level. Hence, the effect of oil concentration in the mixture on oil loss was determined to study the industrial relevance of current technology.

#### 3.2.3.2.3. Oil loss

The percentage of oil loss was highest in PES membranes, followed by polyamide and PTFE membrane (Figure 3.7). As the percentage of oil in the mixture reduced to 10% (w/v), the oil loss increased to 65%, 45.6% and 30% in PES, polyamide and PTFE membrane respectively (Figure 3.7). The better performance of PTFE membrane was due to the hydrophobic nature of membrane surface, which results in the efficient passage of oil molecules while hindering ethanol. Hence, a clear and complete separation of oil/ethanol mixture was obtained. This is consistent to the results obtained by Rao et al. (2013) when deacidification of coconut oil was performed by using polymeric membranes.

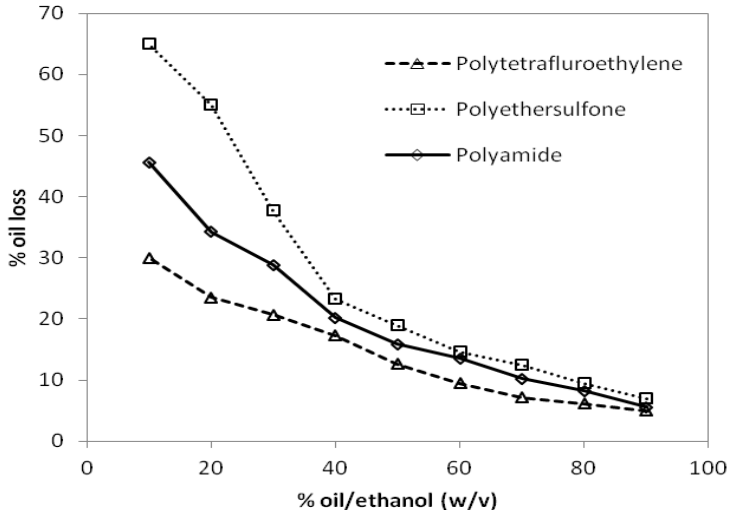



**Figure 3.7 Effect of oil/ethanol ratio on the percentage oil loss during membrane process at 3.5 bar and 200 rpm.**

In addition to oil loss and FFA reduction, the efficiency of membrane process was further calculated by analysing the percentage of solvent traces after separation through membrane process. The overall performance of the three membranes was analysed by calculating the FFA reduction, oil loss and solvent traces in the membrane refined oil and the results are tabulated in Table. 3.4.

**Table 3.4 Characteristics of membrane assisted deacidified oil**

| **Parameters analyzed** | **Polyamide** | | **Polyethersulfone** | | **Polytetrafluroethylene** | |
|---|---|---|---|---|---|---|
| | **Control*** | **Test[a*]** | **Control** | **Test[a*]** | **Control** | **Test[b*]** |
| % Reduction in FFA | NA | 32.16 | NA | 31.67 | NA | 51.96 |
| % Oil loss | NA | 8.20 | NA | 7.56 | NA | 1.45 |
| Amount of Solvent (%) | NIL | 6.66 | NIL | 8.92 | NIL | 0.53 |

Control – plain degummed oil passed through the membrane; Test[a*]- deacidified oil as retentate; Test[b*] - deacidified oil as permeate.

It was noted that the amount of trace solvent for PES membrane was highest followed by polyamide membrane. However, a complete separation of oil and solvent was seen in PTFE membranes with only 0.5% of solvent traces in oil after separation (Table 3.4). This indicates the higher selectivity of PTFE membranes to oil which could be attributed to its hydrophobic nature. This is similar to the results obtained by Firman et al. (2013), when PTFE membranes were used to separate soybean oil from hexane-oil micelle with higher selectivity. However, polyamide and PES membranes failed to show the same selectivity as the separation through these membranes was based on the particle size. Hence, it is highly possible that oil permeates along with ethanol, thus leading to increased oil losses. Thus, the screening of membranes for the application of sardine oil deacidification indicated the PTFE membranes consistently showed higher performance. It was also seen that properties like flux, FFA reduction, oil loss and also solvent traces in oil after membrane process was strongly influenced by the pressure and percentage of solvent in the feed.

### 3.2.3.2.4. Study of PTFE membrane performance for oil/acetonitrile mixture

From the study on the performance of three different membranes, it was noted that PTFE membrane performed with higher efficiency in all aspects. Similarly, while analysing the choice of solvent for FFA reduction, it was identified that methanol and ethanol showed highest reduction while acetonitrile showed lowest reduction. However, the percentage of oil loss was found to be minimal in case of acetonitrile. Hence, the possibility of using acetonitrile oil mixture for deacidification of sardine oil was also explored.

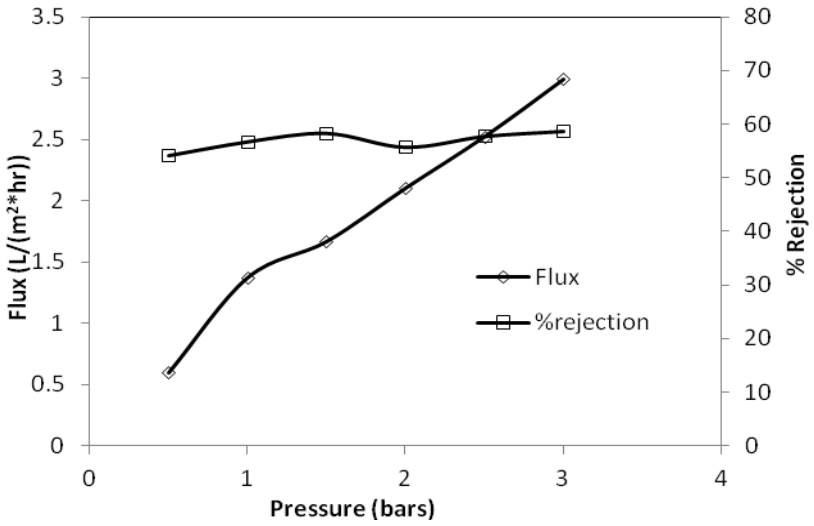



**Figure 3.8 Effect of pressure on flux and percentage rejection of FFA from 30% (w/v) oil/acetonitrile miscella in PTFE membrane**

The permeate flux and percentage of FFA reduction of sardine oil/acetonitrile mixture was studied at various pressures and depicted in Figure 3.8. It can be seen that the flux was found to increase from 0.598 to 2.99 L/$m^2$ hr with an increase in pressure from 0.5 to 3 bars (Figure 3.8). In case of FFA reduction, increase in pressure did not show significant difference in free fatty acid removal and remained consistent with approximately 60% reduction (Figure 3.8). Thus it can be concluded that, on using oil with lesser FFA it is sufficient to use acetonitrile to achieve the desired final FFA content in oil. However, when the FFA content is high, use of ethanol or methanol can be preferred in a single stage or double stage process. Though few reports are

available on the use of solvents like methanol and ethyl alcohol for deacidification using membrane process, this is the first report to have successfully used acetonitrile as an extractant for removal of FFA to desired levels.

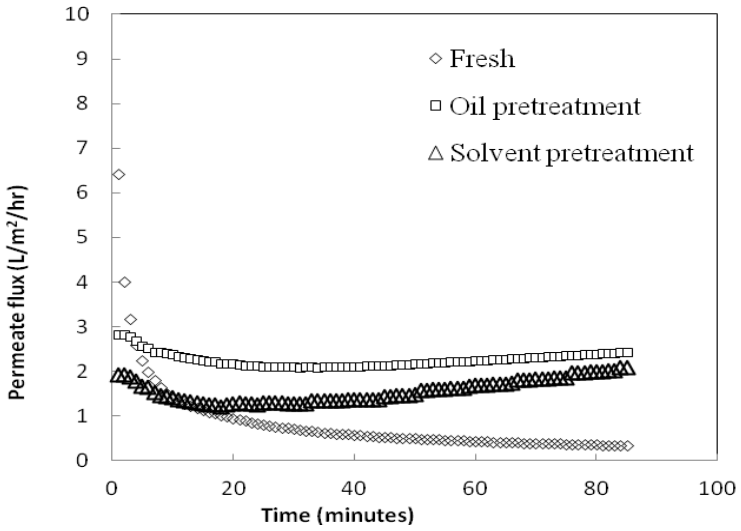



**Figure 3.9 Effect of pretreatment of membrane on permeate flux of polytetrafluoroethylene (PTFE) membranes**

Considering the higher efficiency of PTFE membrane process for the solvent assisted deacidification of sardine oil, further studies were performed to improve the process. Most of the polymeric membranes are dissolved in solvents as a part of their casting process. Consecutively, they either swell or dissolve in the respective solvent leading to changes in flux and separation efficiency. Hence, many studies on the application of membrane process for refining of edible oil have studied the effect of conditioning in the improvement in process (Koris and Vatai 2002, Saravanan et al. 2006, Ribeiro et al. 2008, Rao et al. 2013). In order to understand the effect of oil/solvent mixture on the flux of PTFE membrane, the membranes were kept for conditioning overnight by soaking in oil and acetonitrile, respectively. It was found that the oil pretreated membrane had a higher and consistent flux of 2.5 kg/$m^2$ hr (Figure 3.9). However, in case of solvent pretreated membrane, the initial reduction in flux was noted after which it steadily increased (Figure 3.9). The pre-treatment of hydrophobic PTFE membrane with hydrophilic solvent like acetonitrile contributes to minute changes in

the membrane structure (Koltuniewicz et al. 1995), leading to the initial reduction in the flux after which it steadily increases. Though it is considered that hydrophilic membranes has low tendency for fouling potential than hydrophobic ones, the results have been inconsistent (Zhang et al. 2015). Similar to our results, Zhang et al. (2015) was also unable to establish a clear understanding on the effect of membrane hydrophobicity on permeation rate when a wide range of rough-surfaced membranes were treated with oily sludge. Hence, it becomes difficult to understand the role of membrane hydrophobicity in oil adhesion and membrane fouling and extensive studies has to be conducted to get a brief idea on the effect of oil-solvent mixture on PTFE membranes.

Since, triethanolamine treated oil was used for deacidification stage, the FFA levels reduced to desired value, even with low performing solvents like acetonitrile. Further, the solvent trace in the membranes assisted deacidification of oil/acetonitrile mixture was very low. Membrane conditioning failed to significantly improve the flux. Hence, further preparations of refined oil were done by simultaneous degumming and deacidification using triethanolamine. Additional deacidification was carried out using acetonitrile in PTFE membrane at 40% oil/solvent (w/v) and pressure 3 bars.

### 3.2.4. Bleaching

The main reason for bleaching step is to remove minor impurities and metal ions along with the remaining phospholipids and FFA. The overall quality of the product improves at this stage of refining. When bleaching is carried out with a bleaching agent like activated carbon, these impurities get adsorbed on the surface of the adsorbent at higher temperature. The efficiency of bleaching process is generally characterised by colour reduction and metal ions removal. Hence, it becomes critical to choose an efficient bleaching agent. The use of activated charcoal as bleaching agent for many vegetable oils like soybean oil, rice bran oil, cotton seed oil and rapeseed oil have been reported in literature (Srikaeo and Pradit 2011, Moore and Yeates 1979). Hence, activated charcoal was chosen as bleaching agent in the current study and the process conditions were adapted from Gracia-Moreno et al. (2013).

The degummed and deacidified sardine oil was bleached by treating with 5% (w/w) activated charcoal at 80°C under vacuum for 30 minutes. Since an optimised condition for sardine oil was employed from literature, further optimisation of the process variables was not done, and the overall efficiency of the process is tabulated in Table 3.5. It can be seen that the overall quality of the sardine oil was improved on bleaching. The phospholipid content further reduced to 22 µg/g. As reports suggest preference of some bleaching materials to cationic and polar molecules (Zschau 2001, Silva et al. 2013), significant reduction in phospholipids was seen at this stage.

As FFA content is known to increase the oxidative instability in oil, the efficiency of bleaching was also studied based on FFA reduction. The FFA content of degummed and deacidified sardine oil was 0.6%, which is lower than the acceptable level of FFA content in fish oil (1.8%) (Sathivel et al. 2003). No significant reduction in FFA was noted during bleaching stage (Table 3.5). This could be due to the significant reduction in FFA content in the deacidification stage itself. Since, the FFA content reduced to acceptable levels, further optimisation of bleaching conditions was not performed. The possible reason for lack of reduction in FFA on bleaching could be due to the reduced mass transfer rate of FFA into bleaching agent in the high viscous fish oil (Garcia-Moreno et al. 2013). The lack of increase in FFA during the bleaching stage also indicates that the bleaching process has not resulted in the hydrolysis of triglycerides (Lin and Lin 2005). Additionally, the solvent and moisture traces in the sample were also effectively reduced. Since the bleaching process occurs in vacuum environment, efficient removal of trace water can be noticed during this stage.

**Table 3.5 Physical and chemical properties of Sardine Oil during the course of refining**

| **Properties** | **Crude** | **Degummed** | **Solvent Extracted** | **Membrane Treated** | **Bleaching** |
|---|---|---|---|---|---|
| Phospholipid (µg/g) | 303.03±5.02 | 37.91±0.31 | 38.33±0.82 | 35.01±0. 39 | 22.38±0.11 |
| Acid value | 7.46±0.01 | 2.40±0.24 | 1.77±0.34 | 1.19±0.23 | 0.79±0.01 |

| Properties | Crude | Degummed | Solvent Extracted | Membrane Treated | Bleaching |
|---|---|---|---|---|---|
| % Free fatty acid (w/w) | 3.75±0.01 | 1.21±0.24 | 0.89±0.34 | 0.60±0.23 | 0.4±0.01 |
| Iodine value | 157.70±2.05 | 154.56±0.12 | 156.88±2.98 | 152.96±2.52 | 142.87±1.5 |
| Peroxide value | 7.35±0.01 | 6.42±1.56 | 9.44±0.81 | 9.30±0.42 | 8.80±0.89 |
| %Solvent/ Moisture traces (w/w) | 0.23±0.02 | 0.29±0.08 | 5.9±0.74 | 0.52±0.02 | 0.11±0.45 |
| Density ($g/cm^3$) | 0.9181±0.07 | 0.9206±0.11 | 0.9185±0.04 | 0.9177±0.12 | 0.9165±0.03 |
| Viscosity (mPa.s) | 41.03±.02 | 39.88±0.23 | 38.35±0.12 | 36.18±0.12 | 34.56±.04 |
| Iron (ppm) | 0.23±0.25 | 0.01±0.08 | 0.01±0.03 | 0.01±0.08 | Nil |
| Copper (ppm) | 0.06±0.1 | 0.004 ± 0.2 | 0.004 ± 0.0 | 0.003 ± 0.2 | Nil |
| Zinc | 0.03±0.5 | 0.002±0.1 | 0.001±0.1 | 0.001±0.2 | Nil |
| Nickel | 0.09±0.07 | 0.05±0.03 | 0.06±0.06 | 0.05±0.04 | 0.01±0.05 |

Values are depicted as mean of three values with standard deviation (n=3)

The overall efficiency of the refining process is shown in Table 3.5. Significant reduction in major impurities like phospholipid and FFA was seen throughout the process. In addition to the major impurities which were targeted, the minor impurities like metal ions and trace water were also found to be effectively removed. Additionally, it was also noted that the density and viscosity of the oil reduced significantly (Table 3.5), indicating the efficiency of the process. The aesthetic appearance of sardine oil was also found to improve over the course of refining (Figure 3.10). Visual observation of the colour of refined oil was found to improve drastically during bleaching stage (Figure 3.10). Since, low viscous oil is generally preferred for obvious commercial applications, this process can be considered for

industrial application as well. In terms of oxidation products, it was noted that there was a slight increase in the peroxide value from 7.35 to 8.80. This could be due to various environmental conditions which influence the autooxidation in sardine oil. During the process of refining, sardine oil comes in contact with atmospheric air and light. Even though care was taken to conduct the refining process in dark, contact with atmospheric air significantly increases the oxidation process (Vaisali et al 2016). Though bleaching process targets the removal of oxidation process, the current results suggest that exposure of oil to higher temperatures increased the oxidation in sardine oil. This is similar to the results obtained by Gracia-Moreno et al. (2013), when the bleaching conditions for sardine oil refining were optimised.

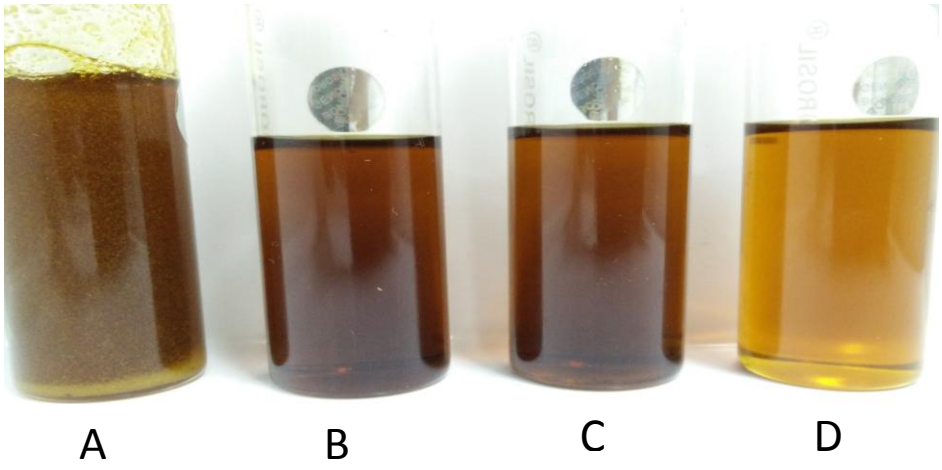



**Figure 3.10 Sardine oil during course of refining (A) Crude (B) Degummed (C) Deacidified (D) Bleached**

Though literature suggests long contact time and increased temperature for maximum bleaching efficiency, the presence of n-3 PUFA compounds reduces the possibilities of using high temperature. Such a result was noted in the current study when the iodine value showed a drastic reduction from 152 to 142 during bleaching. This could be due to the cyclisation and polymerisation of long chain n-3 PUFA leading to the loss of these compounds as polymers or dimmers as degradation products (Fournier et al. 2007). Hence, in order to confirm the effect of refining process on the n-3 PUFA content, the fatty acid composition of the oil during each stage of refining was studied

and tabulated in Table. 3.6. The fatty acids were identified by comparison with a standard mixture of fatty acid methyl esters (FAME) from sigma (Appendix II).

Analysis of fatty acid composition of sardine oil indicated a wide fatty acid profile (Figure 3.11). The major fatty acids in oil indicated that palmitic acid was the predominant fatty acid, followed by myristic acid. In addition to these major fatty acids, stearic acid, oleic acid and lenolenic acid were found in minor quantities in sardine oil. EPA and DHA were found in reasonable quantities with a total of 13.3% (w/w) in sardine oil (Table 3.6).

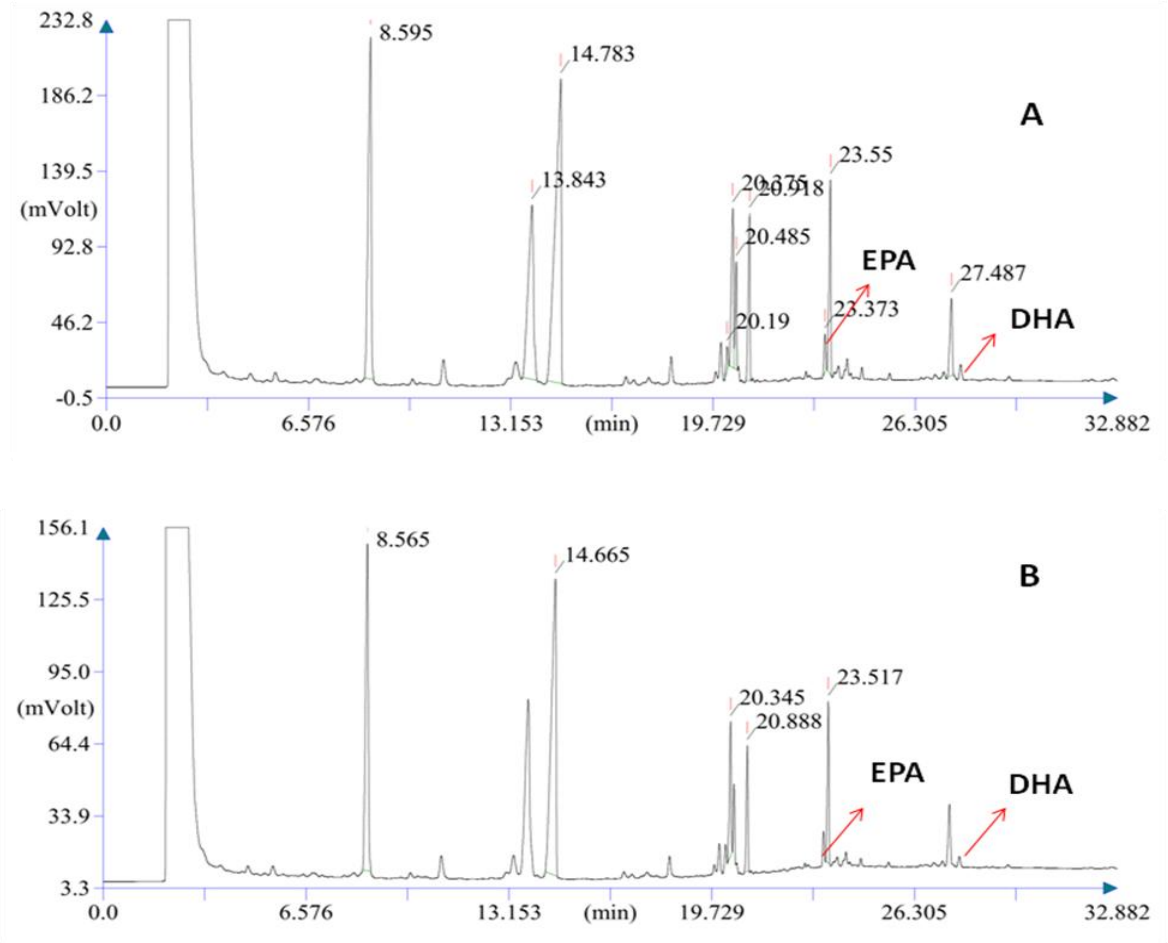



**Figure 3.11 GC Chromatogram of the fatty acid composition of sardine oil (A) Crude (B) Refined**

A significant reduction in all major fatty acids was noted during the degumming stage. As simultaneous reduction in phospholipid and FFA were noted in degumming, loss of many fatty acids was noted at this stage. Thus, it is possible that the palmitic and stearic acid were present in the form of free fatty acids instead as glyceride derivative. Additionally, it was noted that a there was a significant reduction in the EPA content in sardine oil after degumming (Table 3.6). This could be due to the loss of oil noted during this stage. However, it was noted that DHA content was not reduced. During deacidification by either solvent extraction or membranes assisted deacidification, the EPA and DHA content in the oil was found to enhance. This could be due to the removal of free fatty acids, as a result of which the relative weight percent of EPA and DHA in oil was found to increase.

**Table 3.6 Effect of refining process on the fatty acid composition of sardine oil**

| **Type of oil** | **Relative Weight % (w/w)** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **C 14:0** | **C 16:0** | **C 18:0** | **C 18:1** | **C18:2** | **C20:5 (n-3)** | **C 22:6 (n-3)** |
| Crude | 31.860 | 36.440 | 1.106 | 6.083 | 6.006 | 9.363 | 4.092 |
| Degummed | 25.097 | 33.706 | 1.044 | 5.741 | 5.269 | 8.789 | 4.067 |
| Solvent extracted | 20.734 | 33.781 | 1.985 | 6.810 | 6.565 | 8.908 | 4.713 |
| Membrane assisted | 19.598 | 31.069 | 1.012 | 6.805 | 5.626 | 9.668 | 4.958 |
| Bleached | 20.418 | 32.225 | 1.250 | 5.355 | 4.086 | 8.878 | 4.280 |

### 3.3. Summary and conclusions

Edible oil refinery is a major industry and the difference in the crude oil characteristics during various seasons is a major challenge that every refinery faces. Hence, development of a fail-proof technology that will best suit the oil is in demand. The method developed in the current study is one such technology that poses many advantages over traditional methods.

- A novel degumming strategy involving the use of 3% (w/w) ethanolamines resulted in 87% reduction in the phospholipid content with a simultaneous reduction of FFA to 1.21% from 3.75%. The lack of phospholipid reduction by conventional process indicates the complex nature of oil used.
- Further reduction in FFA content was achieved by using solvent extraction. Short chain alcohols showed higher reductions and higher loss of neutral oil, while acetonitrile showed the lower FFA reduction with negligible oil loss.
- Membrane assisted deacidification was found to be better suited for removal of FFA from sardine oil.
- Use of membranes for solvent assisted deacidification indicated that hydrophobic PTFE membrane was best suited for deacidification.
- Bleaching with activated charcoal, resulted in significant reduction in metal ion content, moisture content and phospholipid content. The overall quality of the oil was also found to improve during bleaching stage.
- In case of n-3 PUFA content, a mild reduction in the EPA and DHA quantity was noted during the course of refining.

# CHAPTER 4

# IMPROVING OXIDATIVE STABILITY OF INDIAN SARDINE OIL USING NATURAL ANTIOXIDANTS

The major challenge encountered in the production, storage and distribution of n-3 PUFA rich sardine oil is its poor storage stability. Though refining techniques reduce the oxidation processes considerably, the autoxidation rate of fatty acids increases with degree of unsaturation (Cozzolino et al. 2005, Wijesundera, 2008). To further retard oxidation processes caused by the high degree of unsaturation, use of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), during the processing and storage of oil has been proposed. Although synthetic antioxidants are used widely, their safety has been questioned (Wang et al. 2011), stimulating the exploitation of natural products with antioxidant properties. Recent attempts on the effective use of few natural antioxidants to improve the oxidative stability of fish oil has provided further scope for exploring antioxidants from different natural sources (DeLeonardis and Macciola 2003, Wang et al. 2011, Maqsood and Benjakul 2010, Kindleyside et al. 2012, Pazhouhanmehr et al. 2016). The choice of antioxidants to stabilise fish oils is restricted to few substances, with tocopherols being the most frequently used due to the complex nature of fish oil (Yanishlieva, and Marinova, 2001). However, they do not provide effective protection, especially when the fish oils contain trace metals.

Flavonoids and phenolic acids are the most persistent group of phenolic compounds in plants. Researchers continue to study the chemical structures and effectiveness of such antioxidants from variable sources which may also have anticarcinogenic activity along with antioxidant characters. While phenolic acids are known for their antioxidant mechanism by trapping free radicals, flavonoids can scavenge free radicals as well as chelate metal ions (Brewer 2011). The extent of antioxidant power is greatly influenced by the structure of an antioxidant. Hence, structure-activity relationships could be used to predict the effectiveness of an antioxidant. As the activity of antioxidants in lipids is influenced by numerous factors including nature of lipid, hydrophilic-lipophilic balance of the antioxidant and interfacial interactions (Chang et al. 2003), antioxidants that are effective in one system may not be suitable for other systems. Hence, it is necessary to make a comparison of different phenolics to find the best possible antioxidant for a specific system.

Since oil oxidation occurs in different routes and catalysed by different initiators like peroxides, trace metals, photosensitizers etc. (Shahidi and Zhong 2010), it is necessary to analyse the *in vitro* antioxidant ability to act as radical scavenger, metal chelator or as a reducing agent. This chapter deals with the systematic comparison of the antioxidant capacities of three derivatives of hydroxycinnamic acids, three hydroxybenzoic acids and three different flavonoids by determining the radical scavenging ability using DPPH radical, reducing power and metal chelating activity. Further, sardine oil oxidation in the presence of added antioxidants has been analysed by measuring primary and secondary oxidation products based on peroxide value, p-anisidine value, conjugated diene analysis and TBARS value. Finally, an attempt has been made to compare the effectiveness of an antioxidant in sardine oil system with respect to their ability to act as radical scavengers, metal chelators and reducing agents.

Though search of natural antioxidants might provide effective compounds for improving stability in oil, it might not be sufficient due to the complex nature of oils. Bulk oil is a heterogenous system containing many minor components (Vaisali et al. 2015). The presence of minor concentrations of trace metals and trace water is inevitable. These compounds, along with other surface active compounds in oil, form association colloids. These physical structures act as site of oxidation in bulk oil (Chaiyasit et al. 2007). Hence, studying the role of such compounds for PUFA rich fish oil provides better understanding of the complex oxidation process in such oils. In order to effectively design an antioxidant strategy that acts positively at all conditions, the present investigation was also carried out to explore the role of iron and trace water on sardine oil oxidation. Further, the effectiveness of the antioxidants to act positively on sardine oil oxidation in the presence of iron and moisture was also determined.

## 4.1. MATERIALS AND METHODS

Crude sardine oil with no synthetic antioxidants was purchased from a local seafood industry and was refined by a refining method described in chapter 3. The composition and other physicochemical properties were determined for refined oil and

stored at -20°C. Ferulic acid, Caffeic acid, Sinapic acid, Gentisic acid (2,5-dihydrobenzoic acid), Protochatechuic acid (3,4-dihydrobenzoic acid), Vanillic acid, Catechin, Quercetin, Rutin, 2,2-diphenyl-1-picryl hydrazyl (DPPH), ferrozine, 2,4,6–tripyridyl-*s*-triazine (TPTZ), 1,1,3,3–tetramethoxypropane (malondialdehyde) and p-anisidine were purchased from Sigma-Aldrich (India). Sodium thiosulphate, potassium iodide, starch, thiobarbituric acid, trichloroacetic acid, $FeCl_3.6H_2O$, $FeCl_2$ were of analytical grade and purchased from Loba Chemie. All the solvents used were of analytical grade and was purchased from Merck India.

### 4.1.1. Determination of antioxidant activities

#### 4.1.1.1. DPPH radical scavenging activity

The DPPH assay was performed in triplicate according to Maqsood and Benjakul (2010) with slight modifications. Antioxidant sample (2 ml), with a concentration range of 5-35 µM in ethanol was added to 2ml of 0.1mM DPPH in ethanol. The mixture was mixed thoroughly and allowed to stand for 30 min. The absorbance of the resulting solution was measured at 517 nm using double beam UV/Visible spectrophotometer (Lab India). The measurement was done using ethanol as blank and DPPH without antioxidants as control. The DPPH scavenging activity was expressed in percentage as

$$\%DPPH\ scavenging\ activity = \left[\frac{Absorbance_{control} - Absorbance_{test}}{Absorbance_{control}}\right] * 100 \quad (4.1)$$

#### 4.1.1.2. Ferric reducing antioxidant power (FRAP)

FRAP was determined as described by Benzie and Strain (1996) with few modifications. Stock solutions of 0.3M acetate buffer (pH 3.6), 10mM 2,4,6-tripyridyl-*s*-triazine (TPTZ ) in 40mM HCl and 20mM $FeCl_3.6H_2O$ were prepared. The working solution was prepared freshly by mixing 25ml of acetate buffer, 2.5 ml TPTZ solution and 2.5 ml $FeCl_3$ solution. The resulting FRAP mixture was then incubated at 37°C for 30 min in a water bath. 0.5ml of antioxidant compounds of varying concentration from 2.5 µM to 75µM in methanol was mixed with 3.5ml of

FRAP reagent using vortex mixer and the mixture was allowed to stand in dark for 20 min. The absorbance was measured using spectrophotometer at 593nm. The increase in ferric reducing power was corresponded to the increase in absorbance value.

#### 4.1.1.3. Metal chelating activity

The metal chelating activity of antioxidants was measured according to Maqsood and Benjakul (2010) with slight modifications. 3500 µl of antioxidant in methanol at various levels between 5µM to 35µM were mixed with 40 µl of 2mM $FeCl_2$ and 80 µl of 5mM ferrozine using vortex mixer. The mixture was mixed well and allowed to stand for 20 minutes at room temperature. A control was prepared in the same manner without antioxidant addition. The absorbance was then read at 562 nm. The chelating activity was calculated in percentage as follows

$$\%\ Metal\ chelating\ activity = \left[\frac{Absorbance_{control} - Absorbance_{test}}{Absorbance_{control}}\right] * 100 \qquad (4.2)$$

### 4.1.2. Oxidative stability experiments

For studying the oxidative stability of sardine oil, natural antioxidants having highest capacity were chosen. A known quantity of the chosen antioxidants was taken in vials and dissolved in a small quantity of methanol. The solvent was then allowed to evaporate under nitrogen, after which calculated quantities of sardine oil was added to get a final concentration of 100ppm antioxidants in oil. As antioxidants acts as prooxidants if added in higher quantity, the concentration of antioxidants is a critical parameter. The concentration was chosen based on the reports of Hopia et al. (1996) and Maqsood and Benjakul (2010). The samples were homogenised thoroughly for 15 minutes. Sample were then stored in vials in dark at 37 °C and in contact with atmospheric air. Each oxidation experiment was done in triplicates. Samples were withdrawn every alternate day for the determination of peroxide value, anisidine value, conjugated diene analysis and thiobarbituric acid-reactive substances (TBARS) value.

### 4.1.3. Oxidation in the presence of minor components

In order to understand the effect of antioxidants when the oxidation is influenced by iron, ferrous chloride was added to sardine oil and antioxidant mixture at a level of 10 ppm. The concentration of ferrous chloride was chosen based on the reports by Kapchie et al. (2013). Known quantities of ferrous chloride in solvent were taken in glass vials along with calculated quantities of the chosen antioxidants so that their final concentration in oil is 10 ppm and 100 ppm respectively. Refined sardine oil was then added to these vials and were homogenised thoroughly for 15 minutes. Sample were then stored in vials in dark at 37 °C and in contact with atmospheric air. Each oxidation experiment was done in triplicates. Samples were withdrawn every alternate day for the determination of peroxide value, p-anisidine value, conjugated diene analysis and thiobarbituric acid-reactive substances (TBARS) value.

Similarly, in order to understand the influence of trace water on the antioxidant effects on sardine oil, the existing water content of the oil was adjusted to a constant value. Since refined sardine oil was found to possess a water level of 0.112 %, the water content of the system was adjusted to a constant value of 0.16% by addition of distilled water. It has been reported that 0.02 – 0.1% moisture could be found in bulk refined vegetable oils (Park et al. 2014). However, the trace water levels in fish oils were usually in a relatively higher range from 0.05 to 0.2 % (Bimbo et al. 1998). Hence, the trace water content in the present study was adjusted to a value that fits in this range. The oxidation experiments were then carried out similar to the previous section with 100 ppm of chosen antioxidants.

### 4.1.4. Monitoring lipid oxidation

Sardine oil samples were withdrawn periodically from the storage vials and analysed for the extent of oxidation by measuring the products of oxidation.

#### 4.1.4.1. Peroxide value estimation

Measurement of peroxide value was made in accordance with AOCS (2009). Peroxide value was calculated as follows;

$$Peroxide\ value = \frac{(sample\ titre - blank\ titre) * Molarity\ of\ thisulfate * 1000}{mass\ of\ test\ in\ g}$$

#### 4.1.4.2. p-Anisidine value (pAV) estimation

p-anisidine value was determined according to AOCS (2009) with slight modifications. A known quantity of oil sample was weighed in test tube and dissolved in isoocatane and the absorbance was measured at 350 nm against a solvent blank. 3ml of the above mixture was mixed with 0.5ml of 0.25% p-anisidine in acetic acid (w/v) and after 10 minutes incubation the mixture was analysed at 350nm. The p-anisidine values were calculated as follows

$$p - Anisidine\ value = 25 * (1.2A_s - A_b)/m$$

$A_s$ – Absorbance of fat solution after reaction with p-anisidine, $A_b$ – Absorbance of fat solution, m – mass of sardine oil (g)

#### 4.1.4.3. Conjugated diene (CD) value estimation

The formation of conjugated dienes due to oxidation of fats or oils gives rise to an absorption peak at 230-235nm. The CD value in bulk sardine oil was determined according to (Hopia et al., 1996) by diluting the weighed oil sample with isooctane. The absorbance of the lipid solution was then measured at 234nm. The increase in absorbance was corresponded to increase in oxidation.

#### 4.1.4.4. Thiobarbituric acid reactive substances (TBARS) value estimation

Thiobarbituric acid reactive substances (TBARS) assay is used to monitor secondary oxidation products and was performed as described by Buege and Aust (1978) with slight modifications. The test involves the reaction between malondialdehyde (MAD) from oil oxidation and thiobarbituric acid to give a red colour complex. Oil sample (10 mg) was mixed with 3.5 ml of reagent containing 0.375% thiobarbituric acid, 15% trichloroacetic acid in 0.25N HCl. The mixture was heated in boiling water bath until slight pink colour developed. The samples were then cooled under running water and

centrifuged for 10 minutes. The absorbance of the supernatant was measured at 532 nm. A standard graph was constructed using 1,1,3,3 – tetramethoxypropane (malondialdehyde; MAD) and is shown in Appendix VI.

### 4.1.5. Statistical Analysis

All experiments were triplicated and the analysis was done in duplicates. The data was compared by one way analysis of variance (ANOVA) using MiniTab17 software. Significance was declared for $p < 0.05$.

## 4.2. RESULTS AND DISCUSSION

Based on the available literature, antioxidants were chosen in such a way that they belonged to different categories, viz. hydroxycinnamic acids, hydroxybenzoic acids and flavonoids, and further showed slight variations in the functional group substitution (Figure 4.1 A, B and C).

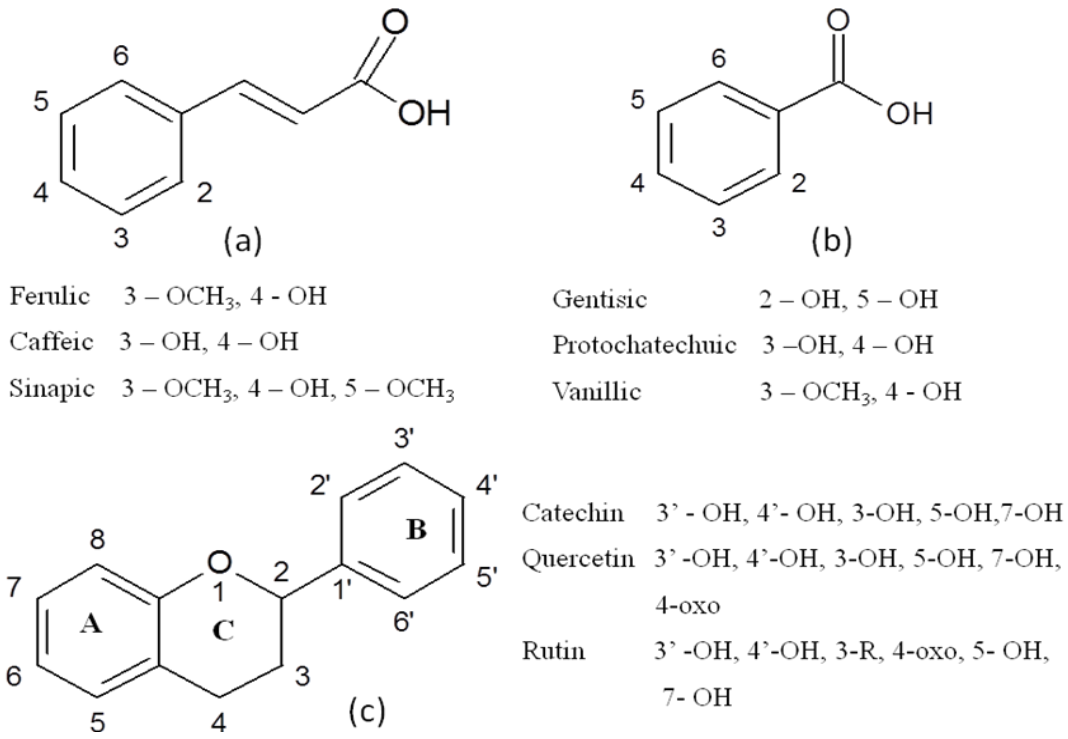



**Figure 4.1 Structure of phenolic antioxidants (A) hydroxycinnamic acid derivatives (B) hydroxy benzoic acids and (C) flavonoids**

### 4.2.1. DPPH scavenging activity

The radical scavenging ability against DPPH radical of a wide range of natural antioxidants (ferulic acid, caffeic acid, sinapic acid, gentisic acid, protochatechuic acid, vanillic acid, catechin, quercetin and rutin) were analysed and presented in Figure 4.2A, B and C. The descending order of the DPPH scavenging activity of the chosen antioxidants was as follows: quercetin = gentisic acid > catechin > rutin > caffeic acid > protochatechuic acid > sinapic acid > ferulic acid > vanillic acid.

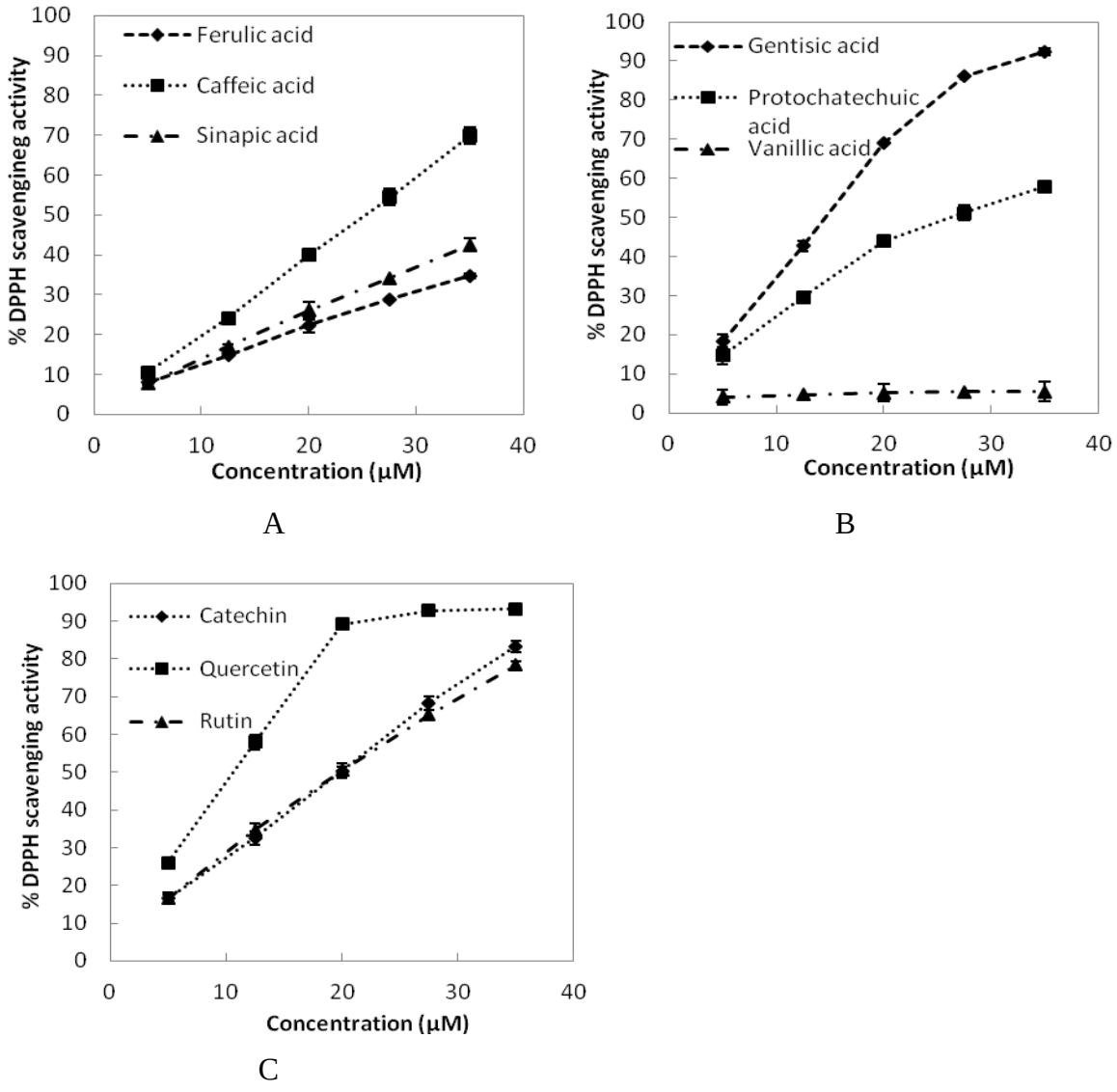



**Figure 4.2 DPPH scavenging activity of phenolic compounds at various levels (A) Hydroxycinnamic acid (B) Hydroxybenzoic acids (C) Flavonoids**

Of the three hydroxycinnamic acids, caffeic acid showed a high radical scavenging activity of 70% (Figure 4.2A) against DPPH radical. The bulky carbon side chain of caffeic acid is responsible for the high activity by stabilizing the resultant phenoxy radical (Choe and Min 2009). Both ferulic acid and sinapic acid showed similar scavenging activity (Figure 4.2A) which was due to the methoxy substitution adjacent to the hydroxyl group in the phenolic ring structure (Figure 4.1A). A methoxy substituent at the ortho and meta position decreased the O-H bond strength by 0.2 kcal/mol and 0.5kcal/mol respectively (Choe and Min 2009). However, sinapic acid showed a slightly higher scavenging activity than ferulic acid, which was due to the presence of another methoxy substitution at the ortho position (Medina et al. 2007, Choe and Min 2009).

In case of benzoic acid derivatives, gentisic acid showed a high scavenging activity of 92% (Figure 4.2B), probably due to the two hydroxyl substitutions at the ortho and meta position (Figure 1B), whereas protochatechuic acid showed reduced scavenging activity of 57% (Figure 4.2B) due to the substitution of one hydroxyl group at the para position (Joshi et al. 2012). Nevertheless, the DPPH radical scavenging activity of vanillic acid was meagre, which is very similar to the results obtained by (Tai et al. 2012). The positive charged substituent at the meta and para-position becomes electrostatically unfavourable and is considered to be the underlying reason for this reduction in activity (Jing et al. 2012).

Among the three flavonoids, quercetin showed the highest DPPH scavenging ability of 93%, followed by catechin 83% and rutin 78% (Figure 4.2C). The reason for quercetin showing high activity was due to the presence of 3,4-dihydroxy substitution in the B-ring (Figure 4.1C), unlike catechin which had 4,5- dihydroxy substitution (Choe and Min, 2009). Though rutin has dihydroxy substitution in B-ring, the replacement of 3-OH by a disaccharide in C-ring (Figure 4.1C) was responsible for further reduction in activity (Farkas et al. 2004). The high scavenging activity of all flavonoids was probably attributed to the polyphenolic structure with high number hydroxyl substituent (Figure 4.1C) to act as electron donating groups (Ali et al. 2013). The replacement of these functional hydroxyl groups by other substituents greatly

reduces the antioxidant activity. This was probably the reason for the high scavenging activities of gentisic and caffeic acid. The antioxidant activity of phenolic compounds, not only depend on the electron donating effect of substituent, but also on the steric crowding around the phenolic OH groups because of the position of substituent (Amorati et al. 2007). This was considered to be the reason for the reduced activity of protochatechuic acid, sinapic acid and rutin.

### 4.2.2. Ferric reducing power assay

Any compound whose reduction potential is lower than the peroxide or alkyl radical can act as an effective antioxidant by donating H atom (Choe and Min 2009). Hence, the ability to reduce TPTZ-Fe(III) complex to TPTZ-Fe(II) complex by different phenolic compounds were tested and the results are shown in Figure 4.3 A, B and C. Among the nine tested phenolic compounds caffeic acid showed the maximum absorbance of 1.73 at 25µM indicating high reducing power. Caffeic acid possesses a marked capacity for iron binding, suggesting that the antioxidant capacity of caffeic acid may be related to iron-binding (Gocer and Gulcin 2011). Of all the hydroxycinnamic acids, caffeic acid showed the maximum ability to donate electrons followed by sinapic acid and ferulic acid (Figure 4.3A). Introduction of a methoxy substitution in ferulic acid reduced the electron donating ability compared to caffeic acid (Medina et al. 2007). In case of sinapic acid the addition hydroxyl function with methoxy substitution increased the electron donating ability to a certain extent in comparison with ferulic acid (Figure 4.3A).

Both gentisic and protochatechuic acid showed almost same reducing power ability which increased with increase in concentration (Figure 4.3B). This was attributed to the lack of hindrance from a methoxy substitution, whereas vanillic acid showed the least reducing power among all antioxidants. This was probably due to the methoxy substitution at the meta position which greatly reduces the activity unlike protochatechuic acid (Figure 4.1B). These results are in agreement with Habeebullah et al. (2010) who reported that potato extracts with high gentisic and protochatechuic acid showed high reducing power.

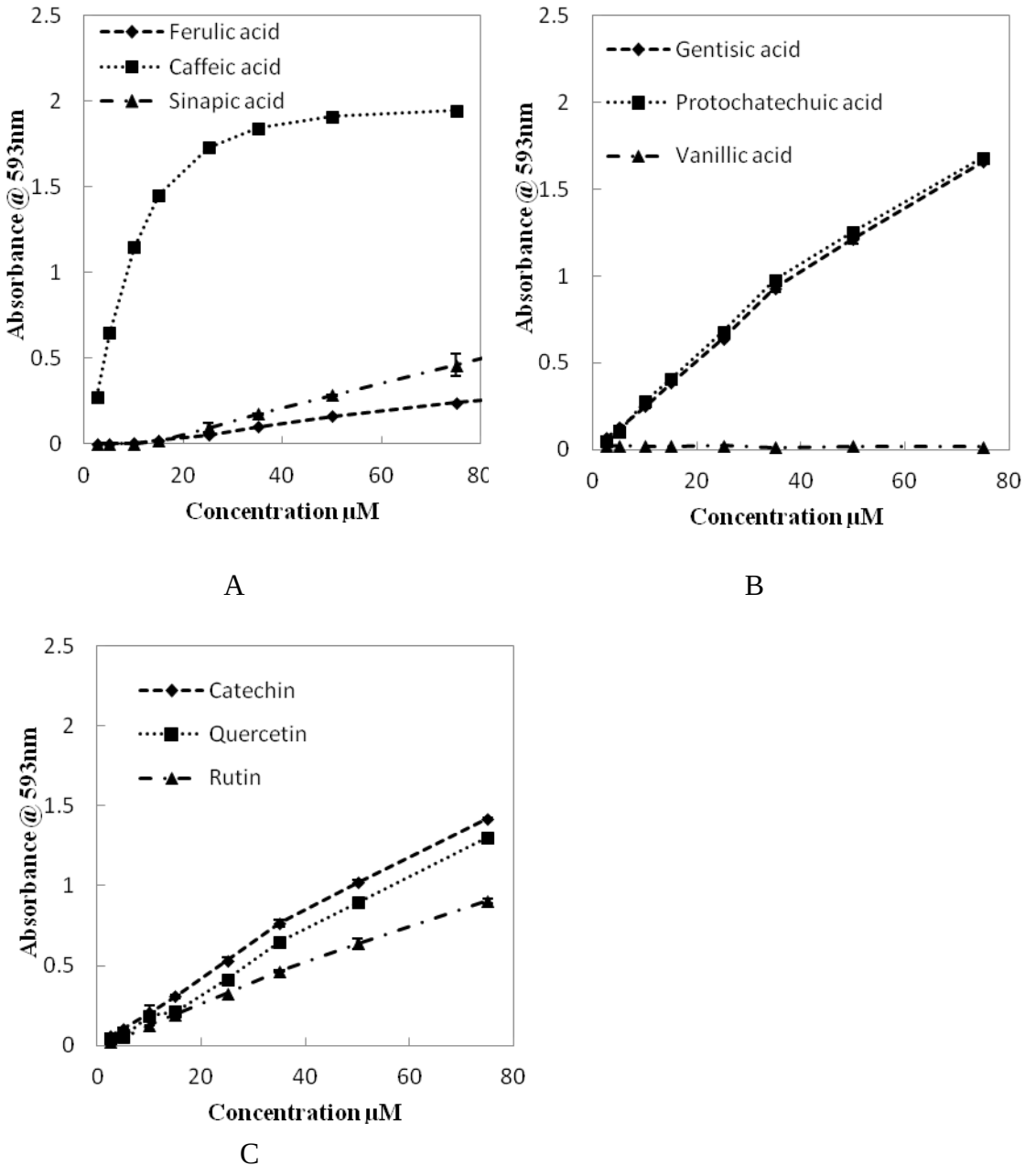



**Figure 4.3 Ferric reducing power of phenolic compounds at various levels (A) Hydroxycinnamic acid (B) hydroxybenzoic acids (C) Flavonoids**

In comparison with other chosen phenolic acids, all flavonoids showed moderate ferric reducing ability (Figure 4.3C), with catechin, quercetin and rutin showing maximum absorbance of 1.019, 0.894 and 0.636, respectively at 50µM concentration (Figure 4.3C). According to DeGraft-Johnson et al. (2007) the presence of catechol structure in antioxidant compounds accounted for their reducing power ability rather

than the number of hydroxyl groups. Since the difference between the reduction potential of peroxide radical and flavonoids are more (670 mV) they exhibit high reducing power (Choe and Min 2009). Recent studies have discussed about the fact that the absolute antioxidant capacity cannot be predicted by determining the number of hydroxyl groups alone (Medina et al. 2007). Hence, multiple antioxidant assays have to be conducted with different mechanisms to understand the effect of structure on the antioxidant activity.

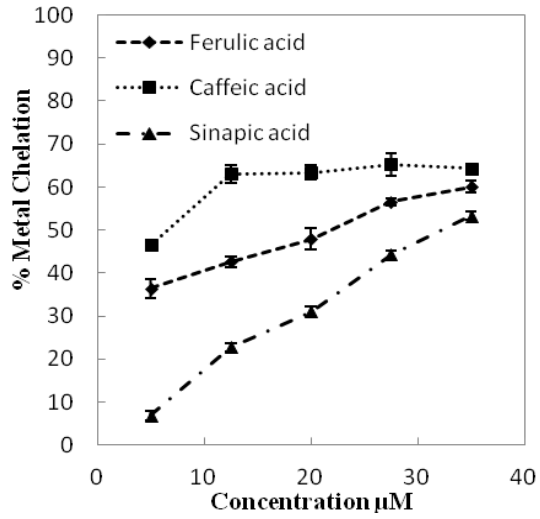
### 4.2.3. Metal chelating ability

The metal chelating ability of the antioxidants at different levels is depicted on Figure 4.4 A, B and C. In case of hydroxycinnamic acids, both caffeic acid and ferulic acid showed activity of 64% and 60% chelation respectively (Figure 4.4A), whereas, sinapic acid showed a mild reduction in activity of 53% due to the methoxy substitution which reduces the iron binding capacity. Though methoxy groups could stabilise phenoxy radicals by donating electron, they do not contribute to metal chelation (Danilewicz 2003).

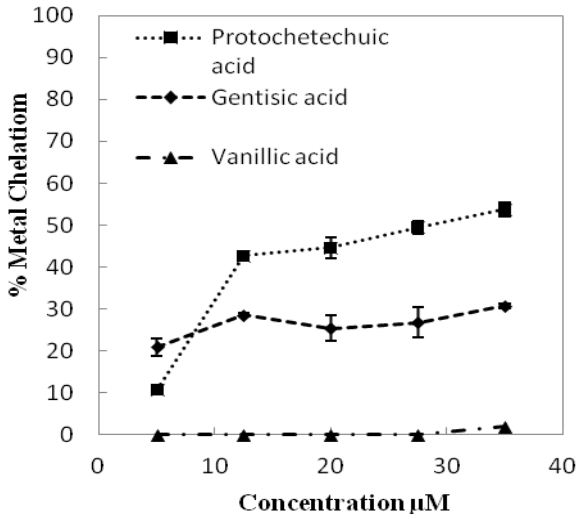
Interestingly, gentisic acid showed a very low chelating ability of 30% (Figure 4.4B) unlike other antioxidant. This is in agreement with the results obtained by Ashidate et al. (2005) who reported that the antioxidant activity of gentisic acid is mainly due to radical scavenging capability. Nevertheless, protochatechuic acid showed a slightly higher chelating activity of 53% and vanillic acid showed negligible activity (Figure 4.4B).

All flavonoids showed a high metal chelating ability with catechin 73%, followed by quercetin and rutin at 64% and 67%, respectively (Figure 4.4C). According to Rice-Evans et al. (1996), the metal chelating potential in polyphenols is dependent on the proximity of the hydroxyl group. Hence, the similar pattern of hydroxylation in the B-ring of all the chosen flavonoids can be considered for the high chelating activity. It has been proposed that the two possible points of attachment of metal ions to a polyphenols are the 3,4 dihyroxy substitution in catechol structure and the 4-oxo or 3-

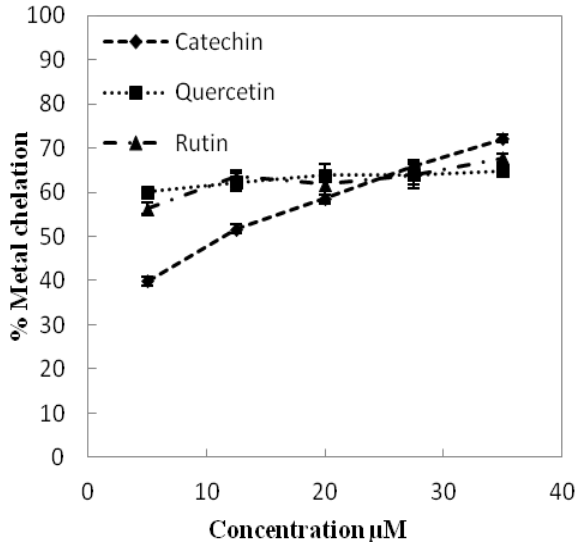
OH or 5-OH in the C-ring (Liu et al. 2014). This explains the difference in the activities of the catechin, quercetin and rutin.




A




B




C

**Figure 4.4 Metal chelating activity of phenolic compounds at various levels (A) Hydroxycinnamic acid (B) hydroxybenzoic acids (C) Flavonoids**

### 4.2.4. Oxidative stability studies

#### 4.2.4.1. Peroxide value

Peroxide value was used as an indicator for the primary oxidation of sardine oil in the presence of several antioxidants. Colourless and odourless hydroperoxides are labile compounds that undergo degradation to produce complex array of secondary products (Poiana 2012). The peroxide value of sardine oil in the presence of nine antioxidants was tested against control and depicted in Figure 4.5A, B and C.

Except samples with catechin and vanillic acid, a gradual increase in the peroxide value was noted in all samples for initial 10 days, after which it increased at a higher rate compared to control. Vanillic acid and catechin showing the maximum peroxide value of 22.09 meq/kg, 21.64 meq/kg and 29.28 meq/kg, respectively. Thus, at the end of 14 days storage period quercetin, gentisic acid, rutin and caffeic acid showed a significant reduction in peroxide value to the extent of 25%, 25%, 24 % and 19%, respectively in comparison with control ($p<0.05$) (Table 4.1).

Among the cinnamic acids tested, caffeic acid showed the highest ability to prevent hydroperoxides formation (Figure 4.5A). The oxidative stability of sardine oil in the presence of cinnamic acids with respect to its peroxide value showed a specific trend which can be correlated to their radical scavenging ability (Figure 4.5A). Ferulic acid and sinapic acid showed a similar effect on reducing the peroxide value, but their efficiency was lower than that of caffeic acid. The ability of caffeic acid to inhibit oxidation in fish oil had been well established (DeLeonardis and Maccio, 2003, Maqsood and Benjakul 2010) and was concluded that caffeic acid can be effectively used to further improve their antioxidant effect (Chalas et al. 2001). However, reports on other potential compounds like gentisic acid and flavonoids are meagre which provides scope for further exploration.

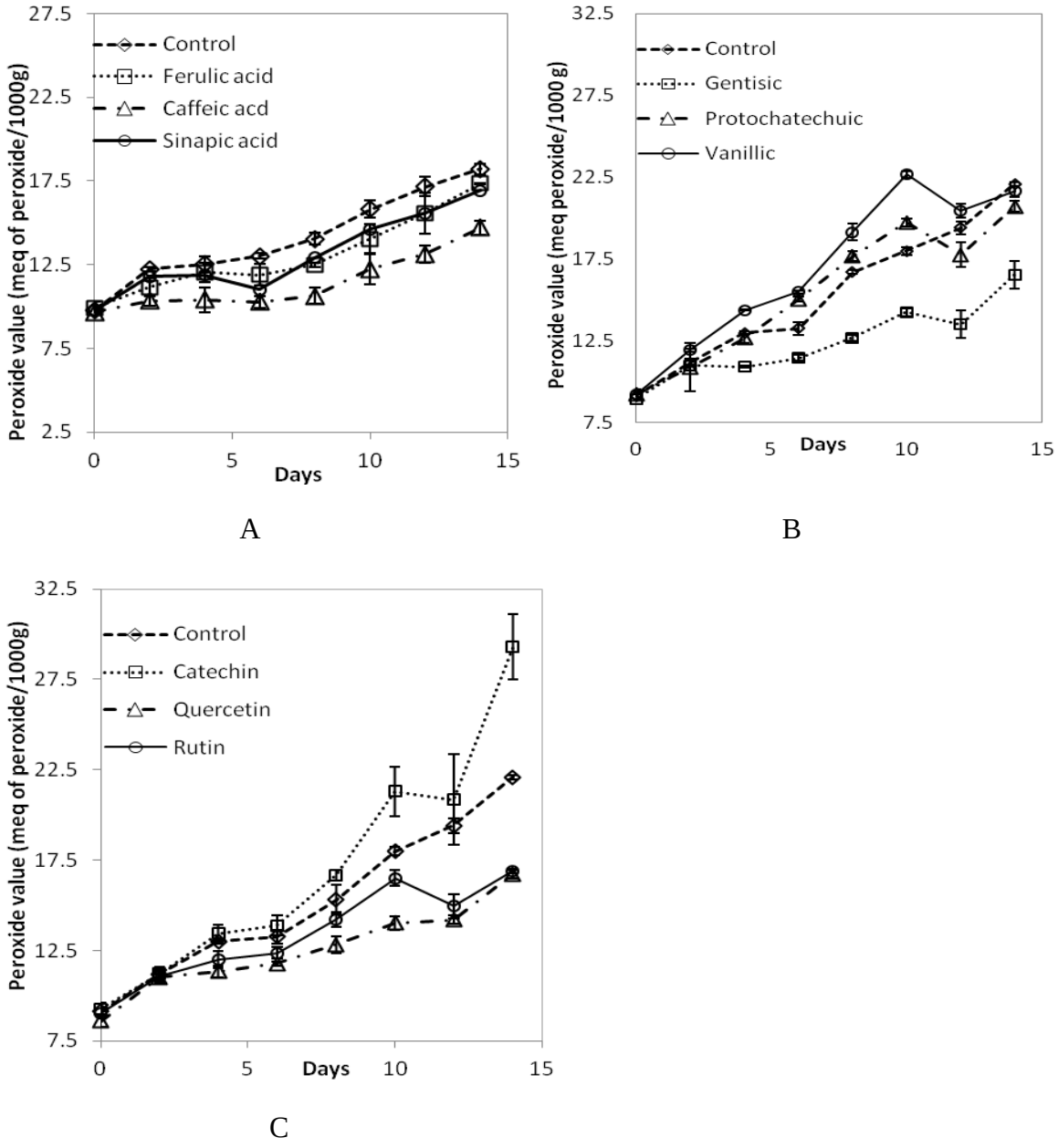



**Figure 4.5 Effect of 100 ppm of phenolic antioxidants on the peroxide value of sardine oil stored at 37 °C for 14 days. (A) Hydroxycinnamic acids (B) Hydroxybenzoic acids (C) Flavonoids. Bars represent standard deviation n=3**

Of all the tested benzoic acids, gentisic acid showed highest reduction in peroxide value of 25.13% (Table 4.1) which is consistent with the results obtained by Ashidate et al. (2005) when tested against human plasma, where cholesterol ester hydroperoxides were effectively scavenged. The reason for the high antioxidant

activity of gentisic acid throughout the 14 days storage period (Figure 4.5B), was attributed to its high radical scavenging ability. The inability of protochatechuic acid to prevent oxidation can be explained by the inefficient association of antioxidant molecules at the sight of oxidation (Marmesat et al. 2009) or due to the steric crowding of bulky peroxyl radicals around the substituent because of adjacent hydroxyl substitution (Choe and Min 2009). Vanillic acid failed to show any significant reduction ($p>0.05$) in sardine oil oxidation but showed a slight prooxidant effect which was attributed to its low radical scavenging ability as a result of methoxy substitution. Thus, methylation of 3-hydroxyl group has reduced the radical scavenging ability and increased the prooxidant effect (Cotoras et al. 2014).

Samples with quercetin and rutin showed a maximum reduction in the peroxide formation than other compounds (Figure 4.5C), that can be correlated to their high radical scavenging ability. However, samples with catechin showed a prooxidant effect ($p>0.05$) on the peroxide formation despite its high scavenging ability. These results are in agreement with Jung et al. (2014), who found that catechins when added at a concentration of 1mM had no effect on the diacyglycerol rich oil. Furthermore, catechin tends to act as prooxidants by reducing transition metal ions and generating hydroxyl radical via Fenton reaction (Maqsood and Benjakul 2010).

**Table 4.1 Effectiveness of each antioxidant on improving the oxidative stability of sardine oil at the end of 14$^{th}$ day storage period**

| Antioxidants | % Decrease in the oxidation of sardine oil | | | |
|---|---|---|---|---|
| | Peroxide value | p-Anisidine value | CD value | TBARS value |
| Ferulic acid | 4.72 ±0.22$^{c}$ | 13.04±2.38$^{d}$ | 5.06±0.79$^{d}$ | 18.86 ±1.87$^{b}$ |
| Caffeic acid | 19.27±2.35$^{a}$ | 26.24±2.14$^{b}$ | 10.38±2.94$^{c}$ | 30.48 ±2.11$^{a}$ |
| Sinapic acid | 6.97 ±1.69$^{b}$ | 17.99 ±0.66$^{c}$ | 10.89±1.50$^{c}$ | 20.23 ±3.40$^{b}$ |
| Gentisic acid | 25.13±3.91$^{a}$ | 11.48±1.84$^{d}$ | 16.43±2.37$^{b}$ | 25.70±3.22$^{b}$ |

| Antioxidants | % Decrease in the oxidation of sardine oil | | | |
|---|---|---|---|---|
| | Peroxide value | p-Anisidine value | CD value | TBARS value |
| Protochatechuic acid | 6.19 ±0.42[b] | Nil | 5.5±0.69[d] | Nil |
| Vanillic acid | 2.05 ±1.53[e] | Nil | 4.8±3.87[d] | Nil |
| Catechin | Nil | 12.71±4.29[d] | 15.05±1.93[b] | Nil |
| Quercetin | 25.21±1.09[a] | 43.27±4.64[a] | 30.64±0.62[a] | 22.23±3.45[b] |
| Rutin | 23.63±0.43[a] | 42.79±4.53[a] | 30.64±1.26[a] | 32.24±1.23[a] |

Percentage decrease was calculated by considering control to be 100% oxidised.

[a, b, c, d] values with the same letter in each column were not significantly different ($p > 0.05$).

[*]Nil – Insignificant

#### 4.2.4.2. Conjugated Dienes (CD) value

During oxidation process, the original pentadienoic double bond systems in some lipid free radicals (primary oxidation products), suffer a rearrangement into isomeric derivatives forming conjugated diene bonds, which shows maximum absorbance at 232 nm (Poiana 2012, Ramadan 2008). The CD value of the samples showed a slight reduction, after which it increased slowly during 14 days of storage (Figure 4.6 A, B and C). As oxidation of lipids occurs in the lipid phase and the surface, a mild reduction in the conjugated diene value can be observed (Figure 4.6 A, B and C), which was probably attributed to the reduction in the surface area exposed to oxidation in the reaction vial used (Schwarz et al. 2000). Since conjugated diene molecules are formed after the formation of unsaturated lipid radicals, similar to peroxide value the conjugated diene value increased rapidly after the 10$^{th}$ day of storage. Quercetin and rutin flavonoids showed the maximum reduction in conjugated dienes (Figure 4.6C). Interestingly, catechin showed effective reduction in the CD value unlike peroxide value. Thus it can be concluded that, catechins are effective in

interacting with peroxide radicals produced out of PUFA, inhibiting the formation of dienes. Thus at the end of the storage, catechin showed 15% reduction in oxidation of sardine oil.

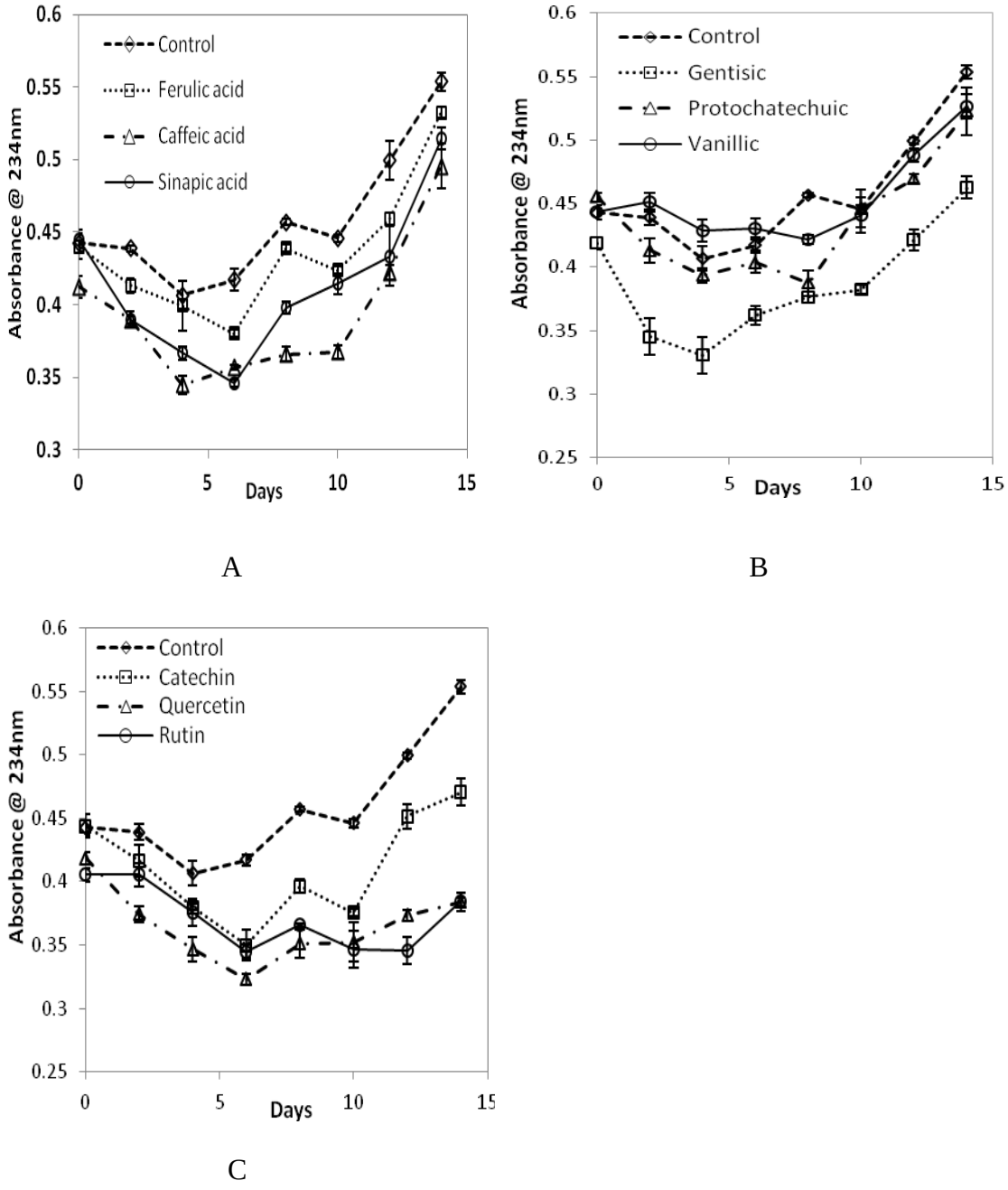



**Figure 4.6 Effect of 100 ppm of phenolic antioxidants on the conjugated dienes value of sardine oil stored at 37 °C for 14 days. (A) Hydroxycinnamic acids (B) Hydroxybenzoic acids (C) Flavonoids. Bars represent standard deviation n=3**

However, in case of cinnamic acids, caffeic acid and sinapic acid showed higher decrease in CD value than ferulic acid (Figure 4.6A), by showing a CD value of 0.495 and 0.524 respectively in comparison with a CD value of 0.532 by ferulic acid. These conjugated dienes are important only after a significant increase in the peroxide value (Ramadan 2008, Marmesat et al. 2009). Hence, both peroxide value and CD value of samples with cinnamic acids followed a trend similar to their radical scavenging ability. The antioxidant effect of gentisic acid was found to be high when compared to all other phenolic acids (Figure 4.6B), which is similar to the results obtained by Hradkova et al. (2013). This high antioxidant activity was related to the formation of hydrogen bond between carboxyl and hydroxyl group (Hradkova et al. 2013). Similar to peroxide value, the samples with vanillic acid showed negligible reduction in the CD value. Since the lipid peroxide molecules get rearranged to form conjugated dienes all potent radical scavengers like quercetin, rutin and gentisic acid showed the maximum activity of 30.6%, 30.6 % and 16.4% respectively (Table 4.1), which is very similar to the results obtained by Maqsood and Benjakul (2010).

#### 4.2.4.3. p-Anisidine value (pAV)

In order to ensure effective monitoring of the oxidation progress in sardine oil, simultaneous measurement of primary and secondary oxidation products is necessary (Poiana 2012). Calculating the p-Anisidine value (pAV) is one of the oldest methods for evaluating secondary oil oxidation. pAV is the measure of secondary oxidation products (aliphatic aldehydes, ketones, alcohols, acids and hydrocarbons) generated during decomposition of hydroperoxides. It is based on the reaction of aldehyde carbonyl bond on the p-anisidne amine group, leading to formation of a Schiffs base that absorbs at 350nm (Wang et al. 2011). The results for the p-anisidine value of sardine oil in the presence of phenolic compounds are shown in Figure 4.7 A, B and C.

It can be observed that the pAV of control is high in comparison to pAV of sardine oil with antioxidants. The pAV steadily increased during first 10 days and showed a sharp increase later, which is very similar to the results obtained by Taghvaei et al. (2014) in case of soybean oil. During the $10^{th}$ day of storage, 100 ppm of quercetin

and rutin showed pAV of 63 and 68 respectively (Figure 4.7C) in comparison with a control value of 147. Though, catechin acted as a mild prooxidant during primary oxidation of sardine oil, it showed slight antioxidant activity during secondary oxidation.

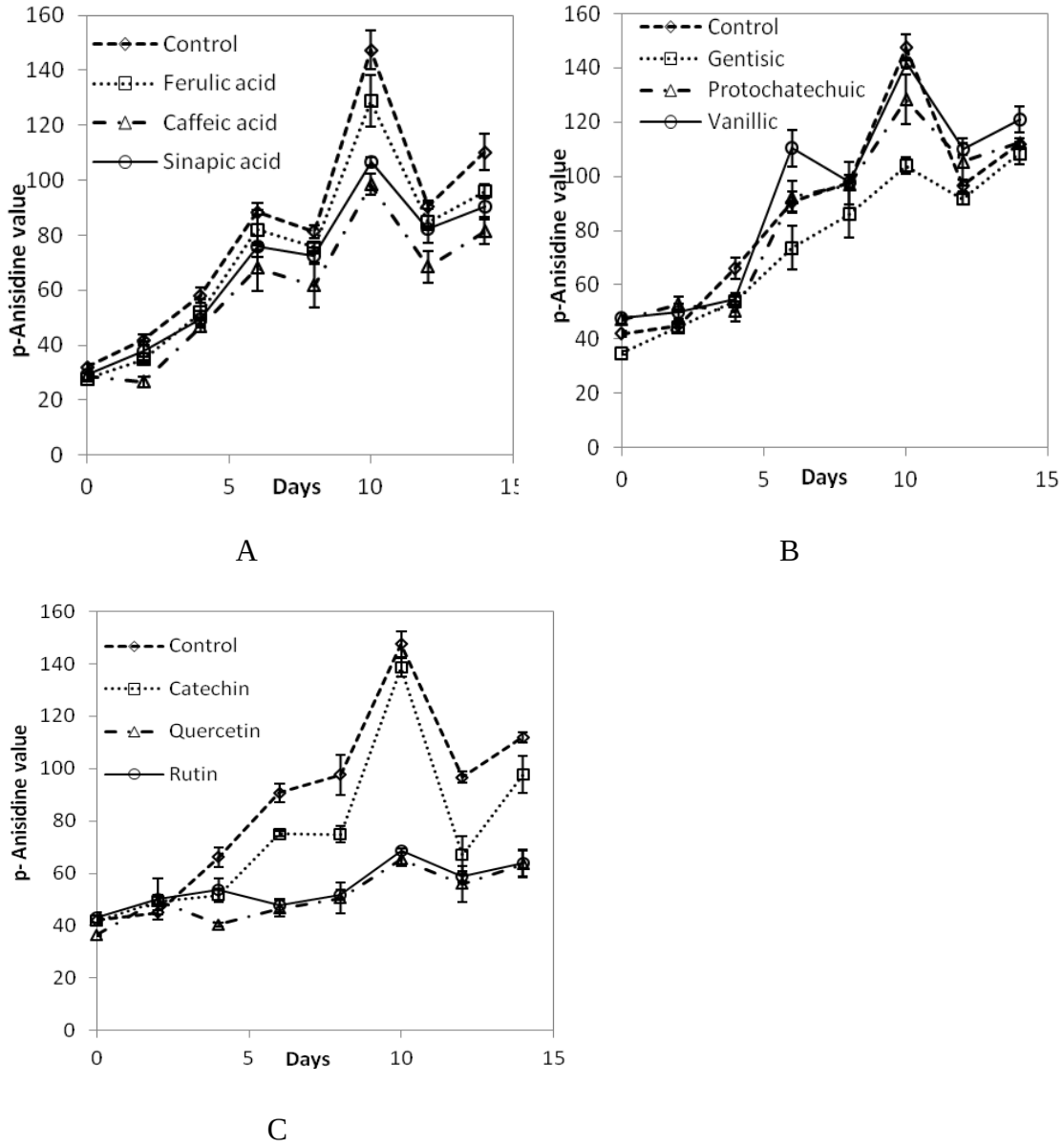



**Figure 4.7 Effect of 100 ppm of phenolic antioxidants on the p-Anisidine value of sardine oil stored at 37 °C for 14 days. (A) Hydroxycinnamic acids (B) Hydroxybenzoic acids (C) Flavonoids. Bars represent standard deviation n=3**

In case of cinammic acids, caffeic acid showed the best reduction in anisidine value of 98 followed by sinapic acid 107 (Figure 4.7A). Nevertheless, only gentisic acid showed significant effect on pAV reduction by 11.48% (Table 4.1) when compared to the other hydroxybenzoic acids tested (Figure 4.7B) at the end of 14 days storage period. Similar to peroxide value, protochatechuic acid and ferulic acid showed moderate activity, while vanillic acid had no significant effect ($p>0.05$). Based on the pAV results, the descending order of oxidative stability of sardine oil with different antioxidants during 14 days storage followed the order: Quercetin = rutin > caffeic acid > sinapic acid > ferulic acid = catechin = gentisic acid > protochatechuic acid > vanillic acid (Table 4.1).

#### 4.2.4.4. Thiobarbituric acid-reactive substance (TBARS) value

The reaction between thiobarbituric acid with malondialdehyde (MAD), produced by lipid hydroperoxides decomposition is employed as the basis of this analysis. TBARS is usually employed as an indicator for monitoring secondary oxidation products (Wang et al. 2011). Together with peroxide value data, a more comprehensive picture on the oxidation of sardine oil can be developed. The results of TBARS value were similar to that of p-anisidine value. As it has been shown in Figure 4.8 A, B and C, the TBARS value showed an increasing trend and peaking at $8^{th}$ day after which it reduces. This reduction was due to the auto-oxidation of secondary oxidation products releasing carboxylic acids or aldehydes (Taghavaei and Jafari 2015). The inhibition of secondary oxidation product formation by quercetin and rutin was almost similar (Figure 4.8C) which was attributed to the 3,4-dihydroxy substitution in B-ring as discussed earlier. However, the replacement of hydroxyl group in C-3 position by disaccharide group in rutin did not seem to have an effect unlike *in vitro* studies. These results were found to be consistent with that of Bilto et al. (2012) who found that querctin and rutin showed similar inhibition of erythrocyte peroxidation when induced by $H_2O_2$, while other flavonoids were not effective.

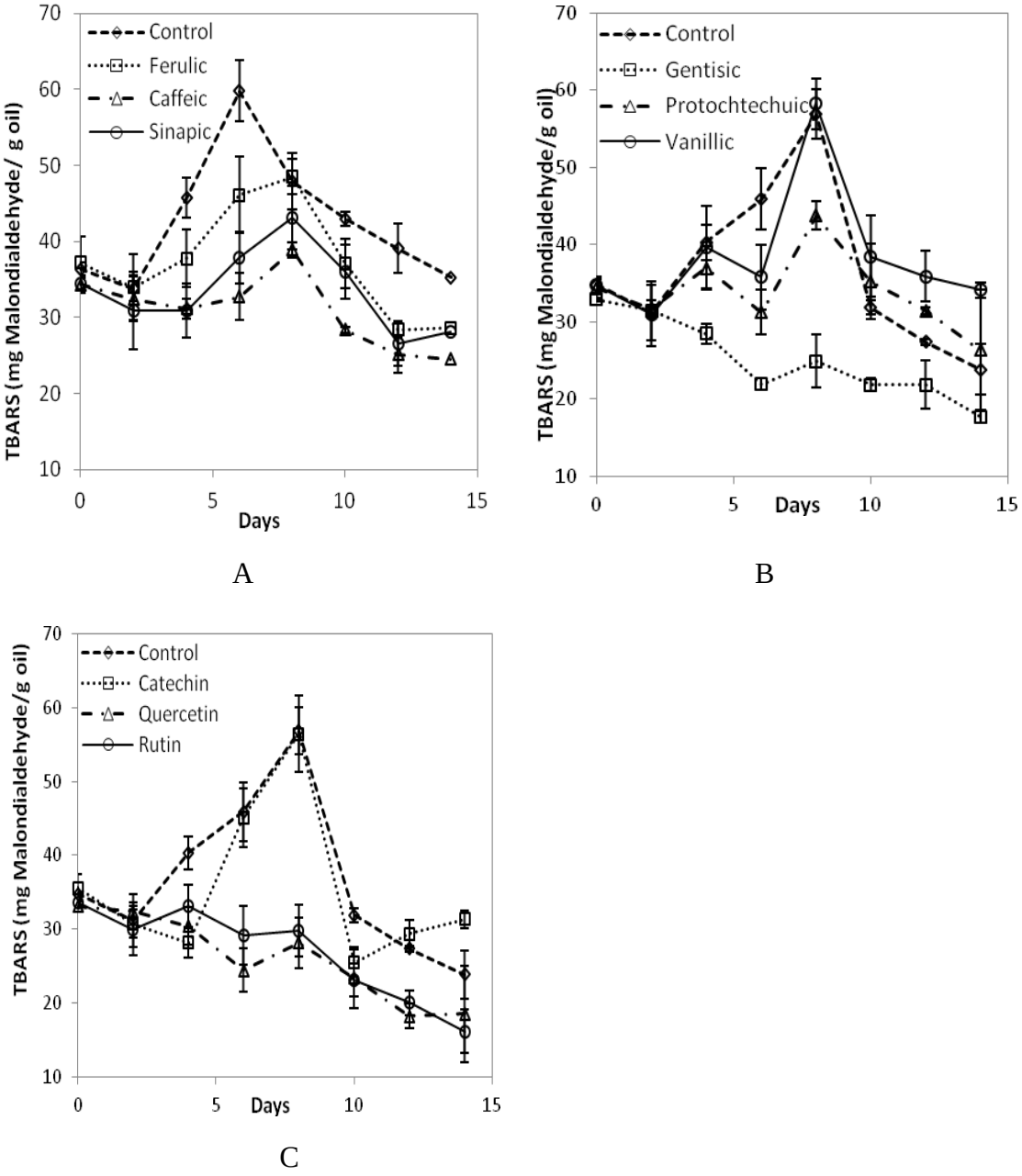



**Figure 4.8 Effect of 100 ppm of phenolic antioxidants on the thiobarbituric acid reactive substances (TBARS) value of sardine oil stored at 37 °C for 14 days. (A) Hydroxycinnamic acids (B) Hydroxybenzoic acids (C) Flavonoids. Bars represent standard deviation n=3**

Of the three hydroxybenzoic acids chosen, gentisic acid showed a maximum of 26% reduction in TBARS value (Table 4.1). A clear antioxidant effect was also seen in

protochatechuic acid until the 8$^{th}$ day of incubation after which the efficiency reduced (Figure 4.8B). Thus gentisic acid and protochatechuic acid showed significant antioxidant activity by reducing the TBARS value to 24.85 and 43.76 respectively, from that of a control value of 56.85 (Figure 4.8B). In case of samples with vanillic acid, no antioxidant effect was seen, which is similar to the results obtained by Jung et al. (2014). In case of cinnamic acids, caffeic acid showed higher reduction of 30.48% followed by sinapic and ferulic acid (Figure 4.8A). This is consistent with radical scavenging behaviour of the cinnamic acids. Since, secondary oxidation occurs as a result of increased lipid peroxides, any potent radical scavenger is expected to have a reasonable effect on the reduction of secondary oxidation. Of all the tested antioxidants quercetin, rutin, gentisic and caffeic acids showed maximum reduction in TBARS value. While ferulic acid, protochatechuic acid and sinapic acid showed slightly high TBARS value in comparison, catechin and vanillic acid failed to show any antioxidant effect (Figure 4.8 B and C) which is consistent with the results obtained by Jung et al. (2014).

In conclusion, quercetin and rutin followed by gentisic acid and caffeic acid were most effective in arresting oxidation of sardine oil, by reducing subsequent liberation of primary oxidation products. The formation of isomeric derivatives of the primary oxidation products (indicated by CD value) and the extent of lipid oxidation (given by TBARS value) were very low for quercetin and rutin, indicating their effectiveness in restraining secondary oxidation as well. Owing to its radical scavenging ability, the overall performance of quercetin was very high in providing oxidative stability to sardine oil during storage unlike catechin (high metal chelating activity) indicating the major mechanism behind oxidation in sardine oil is peroxide radical formation.

However, significant chelating property of catechin played no role in imparting oxidative stability to sardine oil, indicating that the major mechanism behind oxidation in sardine oil was peroxide radical formation. Current study clearly shows that in vitro studies were useful in making an intelligent choice of phenolic compounds for improving the oxidative stability. In view of existence of multiple mechanisms of oxidation occurring in oil and their dependence on the diversity in oil

composition, evaluation of antioxidant performance in different oil is crucial in achieving oxidative stability during storage.

### 4.2.5. Effectiveness of antioxidants in the presence of trace impurities

It is widely known that the degree of unsaturation and the type of oxygen species play a major role on influencing the rate of oxidation in bulk oils containing higher PUFA content (Kim et al. 2014). Along with these major substrates, presence of minor components like free fatty acids, mono and diglyceride, transition metal ions, moisture and phospholipids influence oxidation rate (Chaiyasit et al. 2007). This further affects the quality of the oil leading to formation of oxidation products and volatile compounds at a faster rate. In addition, food safety criterion is compromised, as some oxidation products have been documented to adversely affect human health (Singh et al. 2010). On analysing the best antioxidant for sardine oil oxidation, a rough mechanism of oxidation has been established for the sardine oil used in the current study. However, it is difficult to reproduce the same antioxidant efficiency, owing to the drastic difference in the bulk oil composition obtained from different places and different periods.

Bulk oil is a heterogeneous system containing mono-, di-, and triglycerides along with free fatty acids, phospholipids and water (Vaisali et al. 2015). The presence of amphiphillic mono and diglycerides along with oxidation products like ketone and aldehydes is unavoidable in bulk oil even with subsequent processing of oil (Chaiyasit et al. 2007). These oxidation products form association colloids or reverse miscelles with mono and diacylglycerols, phospholipids and free fatty acids (Chayasit et al. 2007, Kittipongpittaya et al. 2016). These physical structures act as micro or nano-reactors of oxidation reaction and the mechanism at such complex structures still remain unexplored. Further, their presence alters the effectiveness of the conventional antioxidants such as α-tocopherol, torlox etc. (Chen et al. 2012). The heterogeneous nature of oil becomes more significant while considering the site of oxidation. It has been reported that the significance of these minor compounds is higher for oils rich in unsaturated fatty acids (Decker et al. 2010). Hence, studying the role of such compounds for PUFA rich fish oil provides better understanding of the complex

oxidation process in such oils. In order to effectively design an antioxidant strategy that acts positively at all conditions, the present investigation was carried out to explore the role of iron and trace water on sardine oil oxidation. This study of influence of minor components on oxidation might shed some light on how the oxidation in sardine oil can be interpreted in the presence of such association colloids.

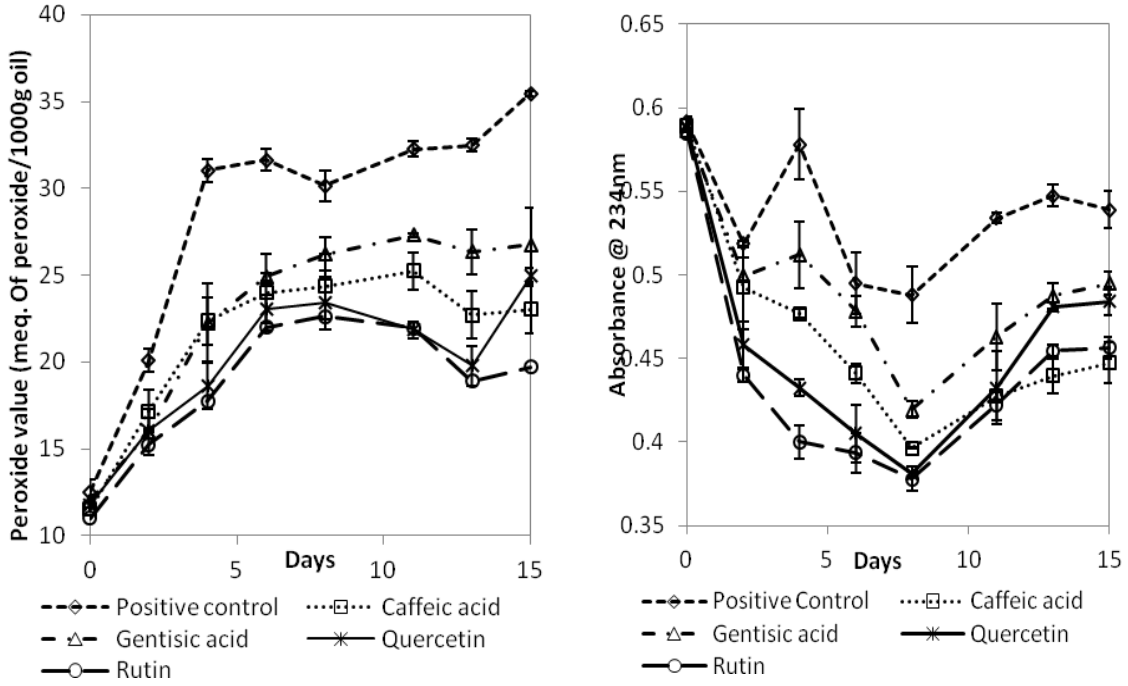
#### 4.2.5.1. Influence of trace metals

Trace metals are common impurities that are found in minor concentrations in bulk oil. During storage and transportation of bulk oil, trace metals get dispersed into the oil from the metal containers. Further, fish oil themselves contain some inherent trace metal content even with subsequent refining process (Bimbo et al. 1998). A common metal ion that is generally found in oils such as iron was chosen to study antioxidants effect (Andersson and Lingnert 1998). Since low valence state iron ($Fe^{2+}$) promotes faster oxidation in food components than ferric iron (Chen et al. 2012; Homma et al. 2016), ferrous chloride was taken as a prooxidant in the current study. As the knowledge on the impact of iron in bulk oil oxidation is limited, it is important to study the effect of iron with and without antioxidant addition. This will provide a brief idea about the stability of bulk oils in presence of metal ions along with the route of oxidation and how it alters the ability of antioxidants.

##### 4.2.5.1.1. Primary oxidation

The four antioxidants which showed good antioxidant activity in refined sardine oil was chosen for analysing the change in their effectiveness when oxidation was influenced by iron. The primary oxidation profile of sardine oil in the presence of iron and antioxidants is shown in Figure 4.9. As can be seen from Figure 4.9A, oil sample added with ferrous chloride showed the highest peroxide value at all times in comparison to oil without iron addition. This could be due to the participation of $Fe^{2+}$ iron in Fenton type reactions (Thanonkaew et al. 2006), thus taking a different and faster route to oxidation. Also, presence of iron Fe (II) is known to activate molecular oxygen to give singlet oxygen and peroxyl radical which are analogous to Fenton and Haber Weiss type reactions (Colakoglu 2007). While positive control without any

antioxidants and with Fe (II) showed maximum peroxide value of 35.46 meq peroxide/1000g (Fig. 4.9A), samples with quercetin and rutin showed 24.93 meq peroxide/1000g and 19.71 meq peroxide/1000g respectively, at the end of 14$^{th}$ day. Whereas, caffeic acid and gentisic acid showed a peroxide value of 23.01 and 26.74 meq peroxide/1000g respectively, after a storage period of 14 days. All antioxidants from our previous screening showed good reduction in the oxidation which could be attributed to their metal chelating property (Vaisali et al. 2016) and radical scavenging ability as well. Since antioxidants were added to the prooxidant initially, there is a possibility that most of the Fe(II) molecules were chelated prior to their participation in oxidation. Also, it has been established that these prooxidant metals and the antioxidants accumulate at the association colloids (Chen et al. 2012) which acts as the site of oxidation in bulk oil.




A B

**Figure 4.9 Effect of quercetin, rutin, caffeic and gentisic acid on the oxidative stability of sardine oil in the presence of ferrous chloride (A) Peroxide value (B) Conjugated diene value**

Similar to peroxide value the measurement of conjugated dienes also indicated the effectiveness of quercetin and rutin to reduce iron catalysed oxidation reaction in sardine oil (Figure 4.9B). Rutin and caffeic acid showed the maximum reduction of 15.30% and 16.97% in conjugated diene followed by quercetin and gentisic acid (Table 4.2). In comparison with the results on the efficiency of antioxidants in refined sardine oil, there was a notable difference in the activity of the antioxidants when iron was added. The probable reason for this could be that iron catalysed oxidation reaction follows a different pathway than the auto-oxidation of sardine oil (Thanonkaew et al. 2006). The antioxidants were found to show better efficiency in reduction of oxidation influenced by iron which could be due to the hydrophilic nature of both antioxidants and ferrous chloride. This further proves the efficiency of chosen antioxidants to act effectively in different pathways of oxidation.

**Table 4.2 Effectiveness of antioxidants in improving the oxidative stability of sardine oil containing ferrous chloride at the end of 14$^{th}$ day storage**

| **Antioxidants** | **% Decrease in the oxidation of sardine oil in presence of 10ppm ferrous chloride** | | | |
|---|---|---|---|---|
| | **Peroxide value** | **p-Anisidine value** | **CD value** | **TBARS value** |
| Caffeic acid | 35.09±0.44$^{b}$ | 10.20±3.12$^{a}$ | 16.97±2.23$^{a}$ | 40.96±2.67$^{b}$ |
| Gentisic acid | 24.54±3.81$^{b}$ | 8.70±2.95$^{a}$ | 8.16±1.31$^{b}$ | 42.22±1.48$^{b}$ |
| Quercetin | 29.69±6.04$^{b}$ | 14.77±2.83$^{a}$ | 10.20±1.57$^{b}$ | 49.57±2.97$^{a}$ |
| Rutin | 44.39±1.30$^{a}$ | 11.69±2.43$^{a}$ | 15.30±1.18$^{a}$ | 51.67±2.97$^{a}$ |

Percentage decrease was calculated by considering control to be 100% oxidised.

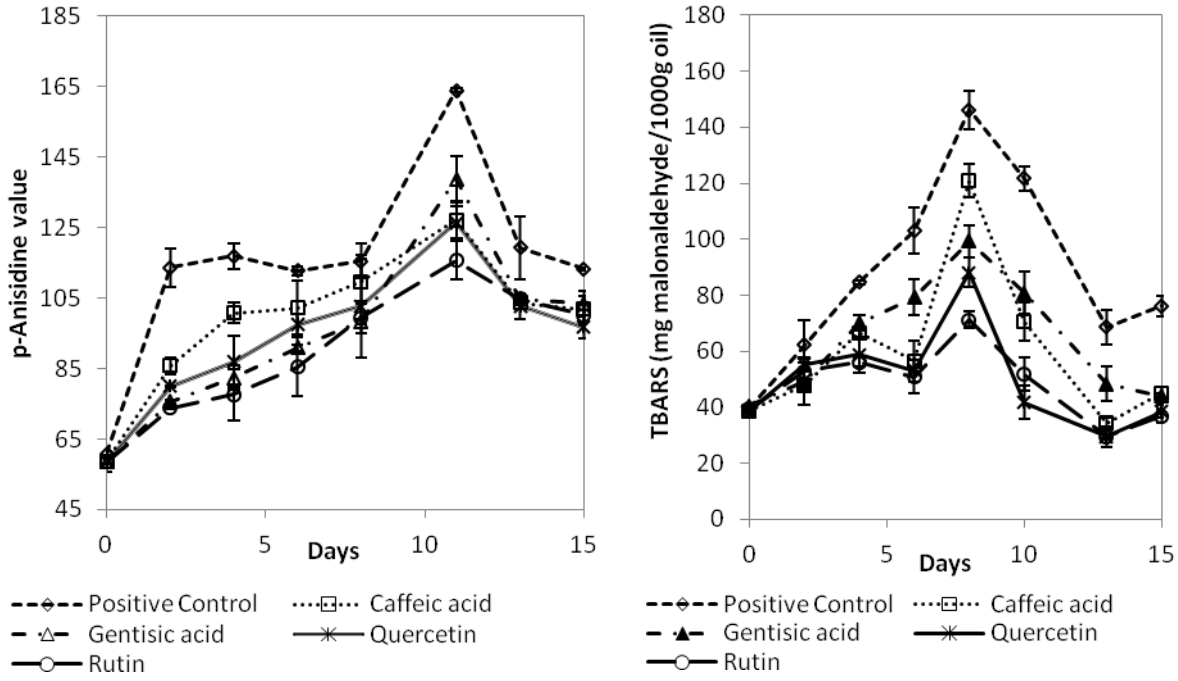
$^{a, b,}$ values with the same letter in each column were not significantly different ($p$ > 0.05).

#### 4.2.5.1.2. Secondary oxidation

While measuring the products of secondary oxidation in sardine oil containing prooxidant iron and antioxidants, it was found that positive control showed the highest generation of secondary oxidation products than other samples with

antioxidants (Fig. 4.10A). While rutin and quercetin gave 95.99 and 100.44 p-anisidine value at the end of 14 days storage, caffeic acid and gentisic acid gave 101.46 and 103.06 respectively, in comparison with a control value of 122.68 (Figure 4.10A). Since secondary oxidation products are formed from primary oxidation products, the trend of antioxidant effectiveness was similar to peroxide value. However, no significant difference in the effectiveness of the antioxidants in reducing p-anisidine value was noted when Fe(II) was added (Table 4.2). This is in contrast to our previous results, where quercetin and rutin were highly efficient in reducing secondary oxidation (Vaisali et al. 2016). The secondary oxidation increased at $8^{th}$ day of storage, due to the faster decomposition of peroxides by $Fe^{2+}$ and the activation energy for the cleavage of O-O bond was less compared to that of O-H bond (Colakoglu 2007).

On analysing the results of TBARS value of oil in the presence of ferrous chloride, it was noted that all four chosen antioxidants showed good reduction in oxidation. Quercetin and rutin showed significantly higher antioxidant effect than caffeic acid and gentisic acid (Table 4.2). Thus rutin and quercetin gave 36.85 and 38.46 TBARS value at the end of 14 days storage, caffeic acid and gentisic acid gave 45.03 and 44.07 respectively, in comparison with a control value of 76.28 (Figure 4.10B). Similarly, TBARS value also showed increase in value till $8^{th}$ day of storage (Figure 4.10B) which it followed a trend similar to previous results (Vaisali et al. 2016) indicating that secondary oxidation in sardine oil is constant, regardless of the prooxidant added or the route of primary oxidation. There was no significant change in the secondary oxidation in sardine oil on addition of ferrous chloride as prooxidant. This is similar to the results obtained by Sorensen et al. (2008), when oxidation of fish oil-in-water emulsion with iron was studied in the presence of caffeic acid and rutin. In case of flavonoids, iron interaction with these phenolic compounds results in the catechol-iron complex formation, while they form nano-particles with phenolic acids (Sorensen et al. 2008). As a result, iron does not participate effectively in the oxidation in the presence of antioxidants. Hence, it can be concluded that proper accumulation of peroxides is the major oxidation mechanism in sardine oil.




A B

**Figure 4.10 Effect of quercetin, rutin, caffeic and gentisic acid on the oxidative stability of sardine oil in the presence of ferrous chloride (A) p-Anisidine value (B) TBARS value.**

### 4.2.5.2. Influence of trace water

Owing to the heterogeneous nature of bulk oil, it was proposed that the initial oxidation in bulk oil occurs at the interface of micellar/ colloidal structures present in oil due to the presence of minor components (Chaiyasit et al. 2007, Shahidi and Zhong, 2011, Budilarto and Kamal-Eldin, 2015a). These dynamic structures are randomly dispersed in oil. The presence of the reverse micellar or lamellar structures in bulk oils has been recently confirmed under X-ray diffraction by many studies (Chaiyasit et al. 2007, Chen et al. 2010). Among the several hydrophilic and amphiphillic minor components in bulk oil, water plays a major role in the formation of these structures. Bulk oils with larger water-oil interfaces oxidise faster and the presence of other amphiphillic molecules further influence this oxidation process (Chen et al. 2010). Additionally, insights on water accumulation and its ability to

enhance micelle formation during oxidation in bulk oils have demonstrated the significance of trace water (Budilarto and Kamal-Eldin 2015b, Chen et al. 2011, Park et al. 2014). This makes it hard to anticipate the antioxidant capacity of a compound in bulk oil during the course of oxidation. Hence, it becomes necessary to study the effect of antioxidants in the presence of water to understand the effectiveness of an antioxidant better.

#### 4.2.5.2.1. Primary oxidation

For the purpose of understanding the influence on trace water on the effectiveness of natural phenolic antioxidants, the trace water content of sardine oil was adjusted to a constant value of 0.16% (w/w). Since the initial trace water content in refined oil was found to vary slightly, the water level in oil was adjusted prior storage experiments. The primary oxidation of sardine oil containing trace water is shown in Figure 4.11. The peroxide value of positive control was high at all times showing a maximum value of 30.98 at the end of 14 day storage period (Figure 4.11A). In comparison with our previous results, samples with trace water adjustment showed slightly higher primary oxidation. This could be due to the mobilization of other prooxidant compounds in the oil by water, which further catalyses the oxidation reaction. However, more studies with varying water levels has to be conducted to confirm the effect of water on increasing primary oxidation. In case of antioxidant effectiveness, rutin was found to be effective of all the antioxidants showing a peroxide value of 22.4 in comparison with a control value of 30.7 meq/kg oil (Figure 4.11A). This is consistent with our previous results indicating the efficiency of rutin (Vaisali et al. 2016). However, quercetin, caffeic acid and gentisic acid failed to show the same efficiency in the presence of moisture. Thus, at the end of 14 day storage period rutin showed maximum of 26% reduction in peroxide value (Table 4.3), followed by quercetin (18%). However, gentisic and caffeic acid showed no significant difference ($p$>0.05) in reducing the peroxide value. This could be attributed to the inefficiency of the antioxidants to associate better at the colloidal structures unlike rutin.

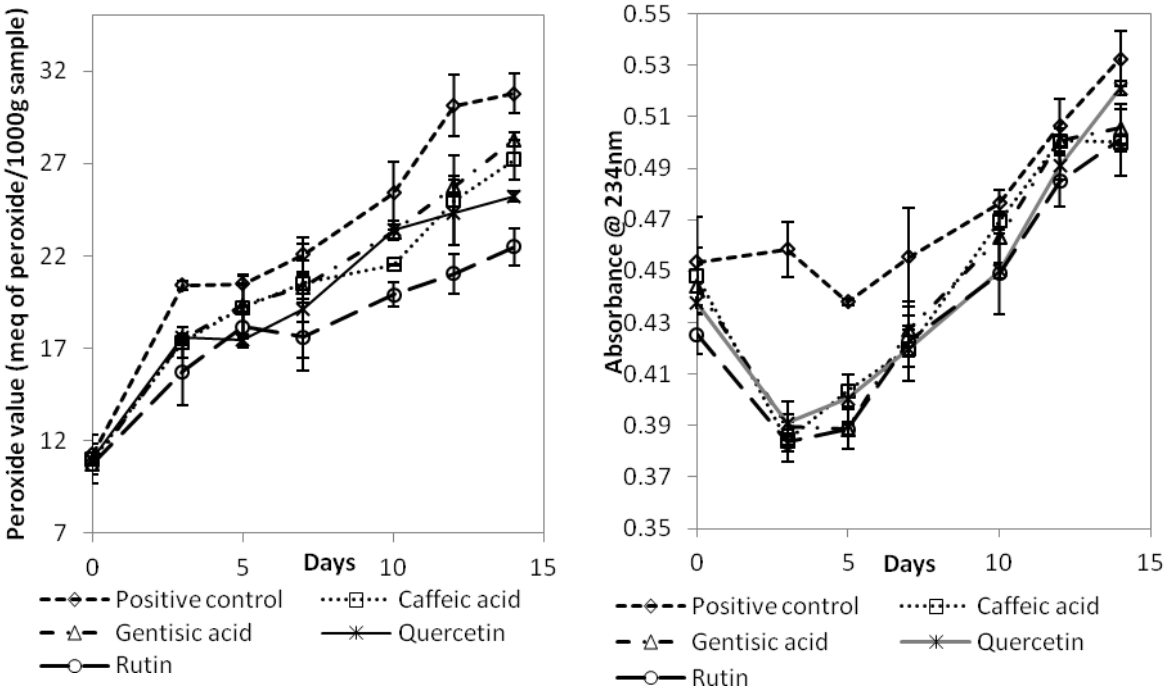



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**Figure 4.11 Effect of quercetin, rutin, caffeic and gentisic acid on the oxidative stability of sardine oil containing 0.16% (w/w) trace water (A) Peroxide value (B) Conjugated diene value.**

On analysing the effectiveness of the chosen antioxidants on conjugated diene value, it was found that all antioxidants gave a reasonable reduction in CD value till 7$^{th}$ day of oxidation (Figure 4.11B). After which, there was a sudden and drastic increase in the CD value, reaching a value similar to the control (Fig. 4.11B). This could be due to the fact that, as the storage time continues, the moisture content either increases or decreases due to relative humidity leading to the inability of antioxidants to associate better (Kim et al. 2015). This further throws some light on the compartmentalisation theory proposed by Pinchuk and Lichtenberg (2016). Thus at the end of storage period, caffeic acid, gentisic acid, quercetin and rutin showed similar reduction in CD value of 6%, 4.9%, 2% and 5.8% respectively (Table 4.3). However, more studies with different weight percent of trace water has to be carried out to confirm the effect of water on oxidation.

**Table 4.3 Effectiveness of antioxidants in improving the oxidative stability of sardine oil containing 0.16% (w/w) trace water at the end of 14th day storage**

| Antioxidants | % Decrease in the oxidation of sardine oil containing trace water | | | |
|---|---|---|---|---|
| | Peroxide value | p-Anisidine value | CD value | TBARS value |
| Caffeic acid | 11.54±3.50[c] | Nil | 6.01±2.39[a] | 14.35 ±2.43[b] |
| Gentisic acid | 8.08 ±1.22[c] | Nil | 4.98±1.72[a] | Nil |
| Quercetin | 18.05±0.93[b] | Nil | 2.06±0.53[b] | 9.18±1.62[c] |
| Rutin | 26.89±3.24[a] | Nil | 5.82±0.26[a] | 28.15±0.81[a] |

Percentage decrease was calculated by considering control to be 100% oxidised.

[a,b,c,d] values with the same letter in each column were not significantly different ($p$>0.05).

Nil – Insignificant

#### 4.2.5.2.2. Secondary oxidation

The secondary oxidation of sardine oil in containing trace and antioxidants is shown in Figure 4.12. The p- anisidine value of all the samples were high regardless of the type of antioxidant used (Fig. 4.12A). Thus at the end of storage period, none of the antioxidants showed any significant reduction in the pAV. This is similar to the results obtained by Sorensen et al. (2008) while determining the oxidation of oil-in water emulsion. Hence, it can be concluded that, addition of water alters the physical properties of oil to behave similar to that of emulsions.

Similarly, the TBARS value of all samples followed the same trend as well (Figure 4.12B). Only rutin showed a mild antioxidant activity of 28.1% at the end of 14 days storage period, while caffeic acid and quercetin showed 14.35% and 9.18% reduction respectively (Table 4.3). However, gentisic acid failed to show any reduction showing the same TBARS value as the control at the end of storage.

Thus it can be concluded that only rutin was able to retain some of its effectiveness in improving oxidative stability in the presence of added moisture. However, it failed to

show any significant improvement in the pAV ($p>0.05$) which suggests that presence of minute amount of water alters the oxidation mechanism. The ability to show the same effectiveness in reducing TBARS value like that in peroxide value could also indicate that some secondary oxidative products results directly from hydroperoxides (Table 4.3)

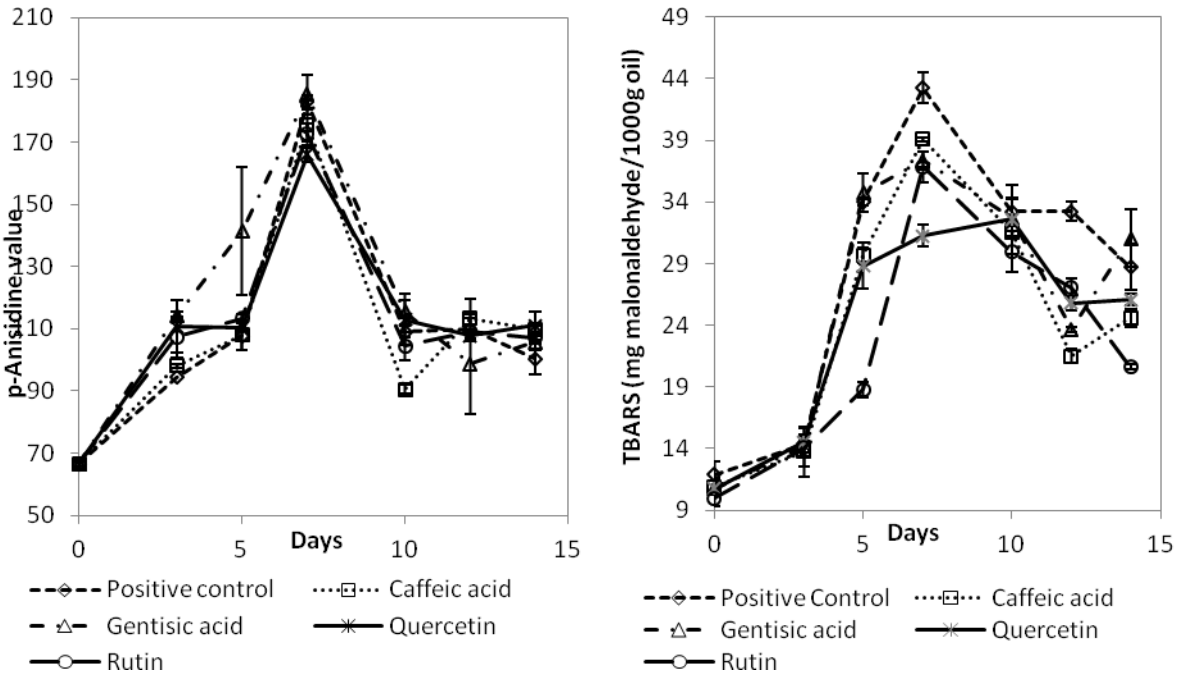


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**Figure 4.12 Effect of quercetin, rutin, caffeic and gentisic acid on the oxidative stability of sardine oil containing 0.16% (w/w) trace water (A) p-Anisidine value (B) TBARS value**

## 4.3. SUMMARY AND CONCLUSIONS

Oxidation is a major challenge for utilisation of n-3 PUFA rich marine oils for human consumption. The existing chemical antioxidants have already lost their scope due to their suspected role in carcinogenesis. Hence search for new antioxidants of natural origin has provided wide research scope and opportunities to improve the oxidative stability of such oils. On studying the effect of several natural phenolic antioxidants

in improving the oxidative stability of sardine oil, the following conclusions were derived.

- Analysis of antioxidant capacity of the nine chosen antioxidants by *in vitro* techniques indicated that all flavonoids were excellent radical scavengers.
- Phenolic acids with hydroxyl substitution showed higher radical scavenging ability.
- Of the nine chosen antioxidants, quercetin and rutin followed by gentisic acid and caffeic acid were most effective in arresting oxidation of sardine oil.
- The overall high performance of quercetin in improving the oxidative stability of sardine oil was attributed to its radical scavenging property.
- The major mechanism of oxidation in sardine oil was concluded to be peroxide radical formation.
- Oxidation in sardine oil seemed to follow a different and faster route to oxidation, when ferrous chloride was added to oil.
- The improvement of oxidative stability by the antioxidants greatly increased when oxidation was influenced by ferrous chloride addition.
- The effectiveness of rutin, quercetin, caffeic acid and gentisic acid in improving the oxidative stability was not affected by prooxidant iron addition.
- While studying the effectiveness of antioxidants in sardine oil containing trace water, it was identified that a total trace water content of 0.16% influenced the antioxidant effectiveness.
- Only quercetin and rutin showed very mild antioxidant capacity in sardine oil containing trace water.

# CHAPTER 5

# ENZYMATIC MODIFICATION OF PHENOLIC ANTIOXIDANTS FOR THEIR APPLICATION IN SARDINE OIL

Results from the previous chapter indicated that, as the heterogeneity of the sardine oil increased, the effectiveness of antioxidants reduced. This was attributed to their inefficient association at the oxidation compartment. Hence, attempts were made to improve the properties of antioxidants for their efficient interaction at the site of oxidation.

The effectiveness of an antioxidant depends on their chemical reactivity and their interaction with food components, which in turn depends on their physical location in the food system (Ramadan 2012). The "polar paradox" theory signifies the importance of physical location of the antioxidant. According to this hypothesis, hydrophilic antioxidants are effective in bulk oil, while lipophilic antioxidants perform well in emulsions (Zhong and Shahidi 2012a, b). However, this theory has recently faced challenges, as some studies report inconsistent results. This was attributed to the wide variation in the fatty acid and triglyceride content in bulk oil systems (Vaisali et al. 2015). The sardine oil used in the current work is one such heterogeneous system.

Since the biggest concern in using natural antioxidants is their solubilisation in lipid systems, the best approach will be to modify the solubility properties of the antioxidant, to interact better at the site of oxidation. The effectiveness of the antioxidants studied in the previous chapter were relatively low when the heterogeneity of the medium increased and it was concluded that it was due to inefficient association of antioxidants at the site of oxidation. Hence, improving the solubility properties of antioxidants could improve their antioxidant activity in oil. This could be achieved by bringing together a fatty acid and the phenolic antioxidant into a single entity (Roby et al. 2015). The resulting phenolic esters were proven to improve solubility properties in oil (Figueroa-Espinoza et al. 2013). Though chemical synthesis of phenolic esters is feasible, the non-regioselective nature of acylation and high reaction temperature (Xiao-na et al. 2012), has rendered this chemical method unpopular.

Hence, this chapter aims at the synthesis of antioxidant fatty ester. Further, the current work endeavours to understand and compare the effectiveness of the native antioxidant with its corresponding lipophilic ester in reducing oxidation in sardine oil.

## 5.1. MATERIALS AND METHODS

### 5.1.1. Materials

*Candida antartica* lipase immobilised on acrylic resin with ≥ 5000 U/g was purchased from Sigma Aldrich, India. Caffeic acid, gentisic acid, quercetin and rutin, 1,1,3,3–tetramethoxypropane (malondialdehyde) and p-anisidine was purchased from Sigma Aldrich, India. Sodium thiosulphate, potassium iodide, starch, thiobarbituric acid, trichloroacetic acid, decanoic acid, acetone and *tert*-butanol, were purchased from Loba chemie, India and were of analytical grade. Molecular sieves 3Å were purchased from Sisco Research Laboratories, India. All solvents were purchased from Merck, India and were of HPLC grade. Water employed for HPLC and LC-MS analysis was of MS grade and purchased from Sigma Aldrich, India.

### 5.1.2. Drying of reaction components

Both rutin and decanoic acid were dried using silica gel in desiccators for more than one week. Acetone and *tert*-butanol was dried for 5 days using 150 g/L of molecular sieves 3 Å. Enzyme was used without any drying.

### 5.1.3. Synthesis of phenolic esters

The synthesis of phenolic esters was carried out similar to Ardhaoui et al. (2004) with slight modifications in 30 mL glass vials with screw cap tubes. Measured quantities of previously dried rutin (50 mM) and decanoic acid (200 mM) were dissolved in known quantities of dried solvents so that the final molar ratio was 1:4 (antioxidant: fatty acid) and the total reaction volume was 5mL. The catalysis reaction was initiated by the addition of 75 mg of immobilised lipase enzyme to the reaction mixture. The mixture was then kept at constant agitation at 150 rpm and 55 °C for a period of 96 hours in orbital shakers. These conditions were maintained throughout the study, unless otherwise specified. All experiments were done in duplicates. Care was taken

to avoid solvent evaporation by sealing the vials. The samples were analysed using HPLC and HPLC-ESI-MS. While studying the effect of process parameters, single parameter was altered at a time by keeping the other conditions constant.

### 5.1.4. Water activity adjustment

The water activity of the system was altered by pre-equilibration of the reactants and enzyme prior to synthesis. The reactants and enzyme were placed in a large closed container consisting of small volume of saturated salt solutions based on the required water activity: KOH ($a_w$ = 0.07), $CH_3COOK$ ($a_w$ = 0.23), NaCl ($a_w$ = 0.75). The reactants were kept in respective containers for a period of three days to reach equilibration.

### 5.1.5. Analytical methods

The synthesis of esters was monitored using HPLC and further confirmed by HPLC-ESI-MS. HPLC analysis was done using Shimadzhu HPLC unit containing quaternary pump system, equipped with online degasser, column heater and a RP- C18 column (250mm × 3 mm and 5µm particle size). The injection volume was kept at 20 µL. The separation of the reaction components was performed using the following gradient system: water (A), acetonitrile (B) and methanol (C) at a flow rate of 1mL/min: 0 min (52.5% A, 30% B and 17.5 % C), 5 min (4% A, 56% B and 40 % C), 7.5 min (4% A, 56 % B and 40% C), 10 min (52.5% A, 30% B and 17.5%C) 15 min (52.5% A , 30% B and 17.5%C). The quantification of the reactants and products were done at 254 nm. The percentage conversion was calculated as the area of ester peak divided by combined area of ester peak and antioxidant peak, multiplied by 100. Following quantification, the product was ascertained by HPLC-ESI-MS detector system (Shimadzhu) with electron spray ionisation (ESI) at both positive and negative mode for identification of fractions. The MS conditions were as follows: interface temperature 350 °C, DL temperature 250 °C, nebulising gas flow 1.5 L/min., heat block temperature 200 °C and drying gas flow 15 L/min. Sampling was averaged over a *m/z* range of 100-1000 amu.

### 5.1.6. Preparation and purification of lipophilic rutin fatty ester

After esterification reaction, enzyme molecules were separated from the mixture by filtration. In order to obtain a relatively pure rutin fatty ester from the mixture containing unreacted rutin and fatty acid along with lipophilic rutin, a two-stage solvent extraction was employed. Initially, the reaction solvent was removed by evaporation under vacuum in rotary evaporator. The mixture was then washed consecutively for three times with 1:1 hexane: water (v/v) at room temperature. The hexane phase was removed after centrifugation at 3000 rpm for 5 mins and the subsequent water phases were pooled. Next, liquid-liquid extraction using ethyl acetate: water (1:2 v/v) was performed. The purity of the product was analysed using HPLC-ESI-MS system by a method described above. Ethyl acetate was then allowed to evaporate under vacuum by rotary evaporator. The purified fatty ester was stored in refrigeration at 4 °C for further use.

### 5.1.7. Oxidative stability studies

The studies on effectiveness of rutin and its ester on improving the oxidative stability of sardine oil and sardine oil with trace water was performed as described in the previous chapter (Chapter 4). All the oxidation experiments were triplicated. The measurement of oxidation was determined by analysing the samples for peroxide value, p-Anisidine value (pAV), conjugated diene and thiobarbituric acid reactive substance (TBARS) value.

## 5.2. RESULTS AND DISCUSSION

Of the several antioxidants available, four antioxidants that performed well in imparting oxidative stability to sardine oil (Vaisali et al. 2016) was chosen for esterification. Modification of phenolic compounds was done to enhance the hydrophobicity in such a way that the modified compounds show better solubility in aprotic media (Figueroa-Espinoza and Villeneuve 2005). Due to their amphiphillic nature, these functionalised phenolic compounds have the ability to accumulate at the oil-water interface, thus increasing the effectiveness of the antioxidants. However,

care was taken to retain the antioxidant activity while improving the hydrophobicity of the antioxidant.

Based on several literatures, immobilised *Candida antartica* lipase was chosen as the catalyst for the synthesis of lipophilic phenolic esters. Considering the ease of separation and non-aqueous nature of the reaction, immobilised lipase was chosen. Though several literatures are available which involve the solvent free synthesis of the desired compounds, the efficiency of conversion in such a system was low (Vosmann et al. 2006, Giraldo et al. 2007, Baeza-Jimenez et al. 2014). Hence, synthesis of phenolic esters was attempted in the presence of solvents.

In case of non-aqueous reactions, the nature of solvents used as reaction medium is of paramount importance. The type of solvent determines the medium hydrophobicity and they affect the enzyme catalysis directly or by influencing the solvation of reactants and products (Adlercreutz 2008). In addition, the influence of the type of organic media depends on the type of substrates used (Chebil et al. 2007b). For biocatalytic synthesis of phenolic esters, the primary concern is to find a solvent that will solubilise both polar antioxidant compound and non-polar fatty acid without denaturing lipase. Hence, two solvents viz. acetone (log *P* value -0.23) and *tert*-butanol (log *P* value 0.6) were chosen based on the solubility data of phenolic compounds and fatty acid (Chebil et al. 2007b). Considering the adverse effect of excess water content in non-aqueous reactions, all reaction components except lipase were dried prior to ester synthesis. Water activity of the reaction system was not controlled during the course of reaction, as molecular sieves were not used. Table 5.1 displays the conversion of four phenolic antioxidants by immobilised *Candida antartica* lipase in acetone and *tert*-butanol solvent system. Though it was endeavoured to esterify all the four antioxidants that performed with relatively better efficiency in sardine oil, only rutin showed good conversion of 51.9% and 14.4% in acetone and *tert*-butanol respectively, after 96 hours of reaction (Table 5.1). The low yields of caffeic acid could be attributed to the structure of the phenolic compound and the position of hydroxyl group participating in the reaction (Guyot et al. 1997, Figueroa-Espinoza et al. 2013).

**Table 5.1 Percentage (%) conversion of different phenolic compounds to their corresponding ester in acetone and *tert*-butanol solvent**

| **Phenolic compound** | **Conversion (%) after 96 hours of reaction** | |
|---|---|---|
| | **Acetone** | ***tert*-butanol** |
| Caffeic acid | Traces | Traces |
| Gentisic acid | 3.4 | 2.9 |
| Quercetin | Traces | 0.21 |
| Rutin | 51.98 | 14.40 |

In case of caffeic acid, the presence of double bond on the side chain conjugated with the aromatic ring and the para hydroxyl group inhibits the lipase (Guyot et al. 1997) and is consistent with the results obtained by Yang et al. (2012). Also, the presence of one or more hydroxyl/methoxy group also reduces the lipase activity considerably (Vosmann et al. 2006). In case of gentisic acid, though literature suggests the lack of esterification reaction due to the inhibiting effect of electron donating substituents (Buisman et al. 1998, Guyot et al. 1997), HPLC profile indicated the formation of gentisic ester product (Figure 5.1A) which was further confirmed by ESI-MS (Figure 5.1B). However, the production was not reproducible with considerable efficiency, and the product formation did not exceed 4% conversion (Table 5.1). This is consistent with the results obtained by Buisman et al. (1998) who obtained only 2% conversion in benzoic acid derivatives.

It was noted that, gentisic acid eluted at 3.5 minutes, while gentisic acid decanoic ester eluted at 5.7 minutes. Since the major basis of separation was size and polarity, the phenolic ester eluted later than native compound in reverse phase chromatography. On analysis of the two peaks in ESI-MS, it was noted that there was a significant peak at the m/z of 307 (Figure 5.1B). On esterification of one molecule of gentisic acid having a molecular mass of 154 g/mol with one molecule of deacanoic acid of molecular mass 172 g/mol, an ester of molecular mass of 308 can be expected along with the removal of water molecule. Thus, a negative m/z peak at 307 indicates the gentisic acid ester presence after the reaction.

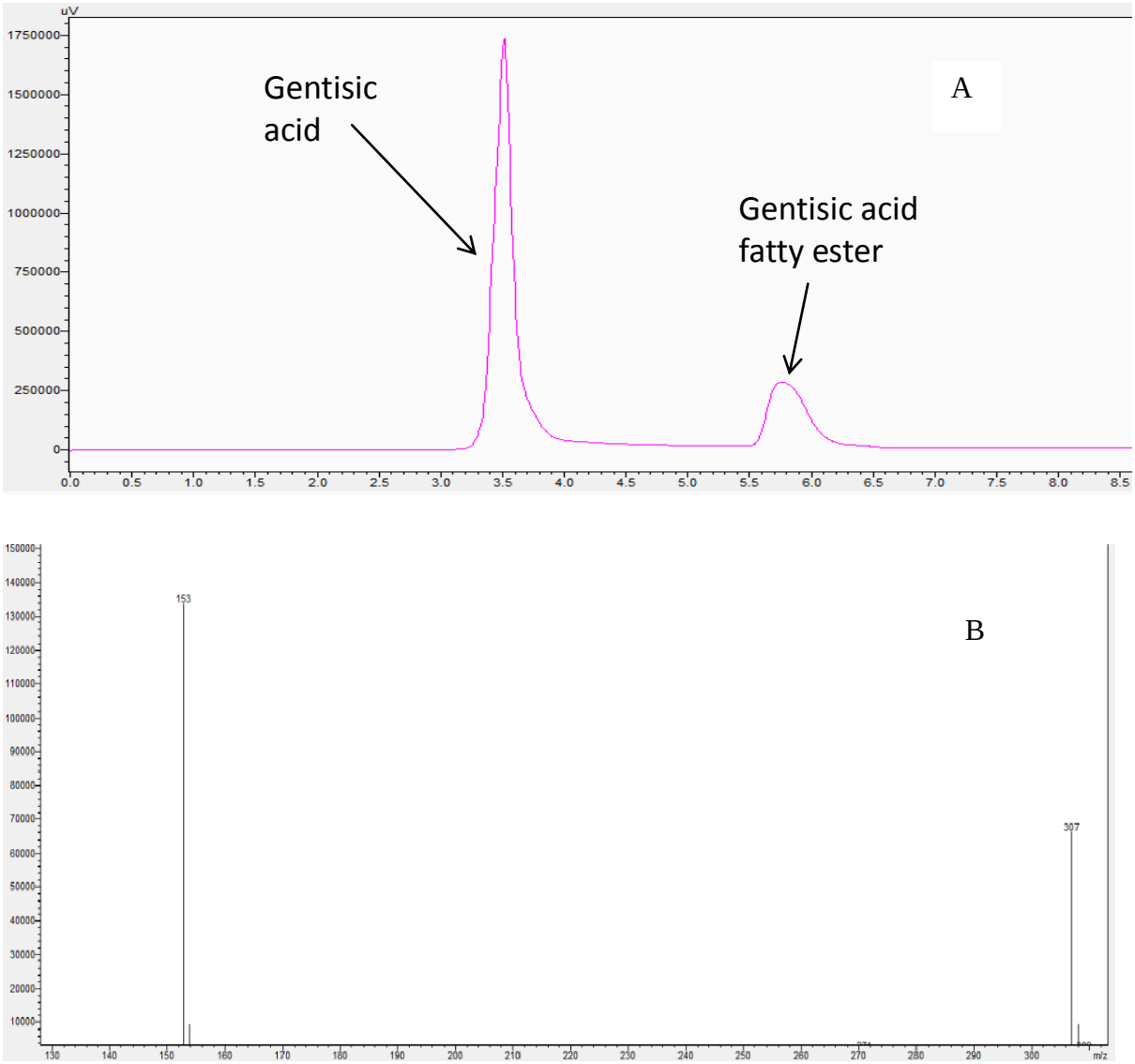



**Figure 5.1 (A) HPLC chromatogram at 254nm of reaction mixture of lipase catalysed esterification of gentisic acid and decanoic acid over a period of 96 hours. Peaks were identified as follows: gentisic acid (3.5 minutes) and gentisic acid decanoic ester (5.6 minutes). (B) Mass spectra of gentisic acid (153 m/z) and gentisic acid decanoic ester (307 m/z) scanned at the negative mode using HPLC-ESI-MS.**

In case of quercetin, product formation was observed only in trace level (Table 5.1) Though several literature are available on the successful enzymatic synthesis of quercetin esters (Xiao-na et al. 2012, Mainini et al. 2013, Kumar et al. 2016), no reports are available on the synthesis of quercetin esters using *Candida antartica*

lipase. This strengthens the fact that *Candida antartica* lipase is effective in acylating only glycosylated flavonoids, while lipase from *Pseudomonas cepacea* and Rhizopus *oryzae* were able to acylate aglycon forms (Chebil et al. 2006; Kumar et al. 2016). Hence, rutin which was the only antioxidant that showed conversion to rutin fatty ester was used for further studies.

For their application in PUFA rich oils, several studies in literature on enzymatic acylation with fatty acids and aromatic acids have been performed (Chebil et al. 2006). The major works on enzymatic acylation of flavonoids has been done with glycosylated flavonoids with meagre reports on aglycon form. The literature on enzymatic synthesis of rutin ester is discontinuous. While some studies focused on the regioselectivity of acylation (Kontogianni et al. 2001; Kontogianni et al. 2003; Chebil et al. 2007a), several others aimed at understanding the nature of substrates, type of lipase origin and operating conditions including water activity of the system on esterification (Ardhaoui et al. 2004, Duan et al. 2006, Chebil et al. 2007a). Considering the significance of this particular work, reports on the purification (Lue et al. 2010) and application of rutin esters (Viscupicova et al. 2010, Mbatia et al. 2011) started emerging. However, the technical gap on the high significance of solvent polarity was never addressed. Such a gap was also reported in a review paper by Chebil et al. (2006) and still remains unaddressed. This ambiguous data in literature emphasize the need for a detailed study on the effect of process variables on the conversion of flavonoid to its corresponding ester with reference to hydrophobicity of solvent system used. As there is a large choice of solvent systems available to carry out enzymatic synthesis of antioxidant esters, finding the influence of hydrophobicity of solvents on the overall synthesis, helps in arriving at a solvent system which is optimal.

### 5.2.1. Enzymatic synthesis of rutin fatty ester

The enzymatic synthesis of rutin fatty esters (Figure 5.2) was performed similar to several existing methods with slight modifications (Ardhaoui et al. 2004, Kontogianni et al. 2003). It has been reported that the conversion rate of phenolic compounds decreased with increasing chain length of the acyl donor (Katsoura et al. 2006, Lue, et

al. 2010). Also, the free radical scavenging activity of the long chain esters were also found to be low. Hence, a medium chain fatty acid, namely decanoic acid (C10) was chosen for the current study. The reaction was monitored using HPLC, and the typical HPLC chromatograms of the reaction mixture are shown in Figure 5.3.

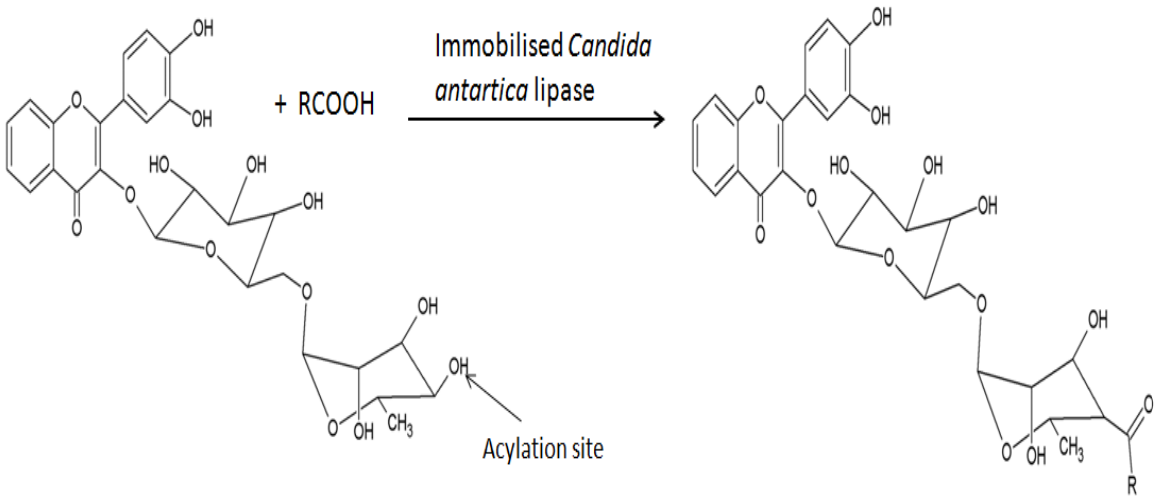



**Figure 5.2 Schematic representation of esterification of rutin with fatty acid**

The rutin peak was identified using standard rutin. Rutin eluted as dual peaks (Figure 5.3), at a retention time of 2.8 minutes. This is similar to the results obtained by Duan et al. (2006), who claimed that one peak corresponded to rutin while the other was an impurity of the flavonoid in the raw material of rutin. The shift in the retention time of rutin after reaction with decanoic acid was an indication of the product formation (Figure 5.3). The retention time of the product was found to be 9.7 minutes. Rutin fatty ester was relatively hydrophobic than pure rutin, that makes the retention time longer in case of a reverse phase chromatography.

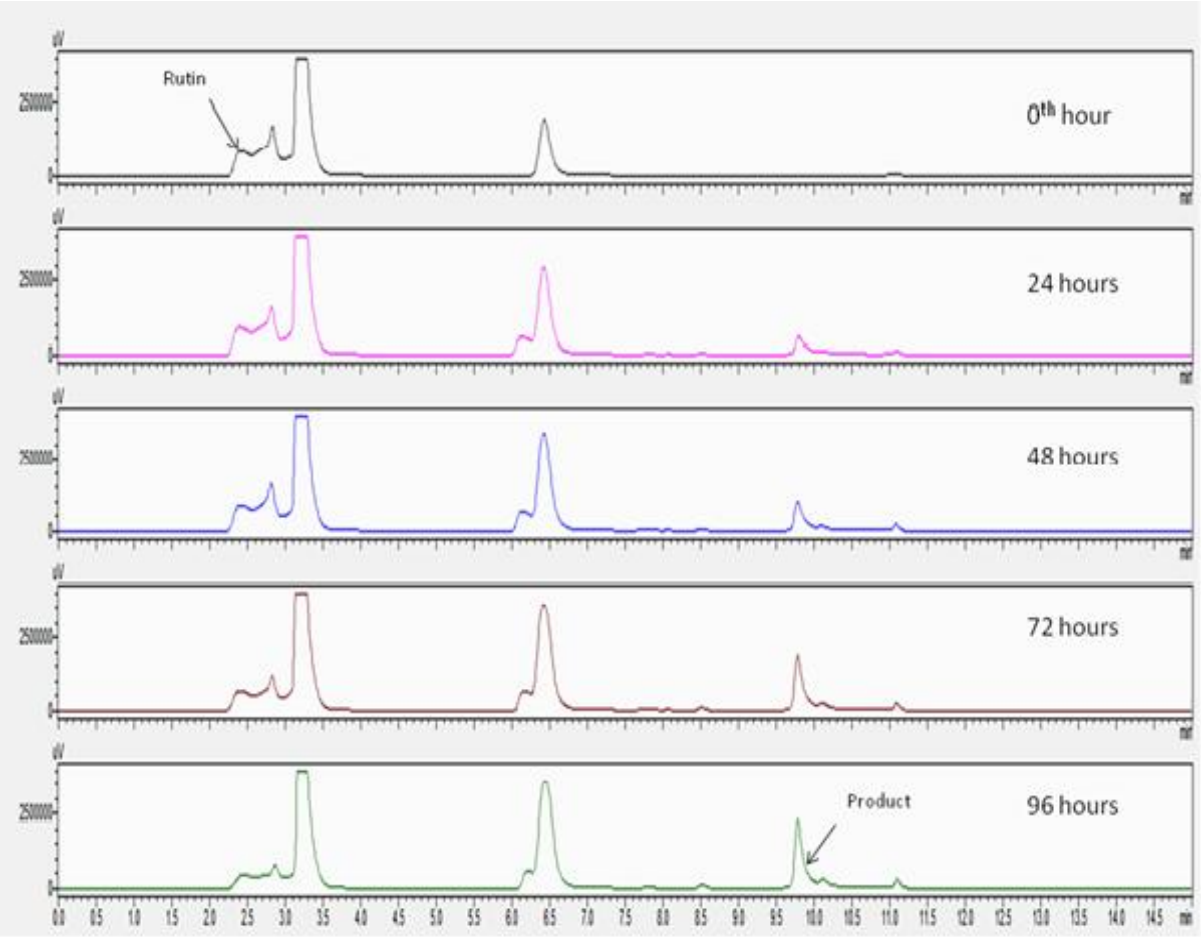



**Figure 5.3 HPLC chromatograms at 254nm of reaction mixture of lipase catalysed esterification of rutin and decanoic acid over a period of 96 hours. Peaks were identified as follows: rutin (2.8 minutes) and rutin decanoic ester (9.7 minutes)**

The product was further ascertained by HPLC-ESI-MS (Figure 5.4). Based on the electron spray ionisation mass spectroscopy (ESI-MS), the synthesised product was found to be monoacylated rutin ester (Figure 5.4). The MS data of the rutin peak indicated a principle signal at *m/z* 609 and two significant fragments at *m/z* 301 and *m/z* 463 (Figure 5.4) indicating the presence of a quercetin and quercetin 3-o-rhamnoside respectively in rutin structure. The analysis of the product peak gave a strong signal at *m/z* 763, indicating that rutin was acylated with single decanoic acid molecule, preferably at the hydroxyl group of the second rhamnoside group (Figure 5.2) (Mbatia et al. 2010, Razak and Annuar 2015).

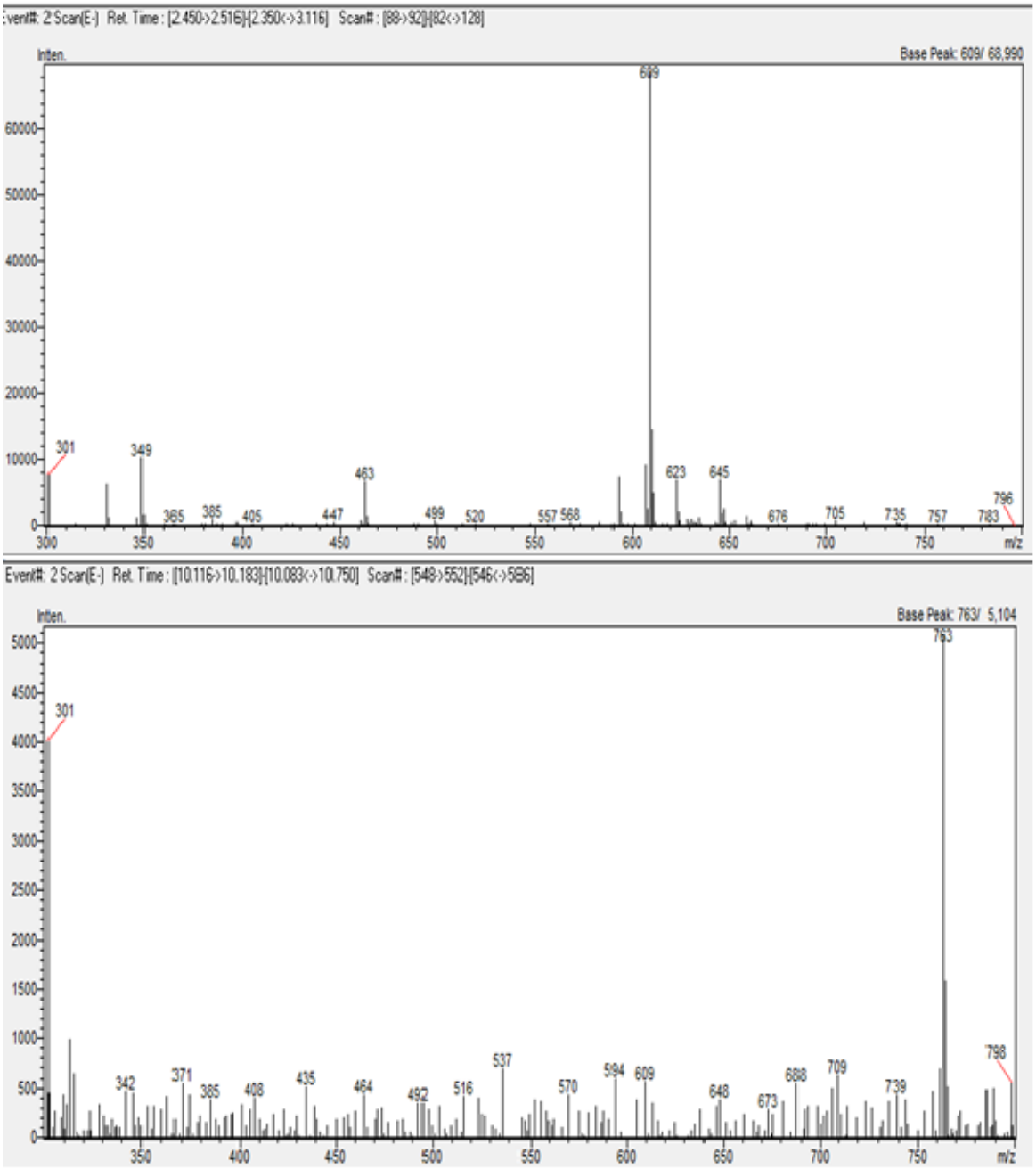



**Figure 5.4 Mass spectra of (a) rutin (b) rutin decanoic ester scanned at the negative mode using HPLC-ESI-MS.**

### 5.2.1.1. Effect of solvent

The reaction rates of rutin ester synthesis varied depending on the solvent, with acetone showing higher reaction rates that increased till 96 hrs of reaction after which

it remained constant (Figure 5.5). Additionally, the percentage conversion was 52.14% and 13.02% in acetone and *tert*-butanol solvent respectively after 96 hours of reaction (Figure 5.5). The main determining factors on solvent influence are the log *P* and dielectric constant. It was noted that there was a negative correlation of solvent polarity with conversion yields which is consistent with the results obtained by Li et al. (2015) when dihydromyricetin was esterified with vinyl acetate using immobilised lipase from *Penicillium* sp. Furthermore, it has been indicated that high dielectric constant is favoured for higher conversion (Chebil et al. 2007a). These observations in literature are consistent with the results obtained in the current study, as the more polar *tert*-butanol with lower dielectric constant gave lower reaction rate and lesser conversion in comparison to acetone.

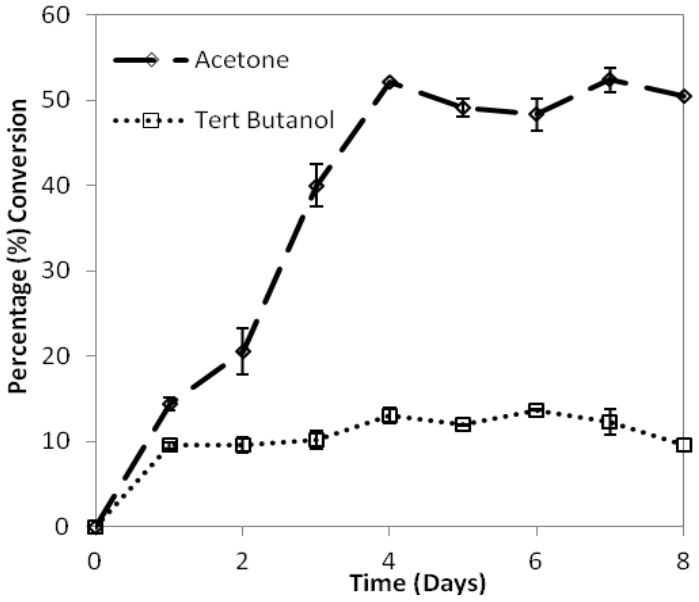



**Figure 5.5 Lipase-catalysed esterification of rutin with decanoic acid over a period of eight days at 55°C, 150 rpm with 15mg/mL enzyme load and 1:4 fatty acid: rutin molar ratio without setting water activity. Bars represent standard deviation (n=2)**

In acetone, the conversion to rutin fatty ester increased during first 96 hours of reaction after which it remained constant (Figure 5.5). This is similar to the results obtained by Karboune et al. (2005), where the conversion to cinnamic ester increased

during the first 3 days of reaction. Nevertheless, the synthesis of rutin ester increased in the first 24 hours in *tert*-butanol media (Figure 5.5) after which it remained constant with no significant change.

### 5.2.1.2. Effect of enzyme load

The effect of enzyme load (2.5 - 20 mg/mL) on the acylation of rutin with decanoic acid was evaluated. With increasing lipase load from 2.5 to 10 mg/mL, enhancement to the extent of 42.36% to 58.63% conversion in acetone system was noticed (Figure 5.6). This was due to the increase in the active sites available for reactants. However, with further increase in the enzyme load to 15 mg/mL, conversion reduced to 40.9 % (Figure 5.6). This could be attributed to the overcrowding of immobilized enzyme beads that resulted in reduction of effective collision among the molecules (He et al. 2012, Wang et al.2015). Thus, the mass transfer rate between substrates and enzymes reduces drastically, yielding poor esterification. Another possible reason could be the increase in by-product water formation with higher concentration of lipase, resulting in the inability of hydrophobic acyl donor to reach the enzyme active site (Giraldo et al. 2007).

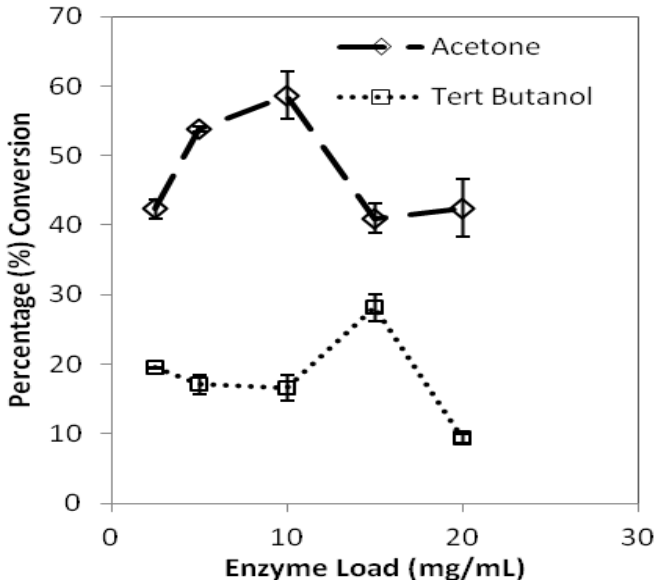



**Figure 5.6 Effect of enzyme load on the rutin decanoic acid ester synthesis**

The optimal concentration of lipase for acetone was found to be 10 mg/mL. In case of *tert*-butanol system, ester formation remained same with no significant difference from 2.5 to 10 mg/mL unlike acetone system (Figure 5.6). Maximum conversion (28.15 %) was found at 15 mg/mL enzyme load (Figure 5.6). This marginal difference in the enzyme load requirement in two different solvent systems could be associated to the water liberated during esterification to act as limiting factor on enzyme catalysis that further depends on the hydrophobicity of the medium.

### 5.2.1.3. Effect of molar ratio

Considering the varying effect of enzyme load in different solvent systems on rutin conversion, the molar ratio of the substrates was verified for each solvent system. Since higher concentrations of acyl donor are generally preferred for esterification in organic media, decanoic acid level was increased by keeping rutin concentration constant.

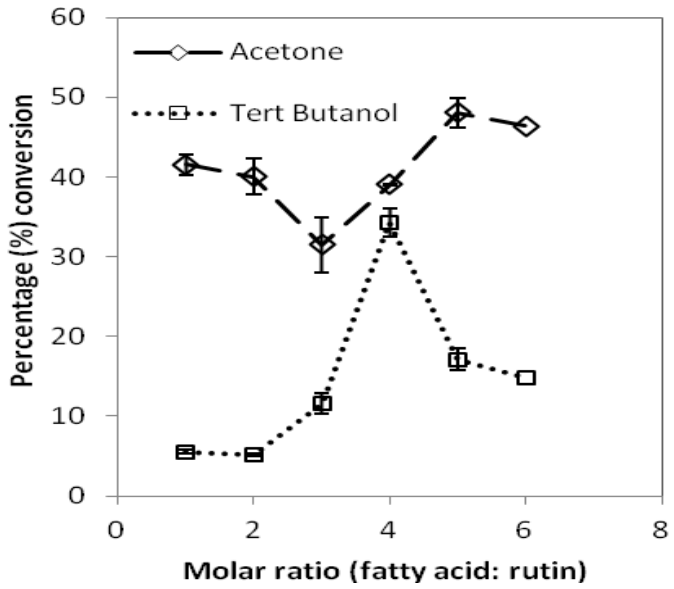



**Figure 5.7 Effect of molar ratio on the rutin decanoic acid ester synthesis**

In acetone solvent system, low percentage conversion with increase in molar ratio upto 3 was noted (Figure 5.7), after which the conversion increased to a maximum of 55.5% at a molar ratio of 5. However further increase in molar ratio resulted in the dip in conversion to 46.4% (Figure 5.7). In *tert*-butanol, increasing the decanoic acid to

rutin ratio from 1 to 4 increased the percentage conversion from 5.53 % to 34.25 % respectively (Figure 5.7). However, further increase in the molar ratio decreased the ester formation to 14.74 %. This is consistent with the results obtained by Wang et al. (2013), who noted that increasing the molar ratio increased ethyl cinnamate production till certain point, after which further increase in acyl donor concentration decreased the production significantly. Though Kontogianni et al. (2001) suggested no inhibition by higher concentrations of decanoic acid during esterification with naringin, some recent studies suggest a possibility of substrate inhibition of *Candida antartica* lipase at higher acyl donor concentrations (Zhu et al. 2014). This indicates the need for a more detailed study on the kinetics of *Candida antartica* lipase for each reaction medium.

When the purified ester was subjected to ESI-MS analysis, spectral profile showed the acylation of single decanoic acid with one molecule of rutin. Interestingly, the degree of acylation did not increase even at higher molar ratio of acyl donor to rutin in both solvents. This is in contrast to the results obtained by Chebil et al. (2007a), where increasing the concentration of vinyl acetate increased the synthesis of di- and tri-acetates of quercetin, with *Pseudomonas* sp. Lipase. This demonstrates the regioselective nature of lipase used in this study. At varying molar ratio, the obtained optimum in acetone and *tert*-butanol was different due to the slight difference in polarity between the two solvents used. As a result, the relatively polar *tert*-butanol shows optimum at lower ratio, due to its inability to dissolve higher concentration of non-polar fatty acid.

#### 5.2.1.4. Effect of reaction temperature

The reaction temperature is generally a significant factor that is responsible for the solubilities of the reactants and products, thermal denaturation of enzymes and also the viscosity of the reaction mixture (Chebil et al. 2006). As immobilised enzyme show higher thermal stability and different optimum temperature for esterification reaction than free enzymes, it is necessary to test the effects of varying temperature on the yield of rutin ester. Hence, for analysing the temperature effects on the

esterification of rutin with decanoic acid, reactions were carried out in the range of 25-60 °C (Figure 5.8) in acetone and *tert*-butanol.

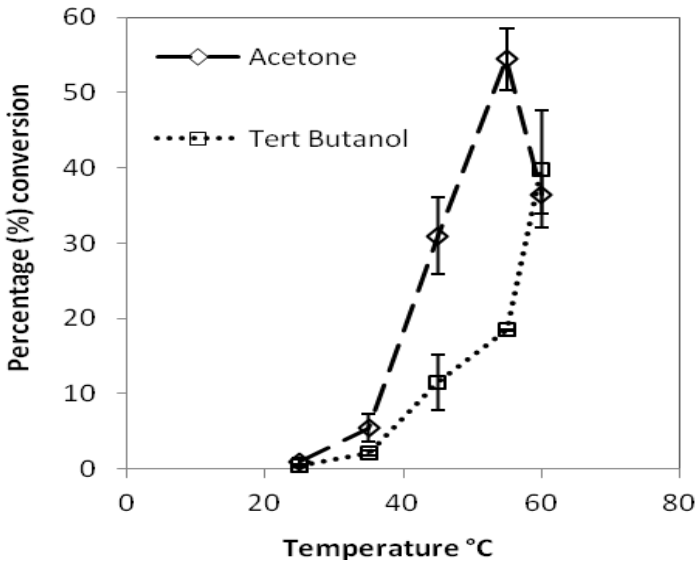



**Figure 5.8 Effect of reaction temperature on the rutin fatty acid ester synthesis**

The ester formation was found to increase linearly with increase in temperature from 25-55 °C for both solvents (Figure 5.8). This is consistent with the results obtained by Badgujar and Bhanage (2004), who established an optimum of 55 °C for the biotransformation of geraniol with vinyl acetate. This could be attributed to the reduction in density and viscosity of the reaction mixtures at elevated temperatures leading to an increase in the conversion at higher temperatures (Wang et al. 2015). From the present data it can be noted that the conversion of rutin ester in acetone system reduced significantly by increasing the temperature beyond 55 °C (Figure 5.8). This could be due to the thermal inactivation of enzyme at higher temperature as suggested by Li et al. (2015). However, *tert*-butanol system showed the maximum conversion at 60 °C, indicating the thermal stability of the immobilised *Candida antartica* lipase. These results indicate the importance of high temperature in such reactions unlike several others. For instance, the conversion was only 5.43% at a temperature of 35 °C, while the conversion increased to 55.44% with further increase in temperature up to 55 °C in acetone (Figure 5.8).

### 5.2.1.5. Effect of water activity

In case of biocatalysis in non-aqueous media, some amount of water is required to maintain enzyme stability, as the water monolayer around enzyme reduces the protein rigidity in enzyme resulting in exposed active sites (Duan et al. 2006). On the other hand, excess water can lead to unfavourable equilibrium shift to hydrolysis reaction (Humeau et al. 1995, Yang et al. 2004, Duan et al. 2006). Therefore, it is crucial to study the influence of water content in the medium that is usually quantified as water activity (Ma et al 2002). The optimum water activity of the system depends on the medium composition. In addition, the water activity of free enzyme tends to differ from that of immobilised enzyme that further depends on the type of immobilisation support. Although many reports are available on the water activity in non-aqueous media, dependence of water activity requirement on polarity of medium used was seldom studied. Hence, it is crucial to examine the molecular events occurring during the interaction between enzyme molecules, its micro-aqueous environment and surrounding organic media. Hence, the enzyme and the reaction components were allowed to equilibrate to specified water activity separately before synthesis reaction.

As the optimum levels of reaction variables varied with the type of solvent, the dependence of product formation over a range of water activity was tested for the two solvents. The effect of three different water activities on the esterification of rutin with decanoic acid in acetone and *tert*-butanol was studied (Figure 5.9). At a water activity of 0.07, the conversion in acetone showed a maximum of 53.96 %, while *tert*-butanol showed a conversion of 16.08% (Figure 5.9). This is in contrast with the results obtained by Kontogianni et al. (2003), who noted a maximum of 50% conversion at $a_w$ less than 0.1, when naringin was esterified with decanoic acid *tert*-butanol using the same immobilized lipase. This indicates the importance of the type of substrate and its influence on the optimum water activity. Thus it becomes crucial to study the influence of each process parameter and water activity that tends to vary depending on the reaction medium components.

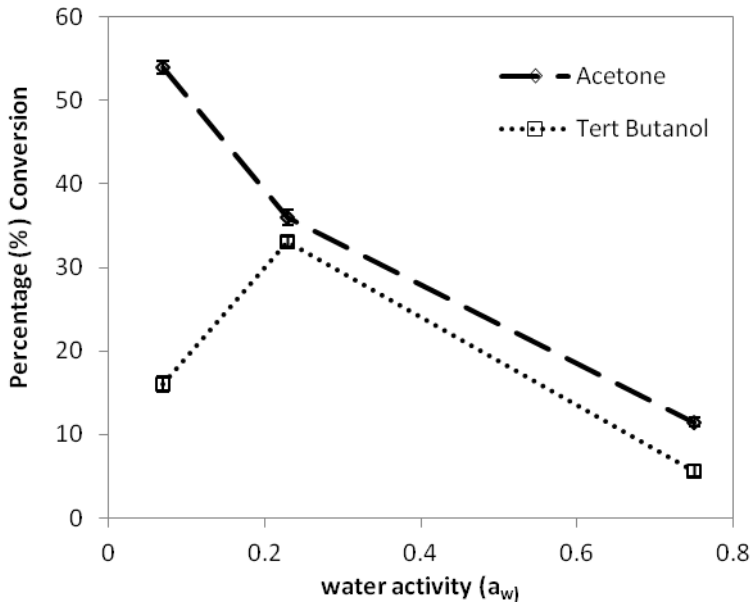



**Figure 5.9 Effect of water activity on the lipase-catalysed esterification of rutin with decanoic acid for 96 h with 15 mg/mL enzyme load and 1:4 rutin: fatty acid molar ratio at 55 °C and 150 rpm. Bars represent standard deviation (n = 2).**

The conversion was reduced to 11.48% and 5.63% at a water activity of 0.7 for both acetone and *tert*-butanol system respectively. From these results, it can be surmised that the $a_w$ of the system prior to setting of water activity was closer to $a_w$ 0.07. The mild difference in the polarity between acetone and *tert*-butanol was the reason for the difference in the optimum water activity for synthesis of rutin ester (Ducret et al. 1998). Though both solvents performed well at lower water activity, a thin monolayer of water is necessary to retain the enzyme activity (Sorour et al. 2012). Hence, acetone showed a higher activity at lowest $a_w$ of 0.07 as the stripping of water monolayer by acetone is relatively low (Figure 5.9). However, due to the polar nature of *tert*-butanol, solubilisation of the bound water monolayer occurs (Zaks and Klibanov, 1988), leading to low reaction rate at a lower activity of 0.07, indicating the need for more water molecules at the enzyme micro environment. This is further confirmed from our results, where *tert*-butanol showed highest conversion at a relatively higher water activity of 0.23 (Figure 5.9). From these results, it can be understood that optimum $a_w$ varies with the reaction medium hydrophobicity, which

in turn is affected by the type of solvent used. Though setting of water activity prior to synthesis reaction led to marginal increase in the product conversion, the water released during the progress of esterification, changes the water activity of the system ultimately affecting the product formation. Hence, it is crucial to remove the liberated water to improve the synthesis of rutin fatty ester.

#### 5.2.1.6. Effect of molecular sieves addition

In case of lipase catalysed hydrolysis reactions, reaction generally occurs in two steps. Initial step involves the formation of covalently modified acyl enzyme intermediate. This intermediate is further attacked by water (hydrolysis) or by another nucleophile (esterification) depending on the reaction medium (Ma et al. 2002). Thus, it becomes critical to maintain optimal concentration of water throughout the reaction for maximum ester synthesis. However, for every single esterification between rutin and decanoic acid, one molecule of water is released. When sufficient esterification reaction occurs, there is a build-up of the by-product water that might shift the reaction equilibrium towards hydrolysis. They further affect the initial reaction rate leading to the decrease in the overall conversion. Hence, to eliminate this problem of by-product effect on the product formation, addition of molecular sieves was proposed. Molecular sieves are generally made of zeolites and they are used to absorb or separate molecules (Duan et al. 2006). The adsorbed molecules are trapped and the pore size of these trap determine the efficiency. For instance, 3Å molecular sieves have a critical diameter less than 3Å and hence water molecules get adsorbed. In the current study, 3Å molecular sieves were added to the reaction mixture at a concentration of 150 g/L.

The lipase catalysed esterification with and without molecular sieves is represented in figure 6B. It can be noted that there was a significant increase in conversion (from 14.4% to 62.1%) in *tert*-butanol solvent system in the presence of molecular sieves (Figure 5.10), which is consistent with the results obtained by Duan et al. (2006) when rutin was esterified with stearic acid. Such increase in the conversion could be due to the adsorption of water released during the initial reactions, maintaining the equilibrium towards esterification. Nevertheless, acetone failed to show any increase

in conversion in presence of molecular sieves, instead showed a profound decrease in the conversion (Figure 5.10). This could be due to the participation of acetone in aldol condensation reaction with molecular sieves leading to self condense (Flego and Perego 2000). Another possible reason for this decreased conversion in acetone could be due to the loss of lipase activity, when the hydration layer around the enzyme is removed by molecular sieves (Sharma et al. 2014) resulting in degradation reactions. Also, the by-product water did not seem to affect the reaction rate in case of acetone, as the conversion of rutin ester was consistently high even without molecular sieve addition indicating the importance of polarity of solvent in such reactions.

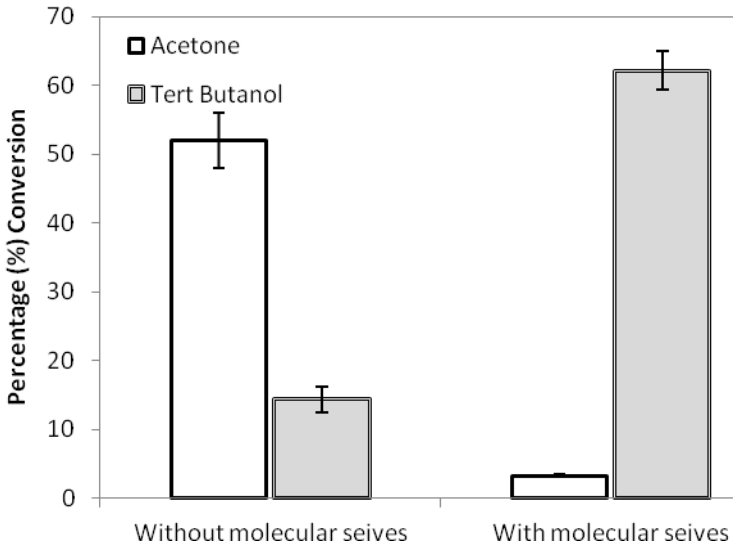



**Figure 5.10 Effect of addition of molecular sieves on the lipase-catalysed esterification of rutin with decanoic acid for 96 h with 15 mg/mL enzyme load and 1:4 rutin: fatty acid molar ratio at 55 °C and 150 rpm. Bars represent standard deviation (n = 2)**

Thus, on validation of the optimum process parameters, acetone system gave a conversion of 60.74%, when the reaction system consisted of 10mg/mL enzyme with 1:4 molar ratios of rutin and decanoic acid incubated at 55 °C for 96 h, without setting the water activity and devoid of molecular sieves (Table 5.2). However, in case of *tert*-butanol prior adjustment of water activity to 0.23, along with molecular sieve

addition of 150 mg/mL increased the conversion to 65.73% at 15 mg/mL enzyme load, 1:5 molar ratios of rutin and decanoic acid incubated at 60 °C for 96 h. From this it can be inferred that maintaining optimal water activity becomes crucial as the reaction systems becomes less hydrophobic. Hence the removal of released water by using appropriate amount molecular sieves is crucial in those systems.

**Table 5.2 Optimum conditions for maximum ester synthesis in acetone and *tert*-butanol**

| | **Optimum Conditions** | |
|---|---|---|
| **Process parameters** | **Acetone (log *P* -0.23) 60.74 % conversion** | ***tert*-butanol (log *P* 0.6) 65.73% conversion** |
| Enzyme load | 10 mg/mL | 15 mg/mL |
| Molar ratio (rutin: fatty acid) | 1:5 | 1:4 |
| Temperature | 55 °C | 60 °C |
| Water activity | 0.07 | 0.23 |
| Molecular sieves | Nil | 150 mg/mL |

From the above results it can be concluded that altering the process parameters can lead to increase in the production of rutin fatty ester. Process parameters like temperature, enzyme load and molar ratio of substrates were found to be affected by the relatively minor difference in polarity of the solvent system. This was attributed to the significant role played by minor concentration of water in enzyme catalysis. This profound effect of water on esterification was more clearly understood by the difference in optimal water activity of two closely polar solvents. Hence, it can be concluded that a more polar solvent like *tert*-butanol require a relatively higher water activity for maximum product formation than acetone. Attempts to increase the conversion by removal of by-product water lead to a two-fold increase in the product formation in *tert*-butanol system. However, the generated water failed to have higher effects in acetone, which could be attributed to its relatively less polar nature. Hence, the optimum conditions for acetone involved no manipulation of water activity resulting in 60.74% conversion, while *tert*-butanol system gave a maximum of

65.73% only on altering water activity and removal of by-product (Table 5.2). Thus the current study illustrates the need for optimisation of each process parameter for chosen organic solvent system to achieve maximum synthesis of ester, catalysed by lipase in a non-aqueous environment.

### 5.2.2. Purification of rutin fatty ester

In order to analyse the applicability of rutin fatty ester in hydrophobic medium, pure form of this compound is necessary. During the initial stages of purification, the immobilised enzyme catalyst was removed by filtration followed by the evaporation of acetone under vacuum through rotary evaporator. Many literatures suggested the use of silica column of mesh size 230-400 (Viskupicova et al. 2010, Reddy et al. 2010, Zhong and Shahidi 2011) and such separation took longer hours. Hence, a two step solvent extraction process was carried out to purify rutin ester.

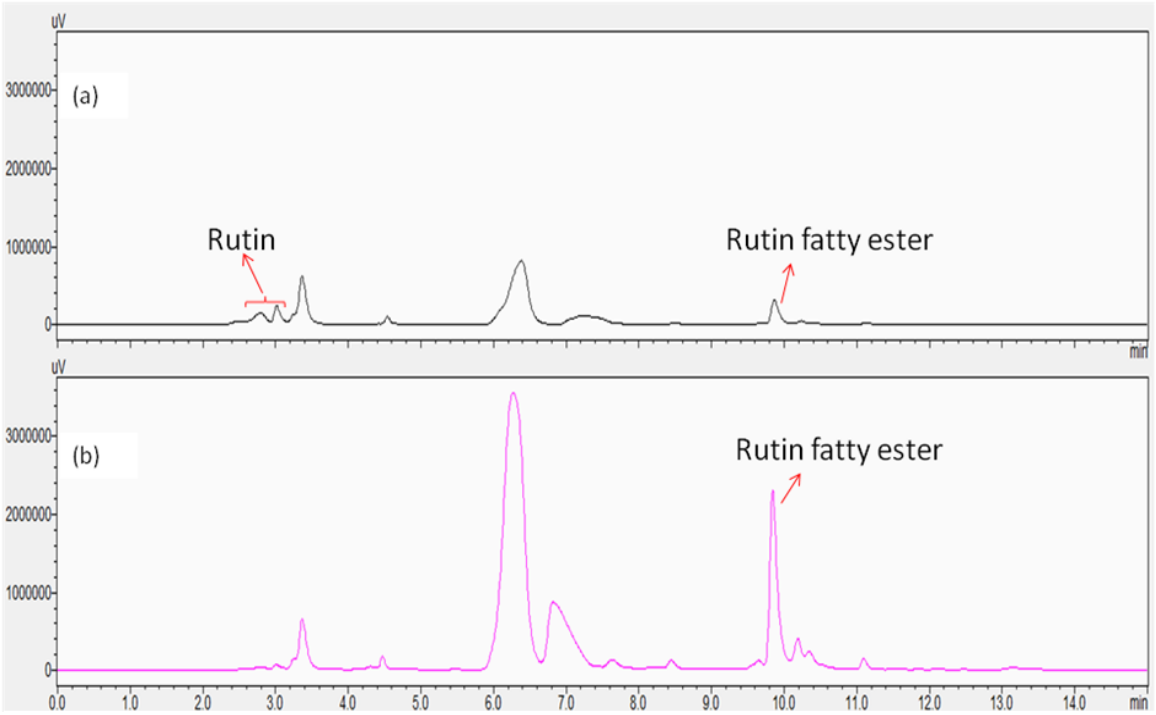



**Figure 5.11 HPLC chromatogram of rutin and rutin fatty ester (a) before purification and (b) after purification by using ethyl acetate: water**

In order to remove the unreacted fatty acid from the reaction mixture, the samples were subjected to liquid extraction at room temperature using hexane/water (1:1 v/v). Thus the unreacted hydrophobic fatty acid extracted into the non-polar organic phase. Though the same procedure could be utilised for removal of residual rutin, a report by Lue et al. (2010), indicated the inefficient separation of rutin fatty ester into the organic phase. The separation of unreacted rutin and its lipophilic ester poses many challenges unlike the separation of hydrophobic fatty acid from the mixture. This is due to the close difference in polarity of the two compounds and also due to the poor solubility of rutin in majority of the solvents (Razzak and Annuar 2015). Hence, ethyl acetate was chosen based on its intermediate polarity and tested for separating unreacted rutin. The HPLC chromatogram of sample before and after subjecting to ethyl acetate: water (1:2 v/v) extraction is shown in Figure 5.11. It can be noted that the concentration of rutin eluting at 2.8 minutes reduced effectively, while the rutin fatty ester concentration was found to improve with the removal of unreacted substrates. The only remaining peaks were found to be from the solvent traces present in the mixture

### 5.2.3. Oxidative stability studies

Research on the application of lipophilised antioxidants is mostly dedicated to *in vitro* antioxidant assays, model systems and very few vegetable oils. As these systems fail to duplicate the complex oxidation mechanisms found in fish oils during storage, the utility of *in vitro* antioxidant assays and data generated by model systems is limited. Moreover, reports are rather scarce on the effectiveness of lipophilised antioxidants in marine oils.

#### 5.2.3.1. Effectiveness of rutin fatty ester in refined sardine oil

##### 5.2.3.1.1. Peroxide value

The effectiveness of the synthesised rutin fatty esters was initially tested with refined sardine oil. The progress of oxidation in sardine oil fortified with rutin fatty ester was analysed by measuring the products of oxidation. Lipid hydroperoxides are the products of primary oxidation and they are measured in terms of peroxide value based

on their ability to oxidise potassium iodide. The effectiveness of rutin fatty ester was further compared with that of rutin. Primary oxidation of sardine oil in the presence of rutin and lipophilic rutin ester was monitored for a period of 14 days and depicted in Figure 5.12.

The peroxide value of sardine oil showed a gradual increase reaching a maximum of 23.86 meq/kg at the end of 14 days storage period (Figure 5.12). Fortification with rutin and rutin ester resulted in a similar reduction in peroxide value with no significant difference ($p>0.05$).

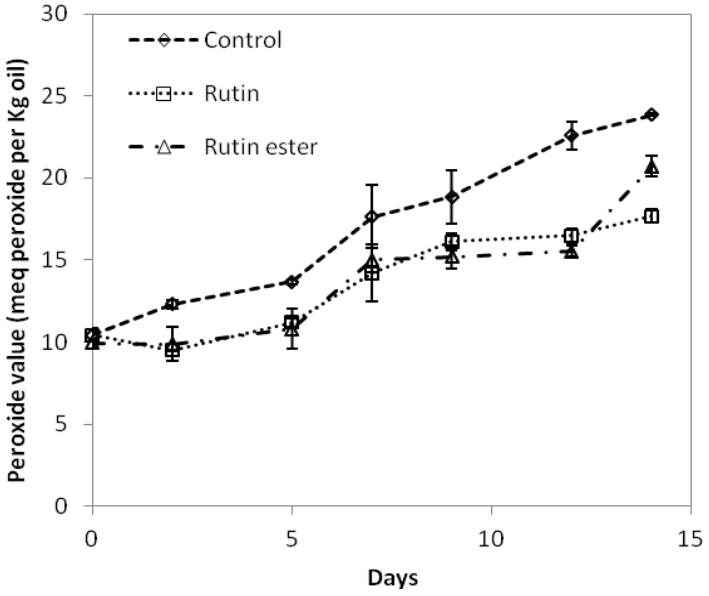



**Figure 5.12 Changes in the peroxide value of sardine oil fortified with native rutin and rutin fatty ester. Control – Sardine oil with no antioxidant addition. Bars represent standard deviation (n=3)**

As the underlying mechanism behind the reduction in oxidation by rutin is radical scavenging (Vaisali et al. 2016), it can be concluded that the radical scavenging ability of rutin was not altered on lipophilisation with decanoic acid (C10). This is similar to the results obtained by Katsoura et al. (2006), when rutin laurate (C12) and rutin showed same antioxidant capacity against low density lipoprotein (LDL) and serum oxidation. A similar conclusion was derived by Lue and team (Lue et al.

2010), when they tested rutin palmitate and rutin laurate for DPPH scavenging activity.

#### 5.2.3.1.2. Conjugated diene (CD) value

Antioxidant activity of rutin and its ester was further tested in sardine oil by analysing their ability to reduce the conjugated diene (CD) formation, that are isomeric derivatives of unsaturated lipid hydroperoxides. Similar to peroxide value rutin showed maximum reduction ($p<0.05$) in CD value throughout the storage conditions (Fig. 5.13). Interestingly, rutin ester failed to display significant antioxidant activity during the 14 days storage period (Figure 5.13). According to Lopez-Giraldo et al. (2009), the loss or reduction in antioxidant capacity of an ester derivative can be attributed to the steric hindrance from the alkyl group during oxidation. However, the ability of rutin fatty ester to exhibit antioxidant activity as indicated by the peroxide value measurement rules out the possibility of steric hindrance.

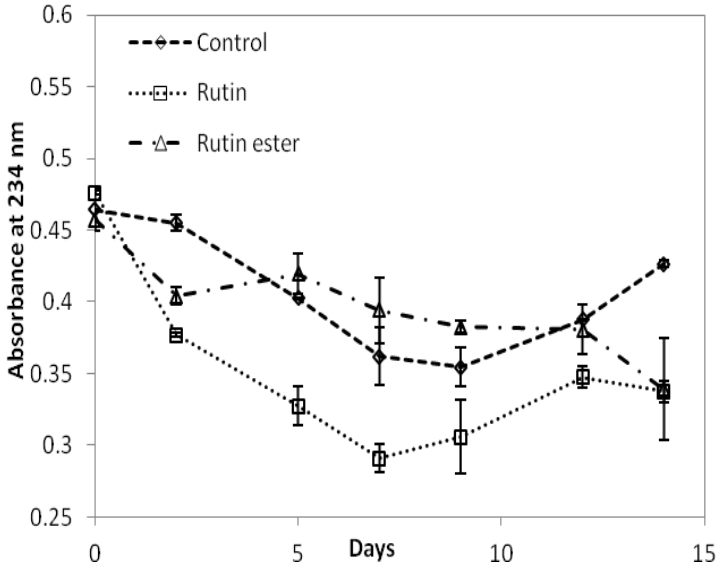



**Figure 5.13 Changes in the conjugated diene value of sardine oil fortified with native rutin and rutin fatty ester. Control – Sardine oil with no antioxidant addition. Bars represent standard deviation (n=3)**

Thus, at the 12th day of storage, rutin ester and rutin displayed an absorbance of 0.380 and 0.347 respectively, in comparison with a control value of 0.387 (Figure 5.13). Based on the results of primary oxidation in the presence of rutin fatty ester, it becomes impossible to conclusively indicate the efficiency or inefficiency of rutin fatty ester in sardine oil oxidation, as the results of peroxide value contradicted that if CD value.

#### 5.2.3.1.3. p-Anisidine value (pAV)

In order to determine the efficiency of rutin fatty ester in inhibiting sardine oil oxidation, secondary oxidation of sardine oil in the presence of these compounds were analysed. The changes in the pAV in the presence of rutin and rutin ester were compared with a control containing no antioxidants (Figure 5.14).

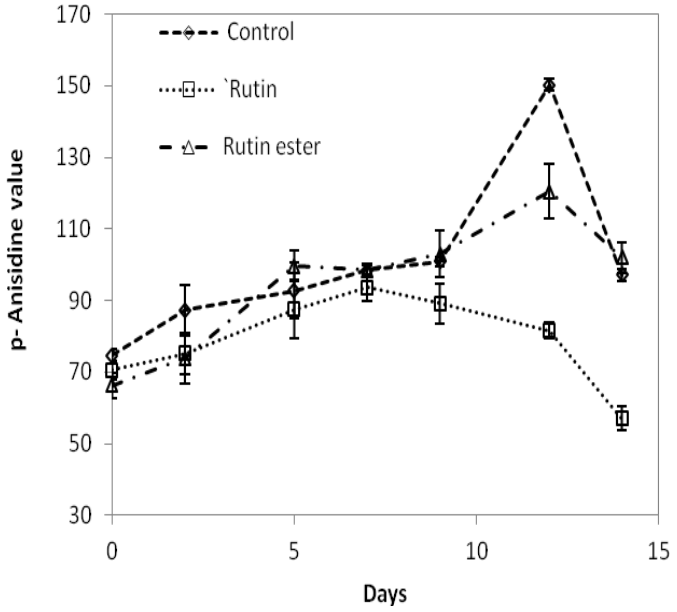



**Figure 5.14 Changes in the p-anisidine value of sardine oil fortified with native rutin and rutin fatty ester. Control – Sardine oil with no antioxidant addition. Bars represent standard deviation (n=3).**

The pAV of control increased in a relatively slow manner for a period of 10 days, after which it showed a markedly higher value. Addition of rutin ester to sardine oil failed to show better efficiency than that of samples with pure rutin (Figure 5.14). Thus under current conditions, samples without antioxidants showed a maximum value of 97.17, while those with rutin and rutin ester showed 56.99 and 101.93, respectively at the end of 14 days of storage period (Figure 5.14).

This is in contrast to the findings by Zhu et al. (2014), who noted a better antioxidant activity of acetylated epigallocatechin gallate (EGCG) than native EGCG in sunflower oil due to the homogenous dispersion of lipophilic antioxidant. Though, the antioxidant activity of rutin fatty ester was nil at the end of storage period, the extent of oxidation was found to be significantly lower ($p<0.05$) based on the peak of oxidation at the $12^{th}$ day of storage. This could indicate the inefficient association of rutin fatty ester during the course of oxidation.

#### 5.2.3.1.4. Thiobarbituric acid reactive substances (TBARS) value

In order to further confirm the efficiency of rutin fatty ester in reducing secondary oxidation of sardine oil, TBARS assay was carried out. From the results of TBARS value, it can be seen that the performance of rutin and its corresponding ester was similar to their activity as seen in peroxide value. During all analysis days, the TBARS value of samples with rutin was significantly lower ($p<0.05$) and consistent with our previous results (Vaisali et al. 2016).

The efficiency of rutin ester was almost similar to that of rutin, but showed a relatively higher TBARS value only during $7^{th}$ and $9^{th}$ day of storage (Figure 5.15). With the decrease in TBARS concentration after $10^{th}$ day, due to volatilisation or autooxidation of secondary oxidation products (Abuzaytoun and Shahidi 2006, Taghavaei et al. 2014), rutin ester showed better antioxidant activity. This could be attributed to the inability of lipophilic rutin ester to effectively solubilise at higher concentrations of the hydrophillic secondary oxidation products. Such an effect was proposed to be due to the compartmentalisation of substrate, reactants and products of oxidation (Pinchuk and Lichenberg 2016, Xenakis et al. 2010, Nielse et al. 2013).

Thus the effectiveness of lipophilic derivative of rutin in refined sardine oil was found to vary over the course of storage under the current conditions. This signifies the importance of solubility of antioxidants, which in turn depends on the micro and nano structures in bulk oil.

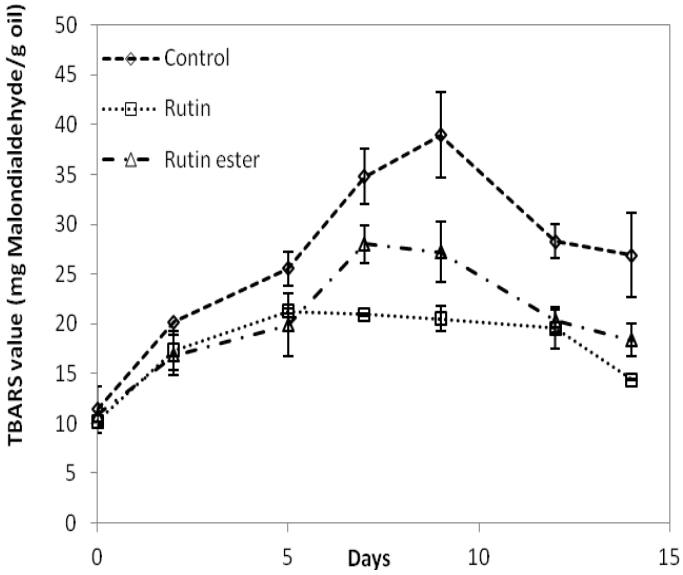



**Figure 5.15 Changes in the thiobarbituric acid reactive substances (TBARS) value of sardine oil fortified with native rutin and rutin fatty ester. Control – Sardine oil with no antioxidant addition. Bars represent standard deviation (n=3)**

### 5.2.3.2. Effectiveness of rutin fatty ester in refined sardine oil containing trace water

The results of experiments testing the efficiency of rutin fatty ester indicated that rutin fatty ester performed with relatively low efficiency than rutin while improving sardine oil oxidative stability. It was also identified that the performance of rutin ester was not consistent during the course of oxidation. This was concluded to be due to the inefficient association of rutin fatty ester at the site of oxidation. In the previous chapter, it was identified from the results of oxidation of oil containing trace water, that the antioxidant effectiveness was greatly reduced when trace water level was increased to 0.16%. Hence, the current work was has been performed to understand

and compare the effectiveness of rutin with its corresponding lipophilic ester in reducing oxidation in sardine oil containing trace water.

#### 5.2.3.2.1. Peroxide value

Primary oxidation of sardine oil containing trace water, in the presence of rutin and lipophilic rutin fatty ester was monitored for a period of 14 days and depicted in Figure 5.16. The peroxide value increased at higher rate after 2nd day of storage indicating the induction period of sardine oil. Thus, the peroxide value drastically increased from 12 meq/kg to 30.23 meq/kg from 2nd day of storage to 9th day, respectively (Figure 5.16).

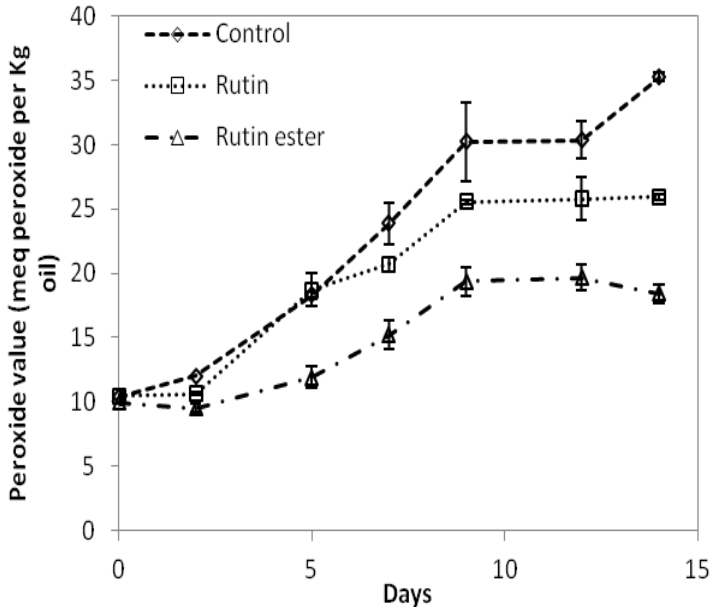



**Figure 5.16 Changes in the peroxide value of sardine oil containing trace water fortified with native rutin and rutin fatty ester. Control – Sardine oil with trace water with no antioxidant addition. Bars represent standard deviation (n=3)**

Fortification with rutin and rutin fatty ester resulted in the latter showing higher performance with 47.76% reduction than the former with 26.37% reduction in oxidation at the end of 14 days (Table 5.3). In the presence of lipophilic rutin ester, the peroxide value reduced significantly ($p<0.05$) in comparison with control. This indicated that rutin fatty ester effectively reduced oxidation. This is consistent with

the results obtained by Sorenson et al. (2011), when the efficiency of rutin was compared to rutin laurate and rutin palmitate.

**Table 5.3 Effectiveness of rutin and rutin fatty ester on improving oxidative stability of sardine oil containing trace water at the end of 14$^{th}$ day storage period**

| **Oxidation parameter** | **% Decrease in oxidation at the end of 14 days storage period** | |
|---|---|---|
| | **Rutin** | **Rutin fatty ester** |
| Peroxide value | 26.37 ± 0.56$^{a}$ | 47.76 ± 2.04$^{b}$ |
| Conjugated diene value | 8.12 ± 1.34$^{a}$ | 29.78 ± 2.37$^{b}$ |
| p-Anisidine value | 10.36 ± 7.0$^{a}$ | 40.11 ± 9.2$^{b}$ |
| Thiobarbituric acid reactive substances (TBARS) value | 22.83± 8.5$^{a}$ | 42.46 ± 8.6$^{b}$ |

Percentage decrease was calculated by considering control without antioxidants to be 100% oxidised

$^{a, b}$ values with different letters in the same row were significantly different ($p<0.05$)

The lipophilic ester showed significant reduction ($p<0.05$) in primary oxidation with a maximum of 18.40 meq/kg in comparison with a control value of 35.24 meq/kg of peroxide value at the end of 14 days storage period. As presence of trace water enhances the formation of micellar structures (Budilarto and Kamal-Eldin 2015b), it can be concluded that the lipophilic nature of rutin fatty ester allows it to effectively associate at the interface of these structures, thus improving oxidative stability. However, its polar counterpart failed to show antioxidant activity during initial days of storage, after which it showed moderate antioxidant activity.

Over the years, many studies were performed to establish a relationship between polarity of antioxidant and their effectiveness in lipids (Sorensen et al. 2011, Zhu et al. 2014, Aladedunye et al. 2015, Ma et al. 2015, Chen et al. 2016). It has been widely established that polar antioxidants are effective than non-polar antioxidants in bulk

oils (Frankel et al. 1994, Zhong and Shahidi 2012b). This phenomenon was reported to be due to the ability of polar antioxidants to associate at the air-oil interface, which dominates the oxidation in bulk oil (Frankel et al. 1994). However, it was noted in the present study that the antioxidant activity of hydrophilic rutin was lower in comparison to lipophilic rutin ester. According to Chaiyasit et al. (2007), the polar lipids and minor components in oil, along with amphiphillic oxidation products self-assemble to form a variety of physical structures and the presence of water alters the structure and characteristic of these association colloids. It is widely accepted that the occurrence of trace amount of water in refined oils is inevitable (Kim et al. 2014, Kittipongpittaya et al. 2016). This trace water has been reported to affect oxidative stability of oils in two ways. They either trigger oxidation by acting as substrates (Kim et al. 2014), or by enhancing micelle formation that acts as site of oxidation (Park et al. 2014).

Presence of water in sardine oil could result in the partitioning of polar rutin into water phase of micelles, leading to a decrease in its activity. Further it has been suggested that micellar structures collapases after sufficient oxidation (Budilarto and Kamal-Eldin 2015a). This could explain the improvement in rutin activity after a particular period of storage. Thus when micellar structures collapses, partitioning of rutin into water phase is reduced. On the other hand, this partitioning effect was not found in rutin fatty ester due to its lipophilic nature nature. Similar results were noted by Kittipongpittaya and others (Kittipongpittaya et al. 2016), when presence of water failed to affect the activity of hydrophobic tocopherol during oxidation of stripped corn oil. Thus lipid oxidation in sardine with trace water seemed to proceed from colloidal formation followed by primary and secondary oxidation, which is consistent with the work by Park et al. (2014), when corn oil oxidation was studied in the presence of moisture.

#### 5.2.3.2.2. Conjugated diene (CD) value

Antioxidant activity of rutin and its lipophilic ester was further tested in sardine oil containing trace water, by analysing their ability to reduce the conjugated diene (CD)

formation, which are isomeric derivatives of unsaturated lipid hydroperoxides. The CD value of sardine oil used in the current study showed that rutin ester was better suited for significant improvement ($p<0.05$) in oxidative stability (Figure 5.17). Whereas, rutin showed only moderate antioxidant activity in oil with a CD value of 0.448 in comparison with the CD value of 0.488 and 0.342 for control and sample with rutin fatty ester respectively, at the end of 14 days of storage (Figure 5.17).

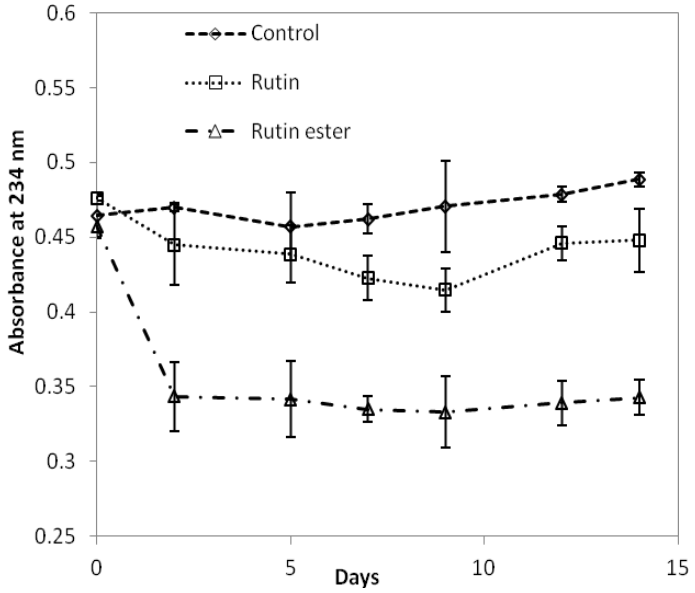



**Figure 5.17 Changes in the conjugated diene value of sardine oil containing trace water fortified with native rutin and rutin fatty ester. Control – Sardine oil with trace water with no antioxidant addition. Bars represent standard deviation (n=3)**

This is in accordance with the results obtained by Zhong and Shahidi (2012b), as lipophilic derivatives of ascorbic acid, gallic acid and epigallocatechin gallate (EGCG) showed higher reduction in CD value of stripped corn oil than their corresponding polar counterparts. Another similar study on the effect of tocopherol on corn oil oxidation indicated that 200 ppm of lipophilic tocopherol was effective in increasing the oxidative stability in the presence of trace water (Jung et al. 2016).

In case of samples with rutin fatty ester, a drastic reduction in the CD value was noted after 2$^{nd}$ day of storage (Figure 5.17). Such a trend was initially attributed to a

decrease in the surface area of oxidation (Schwarz et al. 2000). However, control and samples fortified with rutin failed to show the same profile. The lack of such a trend could represent the oxidation site to be at the interface formed between traces of water and lipid phase unlike the air –oil interface suggested by Frankel et al. (1994). As in peroxide value this further proves that oxidation in bulk sardine oil containing trace water is influenced by these association colloids. Hence, this additionally confirms that lipophilic rutin ester was effective in reducing oxidation influenced by these colloidal/micellar structures.

#### 5.2.3.2.3. p-Anisidine Value (pAV)

The hydroperoxides formed during the primary oxidation are usually decomposed to form an array of secondary oxidation products, the most prominent compounds being those with carbonyl groups. These aldehydes are analysed based on their reaction with the amine group of p-anisidine, resulting in a Schiffs base that shows specific absorption at 350 nm (Wang et al. 2011). Changes in the pAV of sardine oil with trace water in the presence of rutin and its non-polar counterpart was compared with oil samples devoid of antioxidants and presented in Figure 5.18.

It was noted that a more lipophilic antioxidant was required to improve oxidative stability of sardine oil containing trace water. In agreement with our results on primary oxidation, it was noted that rutin fatty ester showed consistently significant reduction ($p<0.05$) in secondary oxidation than hydrophilic rutin. This is similar to the findings by Zhu et al. (2014), who noted a better antioxidant activity of acetylated epigallocatechin gallate (EGCG) than native EGCG in sunflower oil due to the homogenous dispersion of lipophilic antioxidant. A similar conclusion was derived by Zhong and Shahidi (2012a) when lipophilic derivative of epigallocatechin gallate (EGCG) showed better antioxidant activity than hydrophilic EGCG in corn oil. In case of effectiveness of polar rutin, it was noted that it failed to show significant reduction ($p>0.05$) till $12^{th}$ day of storage (Figure 5.18) after which it showed slight antioxidant activity, showing a reduction of 21.25% at the end of 20 days storage (Table 5.3).

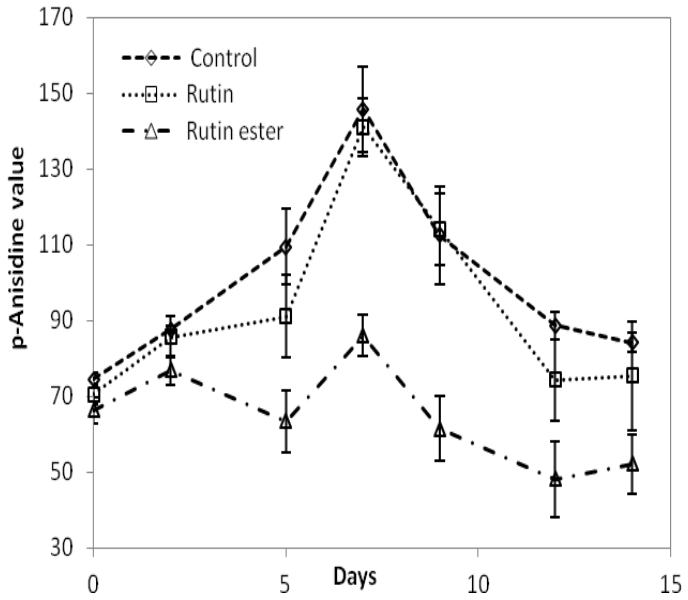



**Figure 5.18 Changes in the p-anisidine value of sardine oil containing trace water fortified with native rutin and rutin fatty ester. Control – Sardine oil with trace water with no antioxidant addition. Bars represent standard deviation (n=3).**

#### 5.2.3.2.4. Thiobarbituric acid-reactive substances (TBARS) value

In order to confirm the effectiveness of the antioxidants, another measurement of secondary oxidation was carried out based on the reaction between thiobarbituric acid and malondialdehyde like substances in oil. From the results of TBARS value, it was noted that native rutin showed mild antioxidant activity throughout the storage period, whereas rutin ester performed with better efficiency showing maximum reduction in TBARS value ($p<0.05$) with 42.46% reduction at the end of 14 days storage (Table 5.3). Thus at the peak of oxidation, control showed a maximum TBARS value of 42.79, while sample with rutin and its ester showed a TBARS value of 37.32 and 18.01 respectively (Figure 5.19).

Additionally, samples with rutin ester showed only a minor increase in TBARS value throughout the course of oxidation ($p<0.05$), denoting a possible delay of secondary oxidation by lipophilic rutin ester. Since, it has been established that oxidation in bulk oil containing minor components is driven by the presence of physical structures

(Chaiyasit et al. 2007), it can be concluded that rutin fatty ester has effectively reduced the progress of oxidation by efficient interaction with colloidal/micellar structures during primary oxidation itself, thus delaying secondary oxidation. As the TBARS value is an indication of secondary oxidation of unsaturated lipids (Decker et al. 2010), it can be concluded that the lipophilic nature of rutin ester provided better protection against the oxidation of hydrophobic PUFA lipids in sardine oil.

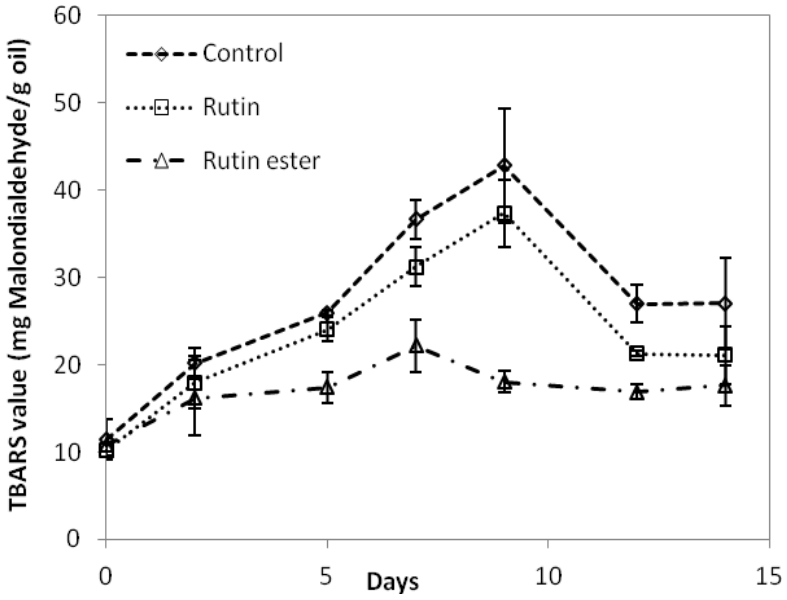



**Figure 5.19 Changes in the thiobarbituric acid reactive substances (TBARS) value of sardine oil containing trace water fortified with native rutin and rutin fatty ester. Control – Sardine oil with trace water with no antioxidant addition. Bars represent standard deviation (n=3)**

Considering the overall oxidation results from the current study, it can be seen that the results obtained contradicts the polar paradox theory, which suggests that hydrophilic antioxidants work well in bulk oil. However, due to the complexity of sardine oil and the presence of trace water, formation of association colloids is inevitable. Many recent studies suggest that these association colloids act as micro/ nano reactors of oxidation as it provides oil-water interface for oxidation. Hence, the progress of oxidation in bulk oils containing these structures proceeds at the oil-water interface

similar to the oxidation in emulsions. As a result, a more lipophilic antioxidant is required to improve oxidative stability.

## 5.3. SUMMARY AND CONCLUSIONS

The results on the synthesis of antioxidant fatty ester indicated that only rutin was capable of participating in esterification, while esterification of caffeic acid and quercetin resulted in no product formation. Similarly, gentisic acid esterification also resulted in lesser product formation. This was concluded to be due to the steric hindrance caused by the hydroxyl functional group and the type of enzyme used.

- Optimisation of the rutin esterification by *Candida antartica* lipase resulted in 60% conversion of rutin to its corresponding ester.
- Process parameters like temperature, enzyme load and molar ratio of substrates were found to be affected by relatively minor difference in polarity of the solvent system due to the significant role played by minor concentration of water in enzyme catalysis.
- Due to the polarity difference in the solvent system used for ester synthesis, different optimal water activity was noted for each solvent.
- Relatively higher water activity was found to increase ester synthesis in polar *tert*-butanol.
- Removal of by-product water led to a twofold increase in product formation in *tert*-butanol system.
- Removal of by-product water by using molecular sieves failed to increase product formation in acetone system.
- The optimum conditions for acetone system to result in maximum rutin fatty ester synthesis was found to be 10 mg/mL, 1:5 rutin to fatty acid molar ratio, 55 °C and a water activity of 0.07
- The optimum conditions for *tert*-butanol system to result in maximum rutin fatty ester synthesis was found to be 15 mg/mL, 1:4 rutin to fatty acid molar ratio, 60 °C and a water activity of 0.23 along with the addition of molecular sieves to the level of 150 mg/mL

- Under optimum conditions, *tert*-butanol system results in a maximum conversion of 65.73%, while acetone system gave a maximum conversion of 60.74%.

In an attempt to improve the lipophilicity of rutin, enzymatic esterification of rutin with decanoic acid was done. Though, acetone and *tert*-butanol were of relatively similar polarity, the yield of rutin fatty ester in *tert*-butanol was considerably low. However, the yield in *tert*-butanol system increased considerably when the process parameters were altered. In addition, both solvent systems followed similar trend on change of water activity. From this it can be concluded that each physical and chemical parameter of the reaction plays a significant role on the water content of the system, which in turn affects the yield of product. This was further ascertained by addition of molecular sieves to the system, which gave a 3-fold increase in yield in *tert*-butanol system. However, acetone system failed to show increase in conversion due to side reaction with molecular sieves.

The successful synthesis of rutin fatty ester has paved way for many applications of food industries. Since, these compounds are amphiphillic their application in many commercial food products can be highly advantageous. On analysing the effectiveness of these compounds in improving the oxidative stability of PUFA rich sardine oil the following conclusions were derived.

- Purification of rutin fatty ester by a two-stage solvent extraction process resulted in a relatively pure ester.
- On comparison of the effectiveness of rutin fatty ester with its native form in sardine oil it was identified that rutin was more effective during oxidation of refined sardine oil, whereas rutin ester showed higher activity in sardine oil containing trace water.
- The effectiveness of rutin and rutin fatty ester was found to change over the progress of oxidation.

Thus it can be concluded that, a more hydrophilic antioxidant is required to delay the onset of oxidation in bulk oil containing less or minimal polar components.

However, as the presence of polar components increases in bulk oil, either due to its inherent properties or due to the progress of oxidation an amphiphillic antioxidant is required to associate better at these locations containing association colloids. Hence, addition of hydrophilic antioxidant along with its lipophilic counterpart could be a more effective strategy to significantly delay oxidation process in bulk sardine oil.

# CHAPTER 6

# SUMMARY AND CONCLUSIONS

The increasing demand for n-3 PUFA rich food sources has made fish oil a promising raw material. Fish oil is generally available in its crude form due to which it shows poor quality and stability. Hence, an effort was made in this study to devise techniques that will remove impurities without affecting the n-3 PUFA content. Another bottleneck in effectively utilising these high value products is their poor oxidative stability. This study is an attempt to develop effective antioxidant strategies using natural compounds to improve the stability of sardine oil. The overall summary and conclusions of the current study is provided below.

### 6.1. Summary

Crude oil characteristics vary with each season resulting raw material of varying quality and composition. Hence, development of a fail-proof technique that will effectively remove impurities from such variable raw material is in demand. Development of such a refining technique in the current study resulted in the following

- A novel degumming strategy involving the use of 3% (w/w) ethanolamines resulted in 87% reduction in the phospholipid content with a simultaneous reduction of FFA to 1.21% from 3.75%.
- Further reduction in FFA content to a value of 0.6% was achieved by membrane assisted solvent deacidification.
- The overall quality of the sardine oil was found to improve during bleaching stage with further reduction in phospholipid, FFA, metal ions and moisture content.
- The n-3 PUFA content in sardine oil was retained without much loss with crude oil showing n-3 PUFA content of 13.3% (w/w), while refined oil showed n-3 PUFA content of 13% (w/w).

Though refining techniques reduce the oxidation processes considerably, the autoxidation rate of fatty acids increases with degree of unsaturation. Recent researches are focused on the search for effective natural antioxidants for

improving the oxidative stability of such oils. On studying the effect of several natural phenolic antioxidants, the following results were obtained

- Of the nine chosen antioxidants, quercetin and rutin followed by gentisic acid and caffeic acid were most effective in arresting oxidation of sardine oil.
- The antioxidants that showed good activity in sardine oil were found to be best radical scavengers.
- In addition to their effectiveness in sardine oil, the four natural antioxidants showed good activity in reducing oxidation influenced by the presence of trace metals. However, their effectiveness was found to decrease when the trace water content in sardine oil was high.

In order to increase the antioxidants availability in sardine oil, lipophilisation of the chosen antioxidants was performed. Thus, a more hydrophobic antioxidant has better solubility in oil that could result in higher antioxidant activity. Enzymatic esterification using *Candida antartica* lipase resulted in the following;

- Among the four antioxidants that were esterified, only rutin showed good conversion to its corresponding ester.
- Initial studies on esterification of rutin in two solvent systems resulted in 52% and 14.4% conversion in acetone and *tert*-butanol, respectively.
- On altering process parameters, *tert*-butanol system resulted in a maximum conversion of 65.73%, while acetone system gave a maximum conversion of 60.74%.

On analysing the effectiveness of these compounds in improving the oxidative stability of PUFA rich sardine oil the following results were noted

- It was found that rutin showed maximum effectiveness in refined sardine oil.

- In case of sardine oil with trace water, rutin ester showed higher effectiveness than rutin with 47.7% and 26.37% reduction in peroxide value, respectively, at the end of $14^{th}$ day of storage
- The effectiveness of rutin and rutin fatty ester was found to change over the progress of oxidation.
- It was identified that rutin was more effective during oxidation of refined sardine oil, whereas rutin ester showed higher activity in sardine oil containing trace water.

### 6.2. Significant findings

The refining technique developed in the current study was found to be highly effective due to the simultaneous degumming and deacidification effect. Also the free fatty acid content in oil was reduced drastically over the course of refining. Since, removal of FFA is the most tedious and expensive process in any fish oil refining, simultaneous reduction of phospholipids and FFA is a major advantage as identified in this study. The n-3 PUFA content was maintained without much loss. This indicates the suitability of this process for n-3 PUFA rich oil. Due to the lack of reports on crude fish oil refining, the process developed in the current study is of high significance.

Based on the results on the oxidative stability of sardine oil it can be concluded that peroxide formation was the major mechanism of oxidation in sardine oil. Hence, all potent radical scavengers like quercetin, rutin, caffeic acid and gentisic acid showed high antioxidant effectiveness. Further it was found that, the antioxidant efficiency reduced with increase in the heterogeneity of sardine oil. On testing the effectiveness of rutin fatty ester in improving the oxidative stability of sardine oil, it was found that rutin was found to be effective for improving stability in refined sardine oil, while rutin ester was effective in reduction of oxidation in sardine oil containing trace water. Further the antioxidants effectiveness changes during the course of oxidation. Thus the current study signifies that the antioxidant requirement for oil changes during storage.

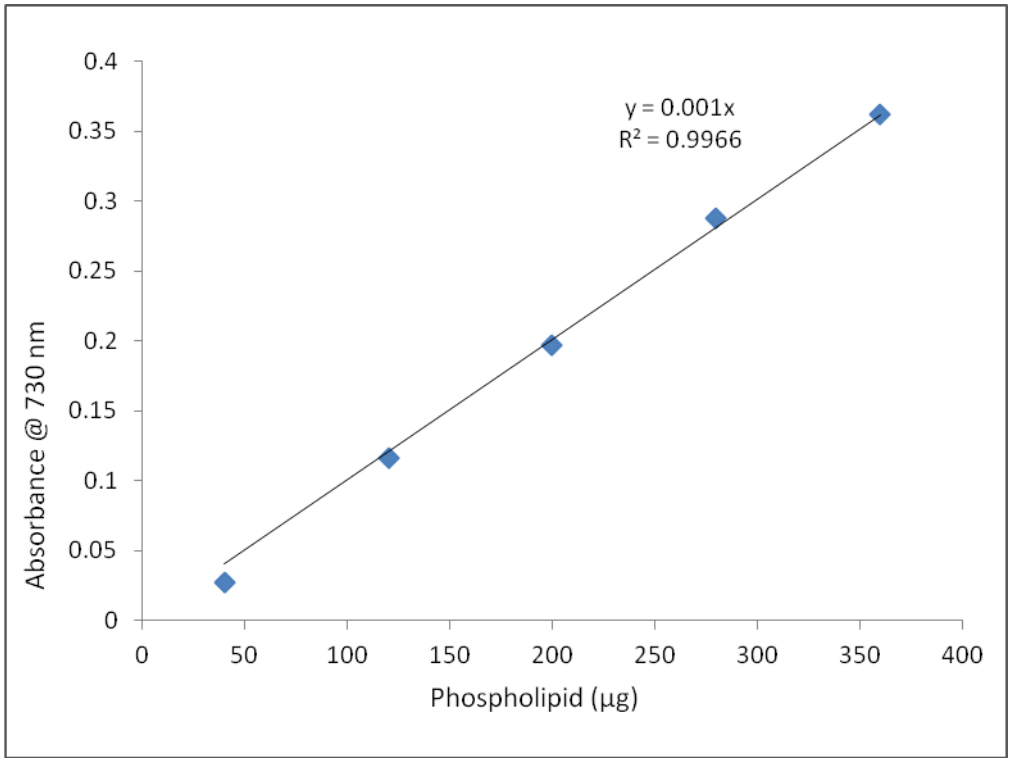
## 6.3. Scope of future work

- ❖ Development of structure-activity relationship for synthesised rutin fatty ester
- ❖ Addition of both hydrophilic rutin and lipophilic rutin ester to meet the antioxidant requirement during oxidation.
- ❖ Study the effectiveness antioxidants of varying polarity based on their concentration.

# APPENDICES

# APPENDIX I

## Standard graph for phospholipid estimation



# APPENDIX II

## GC Chromatogram of fatty acid methyl ester (FAME) standard

| | |
|---|---|
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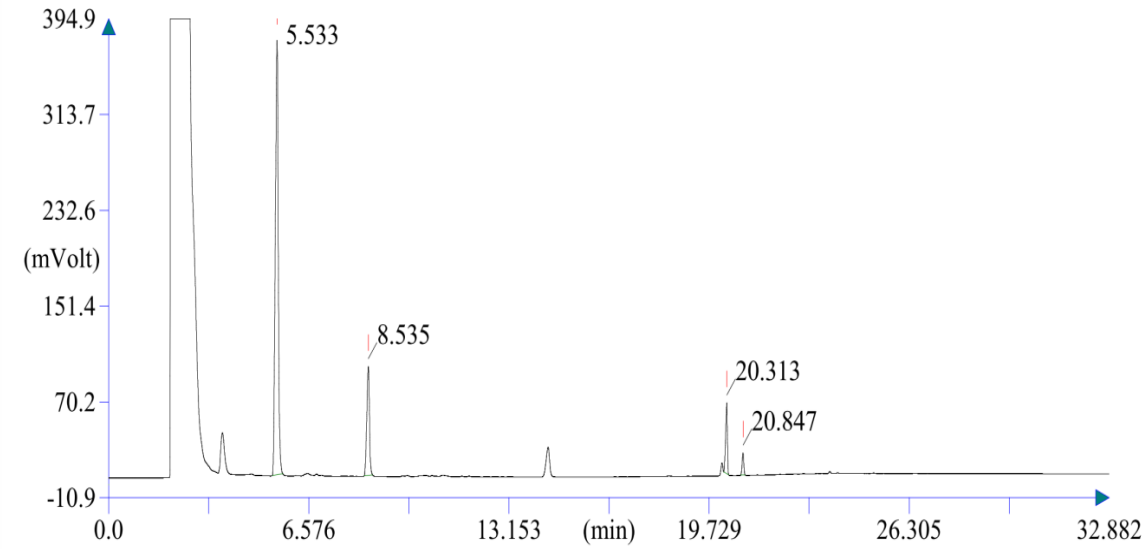
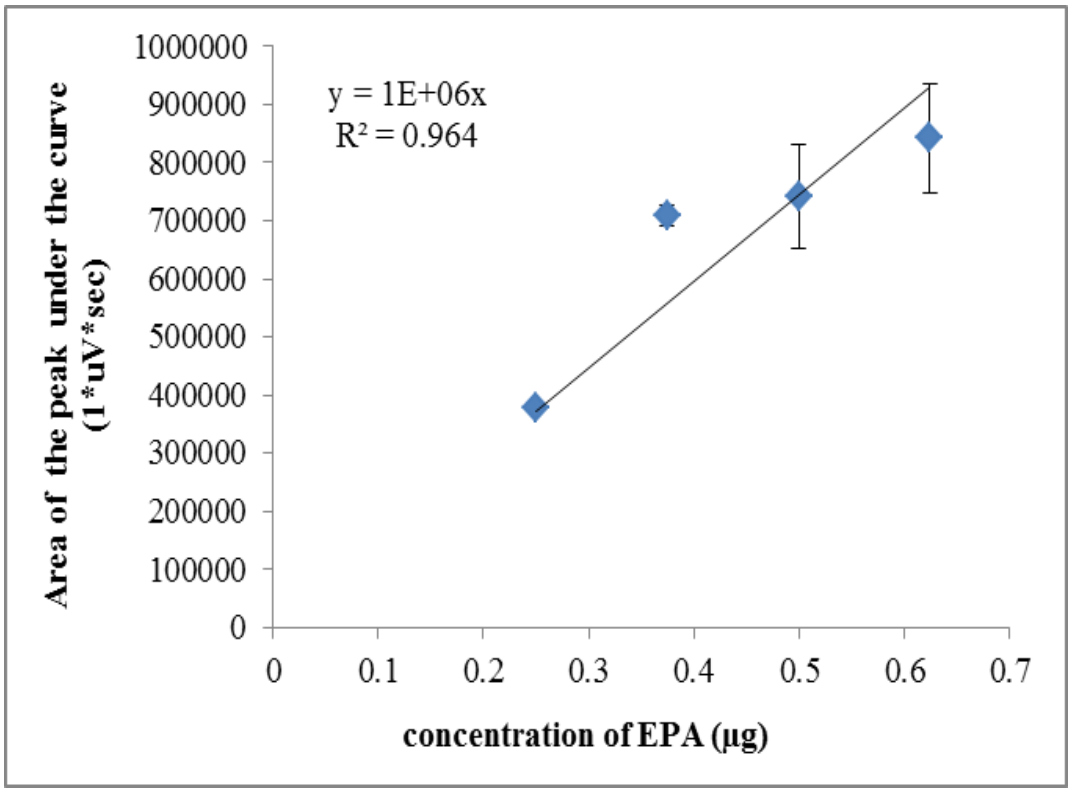
| Company name: | NITK |
| Method filename: | F:\Thermo\Chrom-Card Trace-Focus GC\data\First Run\fame soft meth std mix feb 10.mth |
| Method name: | vais fame feb 10 |
| Analysed: | 02/10/2015 14:32 |
| Printed: | 2/10/2015 15:05 |



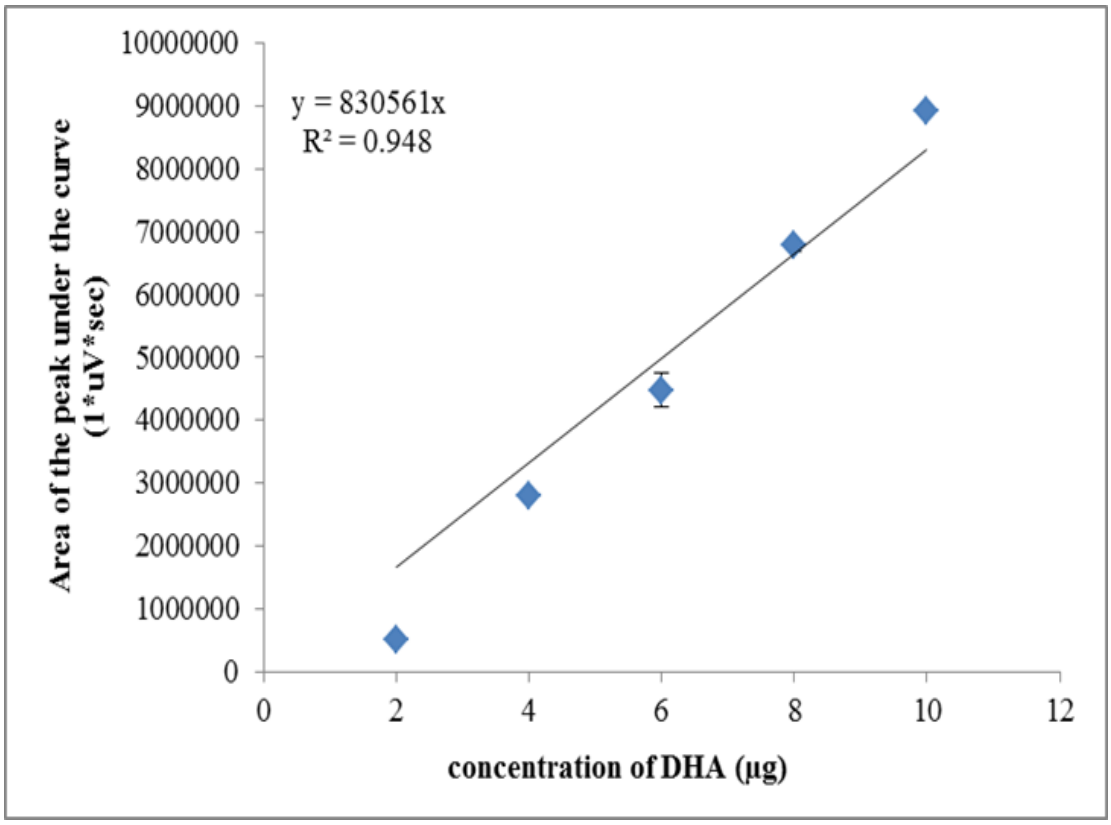
## APPENDIX III

### Standard graph of EPA methyl ester



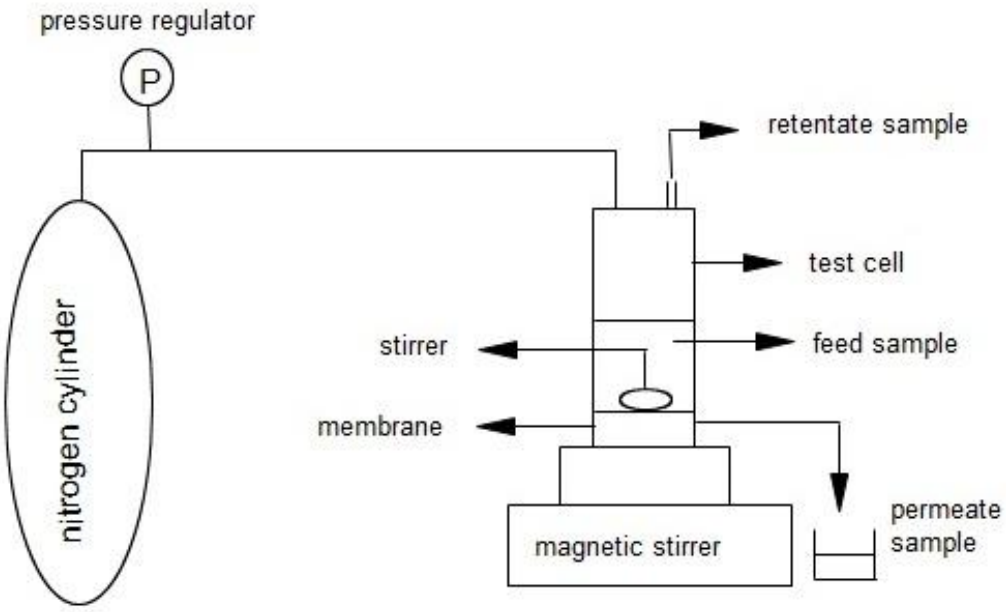
## APPENDIX IV

### Standard graph of DHA methyl ester



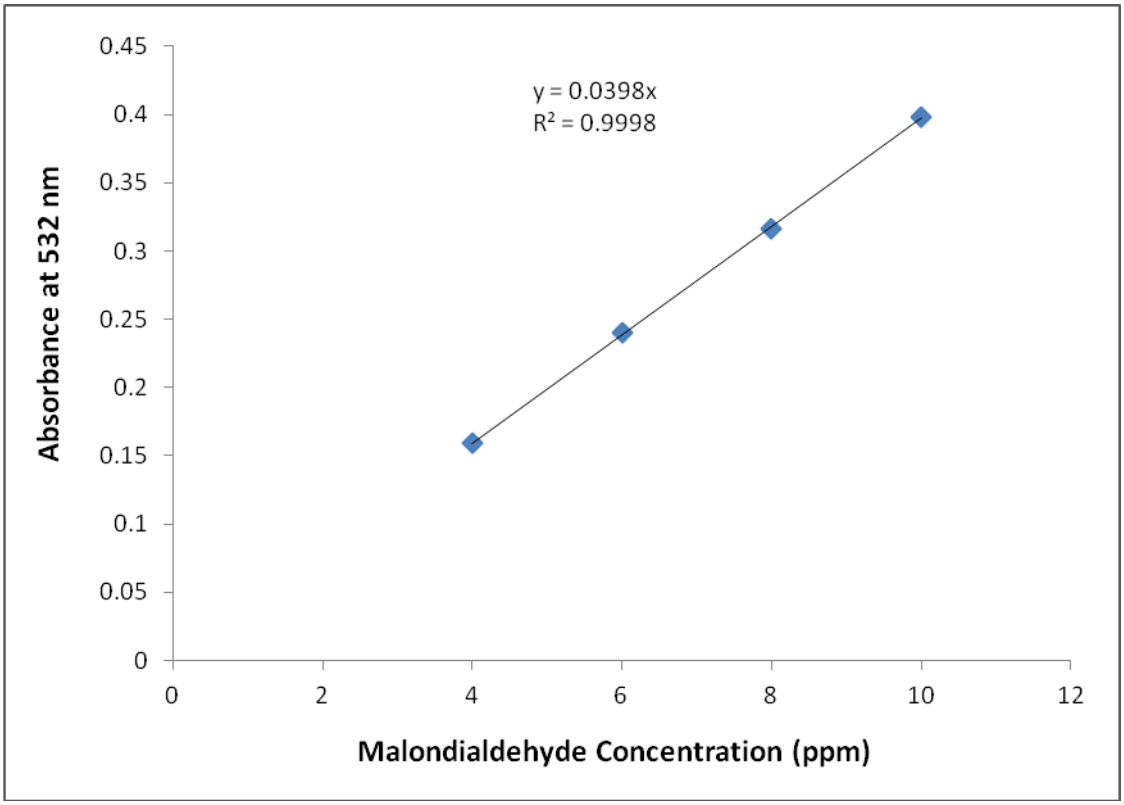
## APPENDIX V

### Membrane test cell



## APPENDIX VI

### Standard graph of Malondialdehyde for TBARS estimation



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# LIST OF PUBLICATIONS BASED ON THE CURRENT WORK

## JOURNAL PUBLICATIONS

1. **Vaisali, C**., Belur, P.D. and Regupathi, I. (2018). "Effectiveness of rutin and its lipophillic ester in improving the oxidative stability of sardine oil containing trace water". ". ***Int. J. Food Sci. Technol,*** 53, 541-548 (Impact factor: **1.64)**
2. **Vaisali, C**., Belur, P.D. and Regupathi, I. (2017). "Lipase mediated synthesis of rutin fatty: Study of its process parameters and solvent polarity". ***Food Chem***., 232, 278-285. (Impact Factor: **4.529**)
3. **Vaisali, C**., Belur, P.D. and Regupathi, I. (2016). "Comparison of antioxidant properties of phenolic compounds and their effectiveness in imparting oxidative stability to sardine oil during storage". ***LWT- Food Sci Technol***., 69, 153-160. (Impact Factor: **2.32**)
4. **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. (Impact Factor: **1.64**)

## BOOK CHAPTERS

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.
2. **Vaisali, C**., Belur, P.D. and Regupathi, I. (2016) Screening of solvents for the deacidification of sardine oil In: Regupathi, I., Shetty, K.V., Thanabalan, M. (eds) Recent Advances in Chemical Engineering". **Springer**, Singapore, pp 75-80.

## 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**.

**CONFERENCE PUBLICATIONS**

1. **Vaisali, C**., Belur, P.D. and Regupathi, I. (2017). "Antioxidant activity of phenolic compounds in improving the oxidative stability of bulk sardine oil influenced by the presence of water and iron". ***Int. Conf. Adv. Biotechnol. Biotherap***., Satyabama University, Chennai, India.

2. **Vaisali, C**., Belur, P.D. and Regupathi, I. (2016). "Effect of hydroxybenzoic acids antioxidants on the oxidative stability of sardine oil". *Technoscape,* VIT, India. (Published as **journal article in *Resource Efficient Technologies***)

3. 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 as **journal article in *Resource Efficient Technologies***)

4. **Vaisali, C.,** Belur, P.D. and Regupathi, I. (2015). "Screening of solvents for deacidification of sardine oil". ***Proc. Int. Conf. Adv. Chem. Eng***., Surathkal, India.

# Bio-Data

**VAISALI.C**

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## ACADEMIC DETAILS

I. **PhD Chemical Engineering** (2013-2018)

National Institute of Technology, Karnataka, India

- Advisor: Dr. Prasanna D Belur and Dr. I. Regupathi
- Thesis: "Development of Novel Refining Techniques and Enzymatic Synthesis of Antioxidant Esters for Improving the Oxidative Stability of Sardine Oil".

II. **Master of Technology in Industrial Biotechnology** - Integrated (M.Tech, 2007-2012).

SASTRA University, Thanjavur, India

- Project title: "Optimization of methods for the extraction of proteins from pigeon pea milling by-products".
- Score: **8.44 CGPA**.

## JOURNAL PUBLICATIONS

1. Vaisali, C., Belur, P.D., and Regupathi, I. (2018). Effectiveness of rutin and its lipophillic ester in improving the oxidative stability of sardine oil containing trace water. International Journal of Food Science and Technology, 53, 541-548.
2. Vaisali, C., Belur, P.D., and Regupathi, I. (2017). Lipase mediated synthesis of rutin fatty ester: Study of its process parameters and solvent polarity. Food Chemistry, 232, 278-285.
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4. Vaisali, C., Charanyaa, S., Belur, P.D. and Regupathi, I. (2015). Refining of edible oil: a critical appraisal. International Journal of Food Science and Technology, 50, 13-23.
5. Vaisali, C. Belur, P.D. and Regupathi, I. (2016) Effect of hydroxybenzoic acids on the oxidative stability of sardine oil. Resource Efficient Technologies, 2, S114-S118.
6. Charanyaa, S., Vaisali, C., Belur, P.D. and Regupathi, I. (2016). Screening of polymeric membranes for the membrane assisted deacidification of sardine oil. Resource Efficient Technologies

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1. Vaisali, C., Belur, P.D. and Regupathi, I. (2017). "Antioxidant activity of phenolic compounds in improving the oxidative stability of bulk sardine oil influenced by the presence of water and iron". International Conference on Advances in Biotechnology and Biotherapeutics, Satyabama University, Chennai, India.
2. Vaisali, C. Belur, P.D. and Regupathi, I. (2016) Effect of hydroxybenzoic acids on the oxidative stability of sardine oil. Technoscape2016, VIT, India.
3. Vaisali, C., Belur, P.D. and Regupathi, I. (2015) Screening of solvents for the deacidification of sardine oil. International Conference on Advances in Chemical Engineering, NITK, India.
4. Charanyaa, S., Vaisali, C., Belur, P.D. and Regupathi, I. (2016). Screening of polymeric membranes for the membrane assisted deacidification of sardine oil. Technoscape2016, VIT, India.

**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.

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1. Vaisali, C., Belur, P.D. and Regupathi, I. (2016) Screening of solvents for the deacidification of sardine oil In: Regupathi, I., Shetty, K.V., Thanabalan, M. (eds) Recent Advances in Chemical Engineering". Springer, Singapore, pp 75-80.

## 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 EXPERIANCE

1. **PhD researcher in National Institute of Technology Karnataka(NITK), Surathkal (July 2013 – present)**
   - Investigated the application of membrane process for the refining of sardine fish oil
   - Experienced in conducting stability studies for food systems.
   - Investigated the effect of antioxidants and the limitations to use the same in food systems.
   - Designed and performed experiments to modify the antioxidants to give a novel compound.
   - Implemented all the chromatographic techniques, for the analysis of the experiments
   - Supervised two graduate students
   - Performed statistical significance of all the experiments by using several statistical tools
2. **Junior research fellow for Ministry of Food Processing Industries (MOFPI) in NITK Surathkal (Dec 2012 to March 2015)**

   **Project: Production of n-3 Polyunsaturated fatty acids concentrate from sardine oil**

- Fatty acid profiling of the various sardine oil was performed using Gas Chromatography (GC) analysis.
- Methods were developed to enrich the omega-3 fatty acid content of the sardine oil
- Various analytical techniques were performed to characterise the physical and chemical properties of oil
- Experienced on enzymology with factors affecting the mass transfer limitations of hydrolysis reaction was gained.

3. **Project Intern on Red Gram milling by-products, Central Food Technological Research Institute (CFTRI), Mysore (Dec 2011- May 2012).**

- Studied the analytical techniques to identify the proteins with nutritional properties, and studied the optimal extraction conditions of a specific bioactive compound
- Investigated the extractability of proteins from pigeon pea milling by-products and preparation of protein isolate from it.

## PROJECTS AND TRAININGS

1. **Training, Department of Biotechnology, Amet University, Chennai**
   Trained on sea food microbiology (Isolation of pathogens from spoiled fish and inhibition by *Lactobacillus* culture isolated from yogurt).
2. **Training, Immunotechnology, Sangenomics laboratory, Bangalore**
   Trained on blood testing, ELIZA test, and culture techniques.
3. **Training, Downstream Processing Lab, SASTRA University, Tanjore**
   Trained on Reverse micellar extraction of proteins from whey.

## LABORATORY SKILLS

- Basic chromatographic skills (HPLC, LC, column packing, HPLC-ESI-MS, HPLC-APCI-MS, GC, FPLC) and other instrumental analysis like thermogravimetry (TG), FTIR, small scale bioreactor

- Biochemical tests, Enzyme assays and inhibitor screening, protein extraction and purification procedures, electrophoresis techniques (SDS-PAGE, Native PAGE) and zymogram.
- Microbiology skills like culture preparation, pure culture isolation, inhibitory studies and microbial-biochemical tests.
- Analytical techniques for performing oxidation studies.