# STUDIES ON THE LIPOPHILISATION OF 3,4-DIHYDROXYPHENYLACETIC ACID AND ITS APPLICATION IN ENHANCING THE OXIDATIVE STABILITY OF FISH OIL

**Thesis**

Submitted in partial fulfilment of the requirements for the degree of

**DOCTOR OF PHILOSOPHY**

By

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# DECLARATION

I hereby declare that the Research Thesis entitled "**Studies on the lipophilisation of 3,4-dihydroxyphenylacetic acid and its application in enhancing the oxidative stability of fish oil**" which is being submitted to the **National Institute of Technology Karnataka, Surathkal** in partial fulfilment of the requirement for the award of the degree of **Doctor of Philosophy** in Chemical Engineering is a bonafide report of the research work carried out by me. The material contained in this Research Thesis has not been submitted to any University or Institution for the award of any degree.

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# CERTIFICATE

This is to certify that the Research Thesis entitled "**Studies on the lipophilisation of 3,4-dihydroxyphenylacetic acid and its application in enhancing the oxidative stability of fish oil**" submitted by **Ms. SELVA SUDHA A (Register Number: 187121CH007)** 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 the degree of **Doctor of Philosophy**.

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# ABSTRACT

Antioxidants are important food additives that can enhance the storage stability by significantly delaying oxidation. Synthetic antioxidants can be potentially toxic and carcinogenic, which has led to the exploration of various natural antioxidants as alternatives. Phenolic acids are hydrophilic in nature and have excellent antioxidative properties in addition to other beneficial effects, including anti-microbial, anti-cancer, and anti-inflammatory properties. 3,4-dihydroxyphenylacetic acid, is one such rarely studied phenolic acid which has not been utilized for retarding oxidation in lipids. The study aims to lipophilise this hydrophilic antioxidant to obtain an ester with improved hydrophobicity for application in fish oils.

Lipases from *Candida antarctica* and *Thermomyces lanuginosus* was used in immobilized form under different reaction conditions for esterification. The enzyme was only efficient in synthesizing methyl ester, while the increase in length of the acyl chain significantly reduced the esterification efficiency. Homogeneous catalyst p-toluenesulfonic acid gave higher yields of the ester but the purity of the ester could not be improved despite our best efforts. The methyl, butyl, and hexyl esters of 3,4-DHPA were synthesized in 81.1%, 69.3%, and 78.% conversion using 15% (w/w) Amberlyst-15 as the catalyst in a binary solvent system comprising n-hexane:2-butanone in 75:25 (v/v) ratio. The methyl ester was obtained through direct esterification in 1:60 molar ratio of phenolic acid: alcohol while the butyl and hexyl esters were obtained through trans-esterification reaction in 1:1 molar ratio.

The obtained esters were studied in refined bulk fish oil for a period of 30 days at 35°C in contact with atmospheric air. TOTOX values of fish oil samples indicated that the antioxidative potential of 3,4-DHPA was on par with the synthetic antioxidant BHT. Among the esters, the methyl ester showed a similar efficacy to that of 3,4-DHPA. The phenolic acid and its esters were also evaluated separately and in various combinations in bulk fish oil containing trace amounts of water. A significant synergistic effect was observed between 3,4-DHPA and its hexyl ester in 2:1 molar ratio in this study. This indicates the role of reverse micelles formed in the presence of trace water in controlling lipid oxidation in a bulk oil system. The relative partitioning of the hydrophilic and hydrophobic antioxidants in a combination among the oxidation sites at the water-oil interfaces significantly impacts their overall efficacy in retarding lipid oxidation.

**Keywords**: 3,4-dihydroxyphenylacetic acid; esterification; fish oil; trace water; oxidative stability.

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# ABRREVIATIONS

| | |
|---|---|
| ALA | α-Linoleic acid |
| n-3 PUFA | n-3 Polyunsaturated fatty acid |
| DHA | Docosahexaenoic acid |
| EPA | Eicosapentaenoic acid |
| DPA | Docosapentaenoic acid |
| 3,4-DHPA | 3,4-dihydroxyphenylacetic acid |
| TBHQ | tert-butyl hydroquinone |
| BHT | Butylated hydroxy toluene |
| FAME | Fatty acid methyl ester |
| PV | Peroxide value |
| *p*-AV | *para*-Anisidine value |
| TOTOX | Total oxidation |
| FFA | Free fatty acid |
| GC | Gas chromatography |
| ICP-OES | Inductively coupled plasma optical emission spectroscopy |
| HPLC | High Performance Liquid Chromatography |
| NMR | Nuclear magnetic resonance |
| TAG | Triacylglycerides |
| DAG | Diacylglycerides |
| MAG | Monoacylglycerides |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| ABTS | 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) |
| FRAP | Ferric reducing antioxidant power |
| ANOVA | Analysis of variance |

# NOMENCLATURE

| **Symbol** | **Description** |
|---|---|
| cm | Centimetre |
| °C | Degree Celsius |
| g | Gram |
| kg | Kilogram |
| L | Litres |
| miLog P | Log P calculated using Molinspiration calculator |
| µg | Microgram |
| m. Eq | Milli equivalents |
| mPa | Milli Pascal |
| mg | Milligram |
| mL | Millilitres |
| mM | Millimolar |
| min | Minutes |
| nm | Nanometres |
| ppm | Parts per million |
| %(v/v) | Percentage volume by volume |
| %(w/w) | Percentage weight by weight |
| rpm | Revolutions per minute |
| s | Seconds |

# CHAPTER 1

# INTRODUCTION

# CHAPTER 1

## INTRODUCTION

### 1.1 PHENOLIC ANTIOXIDANTS

Antioxidants are compounds that prolong the shelf life of foods by protecting them against deterioration caused by oxidation (Karovičová and Šimko 2000). It is useful in preventing the development of rancid off-flavor and taste in food products, thereby improving the quality and sensory appeal. They are important additives in several food, pharmaceutical, cosmeceutical, and animal feed products to prevent oxidative damage. Synthetic antioxidants, including TBHQ, BHT, BHA, and PG, are the most widely used additives in various food products. However, consumer acceptance of these synthetic compounds has decreased significantly due to various toxic effects on health upon consumption (Ji et al. 2023). Hence, the recent focus in antioxidant research has shifted to exploring new alternative natural compounds that have great antioxidative ability (Gulcin 2020; Kumar and Goel 2019; Mishra et al. 2023; Vaisali et al. 2017).

Based on their mechanism, they are classified as primary and secondary antioxidants. Primary antioxidants are basically free radical scavengers that have the ability to donate hydrogen atoms, while secondary antioxidants can effectively neutralize prooxidant catalysts (Zeb 2020). Phenolic antioxidants are primary antioxidants and are a major part of the secondary metabolites of plants (Roleira et al. 2015). They have at least one aromatic ring and one or more hydroxyl groups, which are responsible for the antioxidative activity. These phenolic compounds comprise a vital part of the human diet and are beneficially associated with several chronic diseases, including cardiovascular, atherosclerosis, and cancer (Gulcin 2020).

Phenolic antioxidants are characterized into several groups based on the backbone of the structure. The antioxidative activity of the phenolic compound is dependent on the number and position of the hydroxyl group bound to the aromatic ring and the type of substituent present (Rice Evans et al. 1996). Several phenolic acids, flavonoids, and carotenoids have been explored for their antioxidative properties in vegetable and animal fats and oils (Mishra et al. 2021). Each oil system has different needs in terms of the type of antioxidant and its concentration to efficiently retard oxidation. Animal fats may not be adequately protected from oxidation by an antioxidant designed for vegetable oil. Selecting an appropriate antioxidant while considering the unique requirements of the system is therefore crucial.

3,4-Dihydroxyphenylacetic acid is a lesser-studied phenolic antioxidant with a structure similar to caffeic acid and hydroxytyrosol, both known for their antioxidative properties. This compound shows excellent antioxidant potential, outperforming the synthetic antioxidant Butylated Hydroxytoluene (BHT). It is found abundantly in olive mill wastewater, making it a cost-effective source of natural antioxidants. However, its very low solubility has limited its exploration as an antioxidant in lipid systems.

## 1.2 FISH OIL AND OXIDATION

Fish oils obtained from the body and liver of marine fishes have been widely used in the food, feed, and pharmaceutical industries. It is recognized as a valuable nutrient for both humans and livestock. Fish oils are rich in long-chain unsaturated fatty acids such as EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) when compared to other oils. It plays an important role in human nutrition, and its demand has been increasing because of its beneficial health effects. Fish oil also finds application in food products, poultry and aquaculture feeds and as a functional component in nutraceuticals. It is also available as a dietary supplement in various forms in the market. About 81% of fish oil produced is used as feed in aquaculture and poultry animals (Rizliya and Mendis 2013). Fish oils are produced from targeted fisheries or from by-products from processed fish. Fatty fishes such as anchovies, sardines, mackerels, and menhaden, and the liver of lean fishes such as cod, haddock, and shark are used for the production of fish body oils and fish liver oils.

Long-chain polyunsaturated fatty acids (PUFAs) present in fish oil provide health benefits against cardiovascular and inflammatory diseases (Mozuraityte et al. 2016), rheumatoid arthritis, hypertension, atherosclerosis (Yashodhara et al. 2009), and various cancers (Gogus and Smith 2010). Unlike saturated and mono-unsaturated fatty acids, PUFAs are essential fatty acids that cannot be synthesized in the human body and have to be attained through diet or supplements (Shahidi and Zhong 2010). Fish oils contain high amounts of unsaturated fatty acids and other prooxidant molecules, which makes them susceptible to oxidation. This often leads to the release of undesirable odor and taste during storage, processing, and packaging. This reduces the shelf life of fish oil, causes nutrient loss, and formation of toxic compounds, making the oil unsuitable for use in pharmaceuticals and food products.

The instability of the fish oil is due to the presence of unsaturated double bonds in the fatty acids such as EPA and DHA. These hydrogens are present in bis-allylic positions which makes them vulnerable to oxidation. They are easily removed due to their low bond dissociation energies and can readily form oxidation products such as hydroperoxides (Cao et al. 2006). The hydroperoxides further attack other double bonds leading to a chain reaction of oxidation. The oxidation rate is further accelerated by the presence of factors such as light, oxygen, heat, enzymes, transition metals, metalloproteins, and microorganisms, which act as prooxidants. Oxidized oil may contain trans-fatty acids, which are harmful to consumers. The use of such oxidized oil might affect the industrial application of PUFA-rich oils in terms of both economic and nutritive value. Though several strategies are available to reduce oxidation, the addition of antioxidants is considered the most feasible and economical and is widely adopted industrially.

Improving the oxidative stability of fish oil could be highly impactful given the abundance of fish oil and meal plants in Karnataka, particularly in Mangalore. The South-West Coastal region of India serves as a key hub for fish oil and fish meal production, with Karnataka hosting the maximum number of plants. Fish oil, rich in omega-3 fatty acids, has significant commercial value in various industries, including pharmaceuticals, dietary supplements, and aquaculture feed. The high concentration of production facilities in Mangalore underscores the region's potential to lead advancements in fish oil extraction, processing, and storage technologies. Improvements in yield, quality, and sustainability could help in enhancing India's fish oil industry's economic output and global competitiveness.

The mechanism of fish oil oxidation, factors influencing oxidation, and the development of strategies to mitigate this oxidative damage have been the focus of research in recent decades (Miyashita 2018). The commonly used technique to enhance the shelf life of fish oil includes the addition of antioxidants. While traditional antioxidants like tocopherols have been explored, there is a need for more research on novel, sustainable, and food-safe antioxidants that can better stabilize fish oil over extended periods. Additionally, consumer concerns over the potentially harmful effects on human health associated with the use of synthetic antioxidants also necessitate the exploration of natural sources for antioxidants. The employment of underutilized natural phenolic compounds with significant antioxidative potential should be emphasized more to enhance the storage stability of fish oil.

## 1.3 LIPOPHILISATION OF PHENOLIC ANTIOXIDANTS

Phenolic antioxidants are naturally hydrophilic, and their solubility is limited in hydrophobic systems, which further limits their application in higher concentrations in oils, fats, and emulsions (Arzola-Rodríguez et al. 2022). Lipohilisation refers to the modification of the polarity of the phenolic acids by attaching a lipophilic chain to the hydrophilic head of the antioxidant. An amphiphilic molecule that retains its original functional characteristics with improved lipophilicity can be produced by esterifying the carboxylic acid functional group of phenolic acid with a fatty alcohol using chemical or enzymatic methods (Cruz Figueroa-Espinoza and Villeneuve, 2005). However, selecting the appropriate length of the acyl donor, reaction catalyst, and optimal conditions for the successful esterification of phenolic acids is a challenging task. Several researchers have studied the optimization of suitable reaction conditions for various phenolic compounds for application in oil systems (Arzola-Rodríguez et al. 2022).

Though the polar paradox theory states that polar antioxidants are effective in bulk oils, there are several studies that contradict this theory (Shahidi and Zhong 2011). Designing a potent antioxidant particular to the oil system necessitates consideration of various factors among which lipophilicity might be key. Hence, more research should be focused on the optimization of the esterification reactions to obtain derivatives capable of concentrating at the site of oxidation in any given system. For example, when a trace amount of water is present in bulk oil, the site of oxidation changes from the air-oil interface to the water-oil interface at the reverse micelles formed, which act as nanoreactors of oxidation (Chaiyasit et al. 2007; Villeneuve et al. 2023). In such a system, the ability of the antioxidant to accumulate at these interfaces is vital to reduce oxidative damage. This is often achieved by using amphiphilic antioxidants whose hydrophilic head can be arranged at the core of the reverse micelle while the hydrophobic tail remains at the outer region (Wang et al. 2024).

The antioxidative activity of the resulting amphiphilic esters is often assessed using *in vitro* assays. These assays essentially measure the ability of the esters to scavenge free radicals in aqueous solutions. The activity of the antioxidant esters varies depending on the type of media, the presence of other pro- and anti-oxidants, and environmental conditions. Hence the synthesised esters are evaluated in lipophilic systems rich in n-3 PUFAs to comprehensively understand their efficiency in controlling oxidation under accelerated conditions.

## 1.4 SCOPE OF THE RESEARCH WORK

- Synthetic antioxidants are suspected to have carcinogenic and cytotoxic effects and can induce oxidative stress when used in excessive quantities or incorrectly. This necessitates the use of safer alternatives, including antioxidants of natural origin. However, some natural antioxidants studied recently have relatively lower efficacy than synthetic antioxidants. Thus, there is a scope to identify a potential natural antioxidant that can effectively replace synthetic antioxidants in various pharmaceutical and food products.
- The phenolic acid 3,4-dihydroxyphenylacetic acid represents a potential natural antioxidant with excellent antioxidative properties. However, the utilization of this phenolic acid in fish oils has not been reported. Thus, the study presents an opportunity to study the competence of 3,4-DHPA in retarding lipid oxidation in n-3 PUFA-rich fish oils.
- The major drawback in applying natural antioxidants like 3,4-DHPA in oil-based products is their hydrophilicity. Therefore, there is a scope for studying the lipophilization of the phenolic acid 3,4-DHPA through enzymatic and chemical methods to improve hydrophobicity.

- The structure of the phenolic ester plays a vital role in its effectiveness as an antioxidant. The study presents an opportunity to study the structural characterization of the esters of 3,4-DHPA to evaluate its potential.

## 1.5 OBJECTIVES

Based on the observations from the scope of the study, the research work will focus on the following objectives:

1. Synthesis of 3,4-dihydroxyphenylacetic acid ester of fatty alcohols using enzymatic esterification method.
2. Synthesis of 3,4-dihydroxyphenylacetic acid ester of fatty alcohols and optimization using chemical esterification method.
3. Purification of the fatty ester produced and studies on the characterization of the ester.
4. Oxidative stability studies in refined fish oil during prolonged storage in the presence of 3,4-dihydroxyphenylacetic acid, its esters, and the combination of both.
5. Oxidative stability studies in refined fish oil containing trace water during prolonged storage in the presence of 3,4-dihydroxyphenylacetic acid, its esters, and their combinations.

## 1.6 THESIS ORGANIZATION

The following chapters provide an overview of the objectives that have been addressed in this research work.

### CHAPTER 1: Introduction

This chapter addresses the context, the significance of the proposed work, and the basis for the proposed effort. The chapter also emphasizes the need for lipophilisation of unexploited phenolic acids. The objectives of the current study have been developed and presented, along with the scope of the work.

### CHAPTER 2: Review of literature

An extensive and thorough analysis of the literature relevant to the research work is given in this chapter. A detailed insight into the potential of phenolic antioxidants, emphasizing their mechanism of action and lipophilisation strategies, has been provided. A comprehensive review of the work done on the application of various esters, including 3,4-DHPA in bulk oils and emulsion systems, is given. A concise summary of the main sources, extraction and refining methods, and composition of fish oil has been given. Parameters affecting oxidation in fish oil during storage and the strategies to control oxidation have been discussed.

### CHAPTER 3: Synthesis and characterization of 3,4-dihydroxyphenylacetic acid esters

This chapter discusses the results of the enzymatic method of esterification of 3,4-DHPA using immobilized lipase from *Candida antarctica* and *Thermomyces lanuginosus* under various reaction conditions. The chapter provides the results of the homogenous and heterogenous catalyst-based esterification of 3,4-DHPA under optimal reaction conditions. The efficiency of the different catalysts in the synthesis of the lipophilic esters has been discussed briefly. The studies on the structural aspects of 3,4-DHPA esters using relevant characterization techniques have been presented. The chapter also provides the results of *in vitro* assays carried out to evaluate the antioxidative potential of the synthesized esters.

### CHAPTER 4: Application of lipophilic esters of 3,4-DHPA in oxidative stability studies of fish oil

The chapter deals with the results of the characterization of crude and refined fish oil used for the oxidative stability study. The results of the application of the synthesized esters in reducing oxidation in refined fish oil have been presented and compared with the potential of the native phenolic acid and synthetic antioxidants commonly used.

### CHAPTER 5: Application of lipophilic esters of 3,4-DHPA in oxidative stability studies of fish oil containing trace water

The application of the synthesized esters in different molar combinations with 3,4-DHPA was studied in fish oil containing trace water, and the results are discussed, emphasizing the role of reverse micelles in accelerating oxidation in bulk oils.

## CHAPTER 6: Summary and Conclusions

The work on the lipophilization of 3,4-DHPA and its application in oxidative stability studies of refined fish oil utilizing various molar combinations of the esters is summarized in this chapter, along with important findings. The scope of future work is also outlined in this chapter.

# CHAPTER 2

# REVIEW OF LITERATURE

# CHAPTER 2

# REVIEW OF LITERATURE

## 2.1 ANTIOXIDANTS

In food systems, antioxidants are described as substances that, when present at lower concentrations than oxidizable substrates, can significantly block or delay the oxidation of the substrate in question (Halliwell 1996). It is a vital food additive due to its ability to extend the shelf-life of various food products while maintaining its nutritional properties intact (Gulcin 2020). They can retard the oxidation of lipids and control the formation of oxidation products, thereby contributing to preserving the sensory and nutritional properties of oxidation-prone foods. An efficient antioxidant should be inexpensive, effective, non-toxic, highly stable, and should have good solubility in the food product (Shahidi and Ambigaipalan 2015).

### 2.1.1 Nature of origin

Antioxidants can be classified based on their nature of origin into natural and synthetic antioxidants (Fig. 2.1). They have been in use for decades in various food systems, and the choice of compound added usually depends on the nature of the food matrix, especially its lipid profile. For industrial purposes, synthetic antioxidants such as tert-butyl hydroquinone (TBHQ). Butylated hydroxy anisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate (PG) are highly preferred since they are inexpensive, have high purity and constant activity (Berdahl et al. 2010). They are commonly used to stabilize vegetable oil, fish oils, and other animal fats and oils. BHA, BHT, and TBHQ are oil soluble, while PG is slightly soluble in oils.

TBHQ, BHA, BHT, and PG are hydroxy-substituted phenols (Fig. 2.2) and are reported to have radical scavenging ability through which they retard oxidation (Berdahl et al. 2010). The efficiency of antioxidants in a system is determined by their structure, solubility, and the properties of the matrix to which they are added (Berdahl et al. 2010). Among the synthetic antioxidants, TBHQ was more effective than BHT and BHA due to the presence of 2-para hydroxyl groups in the phenol ring (Shahidi and Ambigaipalan 2015). Further, TBHQ does not change the flavor or odor of the food matrix to which it is added (Dolatabadi and Kashanian 2010). On the other hand, BHT, having two tert-butyl groups, might exhibit steric hindrance compared to BHA (Nanditha and Prabhashankar 2009).

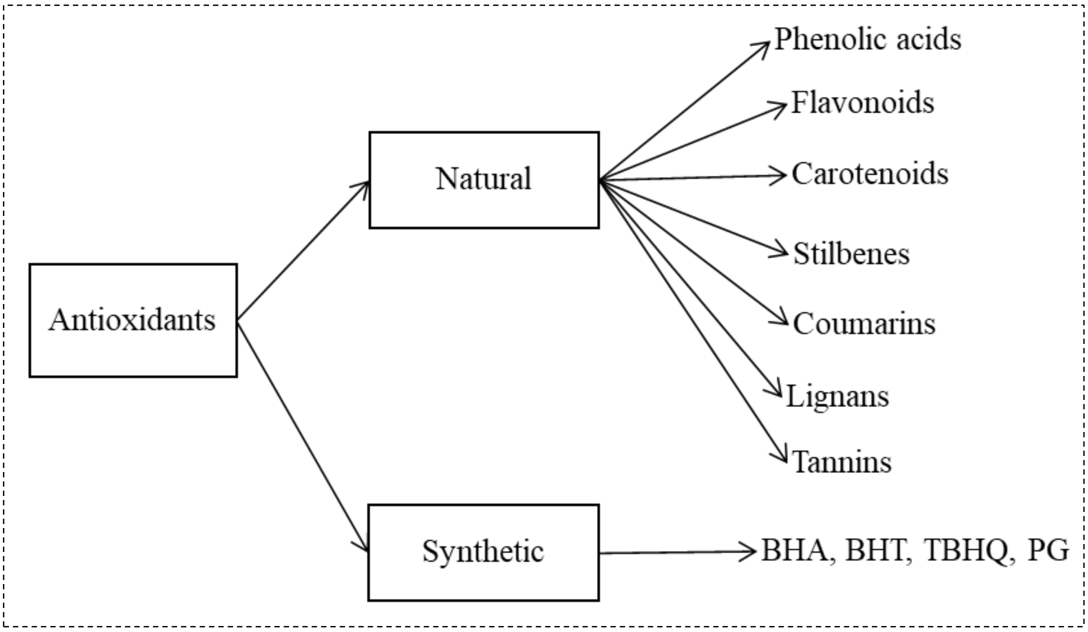


**Fig. 2.1 Classification of antioxidants**

Although BHA, BHT, and PG are considered GRAS (generally recognized as safe) and widely used, recent studies have shown that they have carcinogenic and genotoxic effects when used in higher concentrations (Dolatabadi and Kashanian 2010; Shahidi and Ambigaipalan 2015). Additionally, consumer acceptance of these synthetic antioxidants has diminished rapidly due to the rising concerns about their safety and possible accumulation in various parts of the body. Consequently, the use of antioxidants in foods, drugs, cosmetics, and oils is strictly regulated by various governing bodies, and the usage level and accepted daily intake level varies between countries (Taghvaei and Jafari 2015). According to the US FDA, these synthetic antioxidants (TBHQ, BHA, BHT, and PG) should not exceed 0.02% of fat or oil content in the food (Berdahl et al. 2010). The Food Safety and Standards Authority of India (FSSAI) has regulated the use of these synthetic antioxidants in edible oil to a maximum acceptable concentration of 200 mg/L (Wang et al. 2024). Moreover, in some countries, including Japan, Sweden, Australia, and European countries, the use of BHT in food has been banned.

The restricted use of synthetic antioxidants might limit the performance of these petroleum-derived antioxidants, including BHA and BHT. Consumer demand for products devoid of synthetic components also directs extensive research on alternate sources of antioxidants for usage in lipophilic food systems. Antioxidants of natural origin have gained momentum in recent years due to their excellent potential to retard oxidation on par with synthetic antioxidants. Unlike synthetic antioxidants, the usage of antioxidants derived from natural sources such as plants, herbs, and spices is generally considered safe, and no testing is required for approval for human use (Berdahl et al. 2010). Antioxidants from plants generally consist of an array of secondary metabolites (Fig. 2.3), including flavonoids (rutin and catechin), phenolic acids (rosmarinic acid and carnosic acid), and carotenoids (β-carotene).

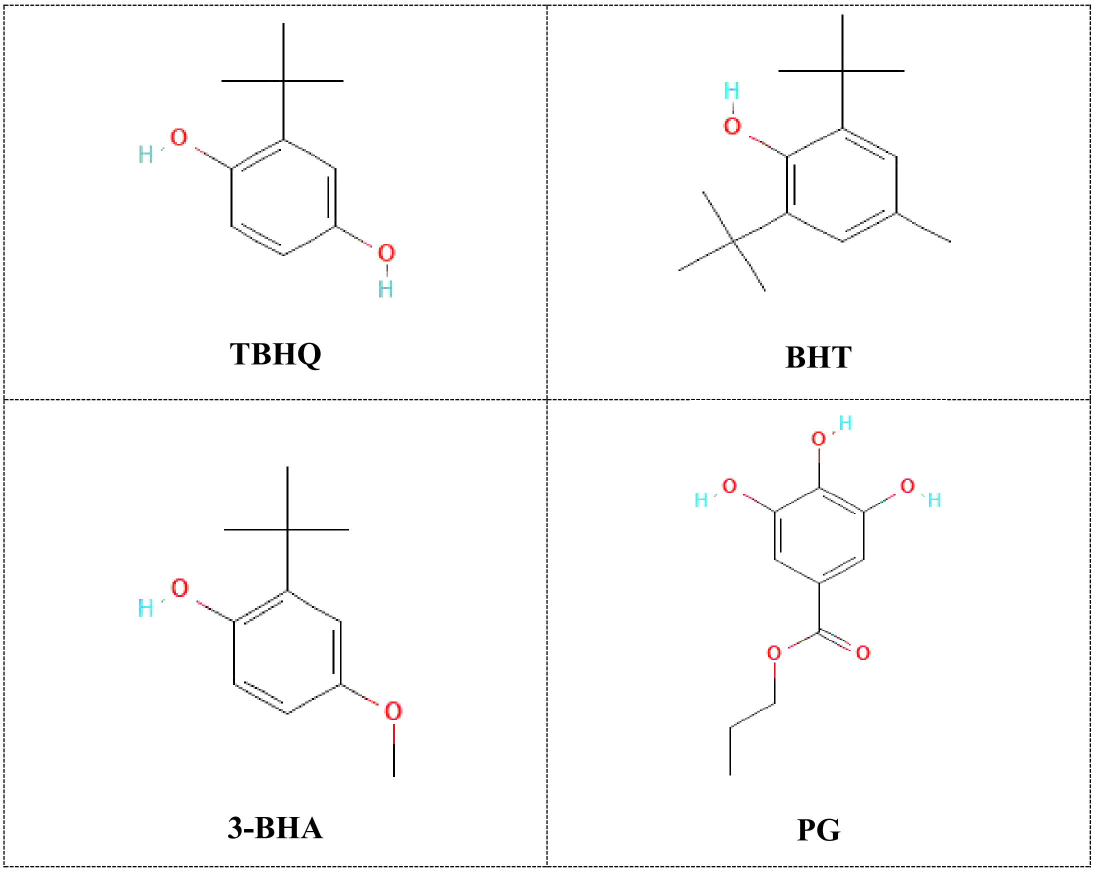


**Fig. 2.2 Structure of synthetic antioxidants**

These phenolic compounds represent a significant portion of the human diet of about 200 mg/day (Clifford and Scalbert 2000). Phenolic acids act as free radical scavengers, while flavonoids can also act as metal chelators. The antioxidative properties of gentisic, vanillic, and protocatechuic acid were studied in sardine oil (Vaisali et al. 2016a). Gentisic acid was found to have superior antioxidative potential while vanillic acid was the least effective. In addition to antioxidative properties, several phenolic acids also exhibit biological potential, including anti-inflammatory, neuroprotective, anti-depressant, anti-hypertensive, and anti-cancer properties (de Araújo et al. 2021).

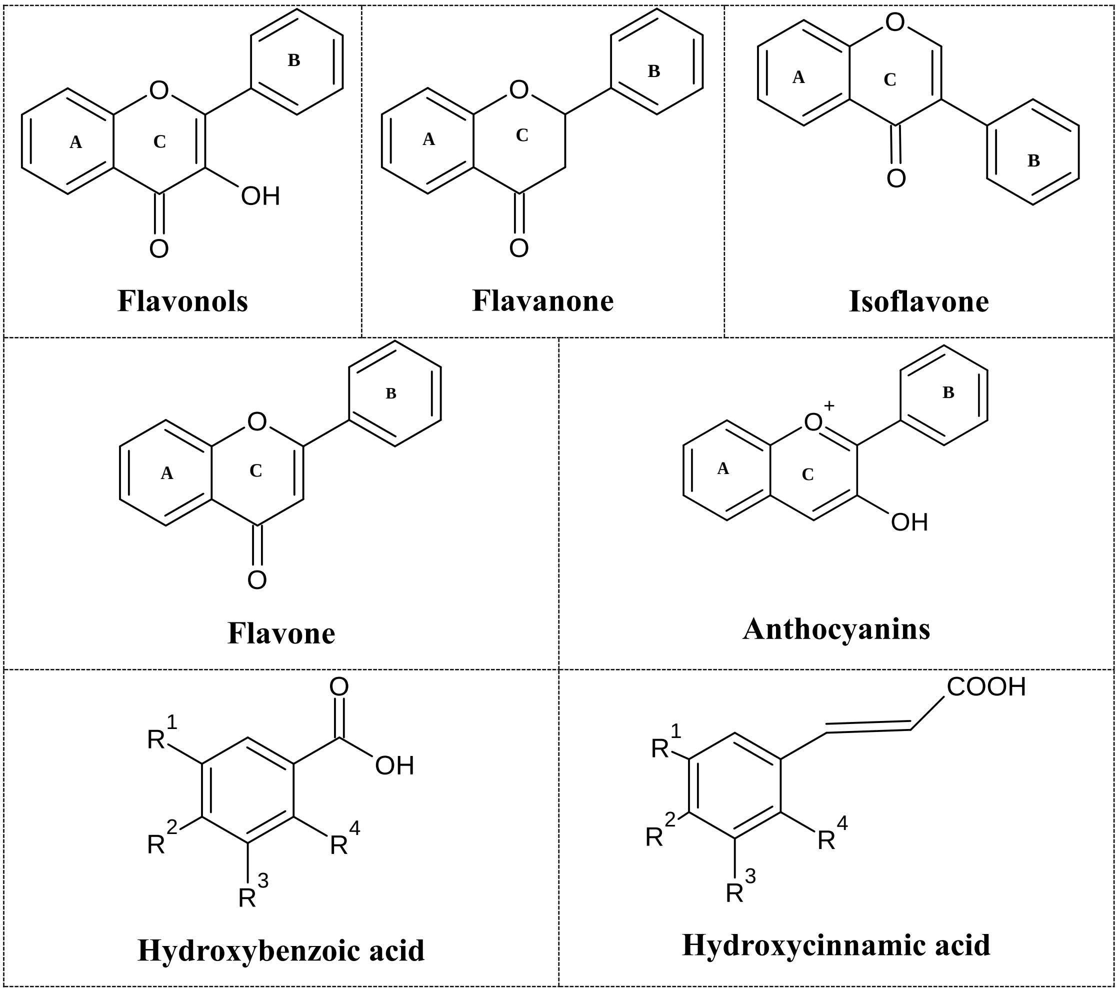


Fig. 2.3 Structure of various classes of polyphenols

The ability of natural phenolic acids and their derivatives to exhibit antioxidative activity depends on various factors. This includes the number and location of hydroxyl groups attached to the aromatic ring, the binding site and mutual position of hydroxyl groups in the aromatic ring, and the type of substituents (Rice-Evans et al. 1996). The presence of different substituents in the phenolic ring influences the antioxidative activity by modulating the radical scavenging ability. When the phenolic ring is unsubstituted, there is negligible antioxidative capacity, while the polyphenolic structure enhances its potential as an antioxidant (Gulcin 2020). The presence of electron-donating groups in the *ortho-* or *para-* positions significantly increases the activity of phenolic acids (Chimi et al. 1991). Phenolic compounds having a catechol group act as chain-breaking antioxidants, and examples include caffeic acid, rosmarinic acid, ellagic acid, etc. (Razzaghi-Asl et al. 2013). The presence of the catechol moiety helps ease hydrogen abstraction from either the 3-OH or 4-OH group, leading to the formation of stable semiquinone radicals (Ordoudi and Tsimidou 2006).

### 2.1.2 Mechanism of action of antioxidants

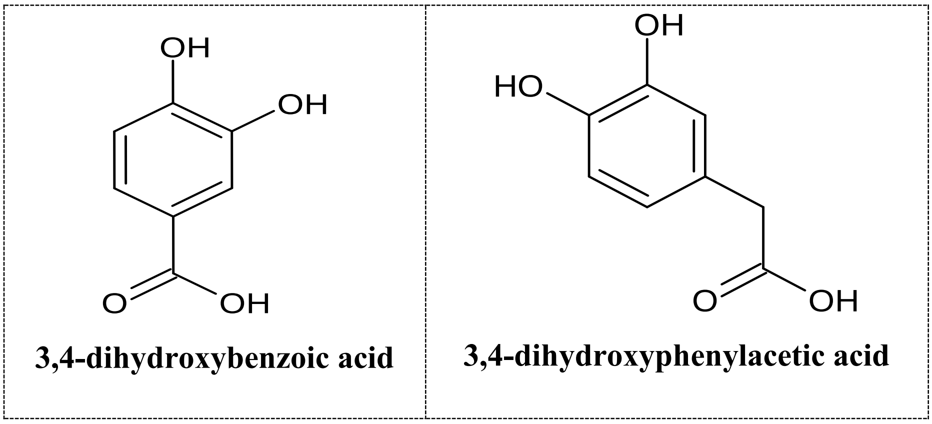
Various kinds of antioxidants can slow down oxidation through distinct mechanisms. Based on the mechanisms through which oxidation is inhibited or retarded, two types of antioxidants are classified (Gulcin 2020). Primary antioxidants act as free radical scavengers since they can donate hydrogen, which helps in prolonging the induction period. This includes phenolic compounds such as α-tocopherols, flavonoids, BHT, BHA, and TBHQ, which react with lipid hydroperoxyl radicals, resulting in antioxidant radicals. Antioxidant radicals produced are more stable and much less active in propagation reactions than lipid hydroperoxides. The antioxidant potency of phenolic compounds depends on the hydrogen donating power of OH groups in *ortho* and *para* positions and their bond dissociation enthalpies (BDE) (Chaiyasit et al. 2007). Secondary antioxidants retard lipid oxidation through mechanisms such as metal chelating, oxygen scavenging, and absorbing UV radiation. Chelating agents include phytic acid, EDTA, and citric acid, and reducing agents such as ascorbic acid belong to the secondary antioxidants.

#### 2.1.2.1 Free radical scavenging antioxidants

Free radical scavenging involves the donation of hydrogen to the free radicals present in fish oils, leading to the production of a rather stable antioxidant radical. Low bond dissociation energy for O-H in antioxidants thermodynamically favors the hydrogen transfer from antioxidants to the peroxy radical in fish oils (Cao et al. 2006) and further stabilizes the antioxidant radical formed. The bond strength of OH is influenced by the substitution of hydrogen in the benzene ring. Alky or hydroxyl group substitution enhances the stability of antioxidant radicals and improves the radical scavenging activity (Shahidi and Wanasundara 2002) by reducing the OH bond strength.

The antioxidative potential of gentisic acid, vanillic acid, and protocatechuic acid in sardine oil was studied (Vaisali et al. 2016a). These derivatives of hydroxybenzoic acids were studied for peroxide value, conjugated diene value, *p*-anisidine value, and thiobarbituric acid reactive substances. Among them, gentisic acid was found to be the most effective antioxidant for sardine oil, while protocatechuic acid and vanillic acid showed little to no effect. This variation is attributed to the position of the hydroxyl group substitution of the phenolic acids. Substituents that are electron withdrawing, such as COOR and COOH at the *ortho* and *para* position, strengthen the OH bond, resulting in the formation of destabilized antioxidant radical and reduced antioxidant efficiency (Rice-Evans et al. 1996). However, the incorporation of an additional -$CH_2$- chain between the phenyl ring and the carboxylic acid group (Fig. 2.4) decreases the impact of the electron-withdrawing carboxylate group and enhances the antioxidant capacity.

The ability to donate hydrogen to food free radicals can be predicted by the reduction potential of antioxidants. Lower reduction potential corresponds to higher hydrogen donating ability (Choe and Min 2005). If the reduction potential of the antioxidant is lower than that of food radicals, then the antioxidant will be able to donate hydrogen to the radicals. The antioxidant radicals thus formed do not catalyze the oxidation of other compounds since they have low reduction potential (Shahidi and Wanasundara 2002). The antioxidative capacity of hydroxycinnamic acid and catechins was studied with minced fish muscle by Medina et al. (2007). Among the antioxidants studied, caffeic acid was found to inhibit lipid oxidation. It was also correlated that the hydrogen donating ability had an influence on the inhibition of the oxidation rather than the metal chelating ability.



**3,4-dihydroxybenzoic acid** **3,4-dihydroxyphenylacetic acid**

**Fig. 2.4 Presence of additional $-CH_2-$ chain in 3,4-dihydroxyphenylacetic acid**

### 2.1.2.2 Metal chelating antioxidants

The presence of minor components, including metals, accelerates lipid oxidation. Metals with high reactivity are efficient pro-oxidants (Gulcin 2020). Transition metals are involved in stimulating lipid oxidation through the Fenton reaction and by decomposing hydroperoxides into peroxyl and alkoxyl radicals (Andersson and Lingnert 1998). Metals can enhance radical formation since they can abstract hydrogen from lipids. Iron is found as $Fe^{3+}$ in foods. Metal chelators retard the lipid oxidation by forming insoluble metal complexes. They may also cause steric hindrance between food components and metals (Graf and Eaton 1990). The pro-oxidant properties might be lost when iron is chelated. Commonly used metal chelators in food include EDTA and citric acid. Crude oil often contains metals such as iron and copper, and the concentration is reduced after refining. Degumming is done in sardine oil refining to remove iron and copper effectively (Charanyaa et al. 2017). The metal chelating ability of flavonoids and their structure-activity relationship was studied (Mira et al. 2002). The presence of a 2,3 double bond and both the catechol group in the b-ring and 3-hydroxyl group were found to have better $Fe^{3+}$ reducing activity. The ability to reduce copper ions was dependent on the number of hydroxyl groups present.

### 2.1.2.3 Singlet oxygen quenching antioxidants

Singlet oxygen reacts at a higher rate with lipids than triplet oxygen. It is a powerful oxidizing agent which can readily react with biomolecules. It is formed in the presence of light and photosensitizers (Gulcin 2020). Compounds such as tocopherols, carotenoids, phenolic acids, and curcumin are able to quench singlet oxygen (Choe and Min 2005). Physical quenching of singlet oxygen happens through its conversion into ground-state triplet oxygen by energy transfer. The process of singlet oxygen quenching by energy transfer is characterized by the absence of oxygen consumption and product formation. In this process, the quencher's energy level is closely aligned with or lower than that of singlet oxygen. The resulting energy transfer effectively quenches the singlet oxygen, preventing it from reacting with other molecules.

$$^{1}O_2 + Q \rightarrow {}^{3}O_2 + {}^{3}Q$$

$$^{3}Q \rightarrow {}^{1}Q \text{ (no radiation)}$$

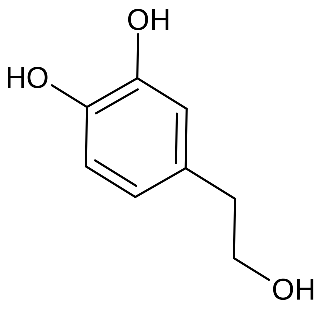
The number of conjugated double bonds present in carotenoids determines their oxygen-quenching capacity (Beutner et al. 2001). Singlet oxygen is quenched through a charge transfer mechanism when the quencher has a higher reduction potential. Examples of such quenchers are amines and phenols, including tocopherols, sulfides, and azides. Chemical quenching occurs through the oxidation of the quencher and the production of oxidation products of the quencher. Chemical quenchers of singlet oxygen are ascorbic acid, β-carotene, peptides, and phenolics. Ascorbic acid reacts with singlet oxygen, resulting in the formation of unstable hydroperoxide (Choe and Min 2009). Tocopherol reacts with singlet oxygen irreversibly and produces tocopherol hydroperoxides. The α-tocopherols react better than the β-tocopherols and δ-tocopherols (Mukai et al. 1991).

### 2.1.3 Structure-activity relationship (SAR) of antioxidants

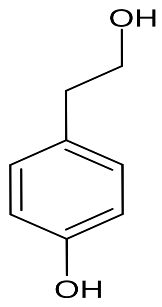
For years, structure-activity relationship studies (SARs) have been helpful in identifying molecules with potential biological activities in various research fields. Analysis of the chemical structure and physicochemical properties of phenolic acids and flavonoids enables the identification of suitable natural antioxidants for a range of food matrices. Lipid oxidation is a complex process that can be influenced by various factors, including the structure of the selected antioxidant. This structure can play a crucial role in enhancing the antioxidant's effectiveness.

### 2.1.3.1 Number and position of phenolic hydroxyl groups

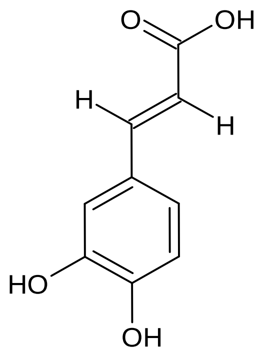
The functional group majorly responsible for the antioxidative activity of phenolic acids is the hydroxyl groups attached to the phenolic rings. Phenolic OH can donate hydrogen to the free radicals, resulting in the formation of a stable antioxidant radical. Therefore, the number and position of the phenolic hydroxyl groups should be the primary feature for selecting antioxidants. The presence of a second OH group at the ortho-position of the phenolic ring (catechol moiety) reduces the bond dissociation enthalpy of the OH bond, thereby increasing the rate of hydrogen transfer to peroxyl radicals (Morales and Lucas 2010). An example was the radical scavenging activity of hydroxytyrosol as compared to tyrosol (Fig. 2.5). The hydroxytyrosol showed better activity and also increased the induction time fivefold in olive oil (Artajo et al. 2006; Mateos et al. 2003).



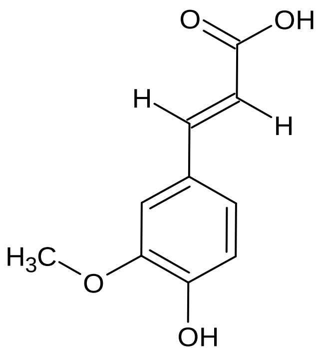
**Hydroxytyrosol**



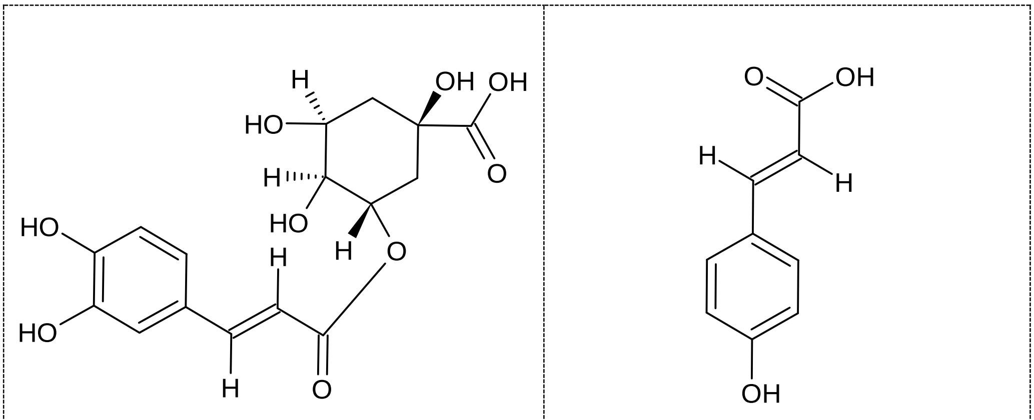
**Tyrosol**



**Caffeic acid**



**Ferulic acid**



**Chlorogenic acid** **p-Coumaric acid**

**Fig. 2.5 Structures of phenolic acids**

The presence of a pyrogallol moiety (3-(3,4,5-trihydroxyphenyl) propenoic acid) enhanced its radical scavenging activity in ABTS and DPPH assays compared to the monohydroxylated caffeic acid (Siquet et al. 2006). However, it should be kept in mind that the efficiency might vary in a lipophilic system, wherein the presence of more hydroxyl groups could make the compound more hydrophilic, decreasing its antioxidant capacity. A similar trend was observed for flavonoids as well (Rice Evans et al. 1996).

The ortho-dihydroxy derivatives of hydroxycinnamic acids, such as caffeic acid and chlorogenic acids, are better at reducing the oxidation of LDL when compared to para-coumaric acid (Laranjinha et al. 1996). Phenolic acids with catechol groups are chain-breaking antioxidants and can form stable semiquinone radicals after hydrogen abstraction from 3- and 4- OH groups (Ordoudi and Tsimidou 2006). In addition, ortho-dihydroxy phenolic acids are known to form intramolecular H-bonds, which is the reason for the stabilization of the intermediate phenoxy radicals (Chen and Ho 1997; Foti and Ruberto 2001).

#### 2.1.3.2 Presence of methoxy groups in the phenolic ring

The introduction of electron-donating groups, such as the methoxy group, to the ortho-phenolic structure could enhance the hydrogen-donating ability. For example, 5-methoxy-protocatechuyl alcohol had better radical scavenging activity than the protocatechuyl alcohol (Torres de Pinedo et al. 2007). This is favored only when the methoxy group does not replace a hydroxyl group, and the effect is still unclear when used in a lipid system. The enhanced activity is a function of the electron-donating ability of the methoxy group that can stabilize the phenoxy radical. When caffeic acid (3,4-dihydroxycinnamic acid) and ferulic acid (3-methoxy-4-hydroxycinnamic acid) were compared in the Rancimat test in a food matrix, caffeic acid exhibited better efficiency than the ferulic acid, resulting in three times longer induction times (Artajo et al. 2006).

#### 2.1.3.3 Presence of acyl groups in the phenolic ring

Natural antioxidants are often esterified to improve their lipophilicity so that they can be incorporated into oil-based systems. the esterification involves the addition of a fatty acyl chain to the antioxidant, which increases the hydrophobicity of the compound. Depending on the position of esterification, the effect on the antioxidative potential varies. Esterification at the phenolic group reduces the radical scavenging activity, whereas the esters, which had intact di-orthophenolic structure, were unaffected by the modification (Morales and Lucas 2010). Though an extra primary alcohol group was present in the fatty acid esters, the potential was lesser than in fatty acid esters with the di-ortho phenolic structure unmodified, implying the importance of phenolic hydroxyl groups. The effect was highly pronounced in the oil system analyzed using the Rancimat test for a series of fatty acid esters of hydroxytyrosol than the DPPH scavenging assay (Grasso et al. 2007; Trujillo et al. 2006). Esterification with short-chain alkyls such as methyl, ethyl, propyl, and butyl of hydroxycinnamic acids indeed reduced the radical scavenging activity, but their lipophilicity has increased, which enables their use in lipophilic media (Gaspar et al. 2010).

#### 2.1.3.4 Length and nature of the alkyl chain

An increase in the length of the alkyl chain connecting the phenolic ring and the carboxylic/alcohol group in phenolic compounds increased the radical scavenging activity and also elongated the induction period in the Rancimat test for vanillic and homovanillic acid (Artajo et al. 2006). A similar increasing trend was observed with the presence of one, two, or three methylene chains in protocatechuyl alcohol, hydroxytyrosol, and dihydrocaffeoyl alcohol, respectively (Torres Pinedo et al. 2007).

This could be attributed to the increase in electron-donating groups with an increase in the alkyl chain, leading to a more stabilized phenoxy radical. The presence of a double bond on the side chain could bring a reduction in free radical scavenging ability. This is due to the electron-withdrawing effects of the double bond present on the side chain. However, the effect varies depending on the medium since the alkene group might fasten the radical side reactions (Morales and Lucas 2010). The hydroxyl group on the side chain might increase the radical scavenging activity since they can interact with free radicals and get oxidized easily.

The consideration of significant functional groups alongside the hydrophobicity of antioxidants serves to deepen our understanding of their behavior in various matrices. This knowledge can be leveraged to select an efficient antioxidant capable of retarding oxidation in the appropriate media. Therefore, it is imperative for researchers and industry professionals alike to acknowledge the value of these factors in the selection process, as it can lead to superior outcomes in the long run.

### 2.1.4 *In vitro* antioxidant activity assays

Assessment of the antioxidative potential of natural antioxidants is essential to make sure that the desired protective effect can be achieved in the food matrix applied. Oxidation progresses through a chain reaction resulting in the release of various products marking the state of oxidation. There are different *in-vitro* methods for the analysis of the interaction of the antioxidant with the reactive species involved in oxidation based on various mechanisms. Since a single antioxidant assay could not completely describe the antioxidant potential, a set of assays based on different mechanisms should be conducted to understand the overall antioxidant profile (Alam et al. 2013). Comparing different antioxidant activity assays can be challenging due to their varying mechanisms of analysis. Given the importance of preserving food quality and safety, it is essential to have a thorough understanding of the mechanisms that underlie the process of lipid peroxidation and the role of antioxidants in preventing it.

Based on the mechanism involved, antioxidant assays could be divided into two categories: HAT (hydrogen atom transfer) and SET (single electron transfer) methods (Gulcin 2020). The SET method involves the transfer of a single electron to reduce reactants such as metals, carbonyl, and radicals, resulting in a change in color as the oxidant is reduced (Apak et al. 2016). HAT-based assays measure the ability of the antioxidant to donate hydrogen and quench free radicals. These methods are solvent and pH independent and are rapid. The hydrogen transfer between antioxidants and free radicals can be expressed as follows.

$$AH + X^{\bullet} \longrightarrow A^{\bullet} + XH$$

The presence of metal ions might cause higher reactivity and is not recommended for HAT-based methods. Assays such as Total radical trapping antioxidant parameter (TRAP), Oxygen Radical absorption capacity (ORAC), β-carotene bleaching assays, inhibition of induced LDL oxidation, and chemiluminescent assays are examples of HAT methods. The relative reactivity of these methods is a measure of the bond dissociation energy (BDE) of the hydrogen-donating group of the antioxidant.

SET methods analyze the ability of the antioxidant to transfer a single electron to radicals, carbonyls, and metals. The reaction could be written as follows.

$$AH + X^{\bullet} \rightarrow AH^{\bullet+} + X^{-}$$

$$AH^{\bullet+} + H_2O \rightarrow A^{\bullet} + H_3O^{+}$$

$$X^{-} + H_3O^{+} \rightarrow XH + H_2O$$

$$AH + M^{3+} \rightarrow AH^{+} + M^{2+}$$

The relative reactivity of SET reactions is governed by deprotonation, and the reactions are usually pH-dependent. The reaction occurs slowly and takes longer time to complete. Hence, the percent reduction in the product is used to measure the antioxidant activity rather than its kinetics. Methods such as Trolox equivalent antioxidant capacity assay (TEAC), Ferric reducing antioxidant power assay (FRAP), 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, 2,2-Azinobis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical scavenging assay, and Cupric ions ($Cu^{2+}$) reducing antioxidant power assay (CUPRAC) all follow SET mechanism. Both HAT and SET mechanisms happen almost always together, while the antioxidant structure and pH are the determinants of the balance between them (Gulcin 2020).

### 2.1.4.1 DPPH radical scavenging activity assay

The free radical scavenging assay is a commonly studied method since it describes the ability of the antioxidant to prevent lipid peroxidation. This process involves antioxidants interacting with peroxide radicals and subsequently scavenging them in order to terminate the chain reaction, consequently increasing the shelf life of food products. By preventing the formation of free radicals and scavenging existing ones, antioxidants can prevent the chain reaction of oxidation that can lead to negative effects on food quality, such as rancidity and color changes. Therefore, the free radical scavenging assay is a crucial tool in food preservation, as it allows us to assess the effectiveness of various antioxidants in preventing lipid peroxidation and thus ensuring the safety and quality of food products.

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) is a stable organic nitrogen radical with a deep purple color. The radical scavenging activity assay involves the reduction of stable free radical DPPH in the presence of an antioxidant molecule in an alcoholic solution resulting in the disappearance of the deep purple color. The loss in color after the addition of the antioxidant is measured as absorbance in a spectrophotometer at 517 nm, and the radical scavenging activity is determined by comparing it with a blank without any antioxidant. The reaction between the antioxidant and the DPPH radical might happen through two mechanisms, namely HAT and SPLET (sequential proton loss electron transfer).

$$\text{ArOH} + \text{DPPH}^{\bullet} \rightarrow \text{ArOH}^{\bullet} + \text{DPPH}_2 \ \textbf{(HAT)}$$

$$\text{ArOH} \leftrightarrows \text{ArO}^{-} + \text{H}^{+} \ \textbf{(SPLET)}$$

$$\text{ArO}^{-} + \text{DPPH}^{\bullet} \rightarrow \text{ArO}^{\bullet} + \text{DPPH}^{-}$$

$$\text{DPPH}^{-} + \text{H}^{+} \rightarrow \text{ArO}^{\bullet} + \text{DPPH}_2$$

The assay is generally conducted using methanolic or ethanolic solutions of the antioxidant and DPPH radical. The DPPH solution is not stable, and hence, it should be prepared fresh. The radical scavenging assay has been used to measure the antioxidative potential of a wide variety of antioxidants. Several plant extracts are reported to have good antioxidant capacity due to the presence of an array of phenolic components and flavonoids (Cömert and Gökmen 2018). The assay serves as a valuable tool for the identification of plant species that exhibit exceptional antioxidant profiles. This information is highly relevant for the purpose of determining the suitability of said species for incorporation into both food and pharmaceutical products. By enabling the identification of such species, the assay can play a pivotal role in enhancing the quality and efficacy of these products.

The radical scavenging activity is usually expressed as the percent inhibition of DPPH radicals and is calculated using the difference in the absorbance before and after the addition of the antioxidant. However, the percent inhibition could not be compared to other similar assays conducted in order to understand the relative antioxidant activities of analogous antioxidants. $IC_{50}$ refers to the inhibitory concentration required to scavenge 50% of the free radicals using an antioxidant and is usually given in micromolar concentrations. This unit of measurement enables the comparison between various phenolic compounds, flavonoids, and synthetic antioxidants to assess their potential as suitable antioxidants. Sharma and Bhat studied the $IC_{50}$ values of common synthetic antioxidants such as BHT, ascorbic acid, and propyl gallate in unbuffered and buffered methanolic solutions (Sharma and Bhat 2009). BHT was found to have a significantly higher difference in the $IC_{50}$ values measured in the two methanolic solutions when compared to ascorbic acid and propyl gallate. This indicates the influence of the medium used for the assay on the resultant activity of the antioxidant. This study also implies that light, oxygen, and pH of the reaction mixture have a significant impact on the DPPH scavenging activity.

The DPPH radical scavenging activity is highly used due to several factors including low cost involved, good reproducibility, applicability at room temperature, ease of performing experiments and automation, and significantly comparable results (Munteanu and Apetrei 2021). Though $DPPH^{\bullet}$ is not a natural radical, the mechanism of reaction with antioxidant is similar to that with peroxyl radicals ($ROO^{\bullet}$). However, its limited solubility in water might limit its application for different antioxidants with varying solubility.

#### 2.1.4.2 Ferric reducing antioxidant power (FRAP) assay

Reducing power of an antioxidant describes the ability of the compound to reduce a reactant by donating electrons (Gulcin 2020). Antioxidants can act as reductants, and the assay determines the increase in absorbance as a measure of reducing power. A reductant refers to a compound that can reduce an oxidant by donating electrons. All antioxidants are reductants, but all reductants might not necessarily be antioxidants (Prior and Cao 1999). FRAP is a SET-based method wherein the reduction of ferric ions to ferrous ions in an acidic media in the presence of an antioxidant is measured. A ligand complex of $Fe^{3+}$ with 2,4,6-tripyridyl-s-triazine $[Fe^{3+}\text{-}(TPTZ)^{2}]^{3+}$ is reduced to form an intense blue-colored $[Fe^{2+}\text{-}(TPTZ)^{2}]^{2+}$ resulting in the increase in absorbance at 593 nm. The increase in absorbance corresponds to the increase in antioxidant power.

The FRAP assay is carried out under acidic conditions with a pH value of 3.6 in order to ensure the solubility of iron during the reaction. At this low pH, the ionization potential that drives electron transfer is reduced, leading to an increase in the redox potential and a consequent shift in the dominant reaction mechanism (Hagerman et al. 1998). FRAP values measured as an increase in absorbance are compared with ferrous ion standard ($FeSO_4$) or with a standard antioxidant solution to comprehend the individual antioxidant power. The assay was initially designed by Benzie and Strain to measure the ferric-reducing ability of antioxidants in plasma samples, and it was later adopted for other samples (Benzie and Strain 1996). A comprehensive profile of several natural and synthetic antioxidants as measured by FRAP and DPPH scavenging assay is given in Table 2.1. Maqsood and Benjakul measured the antioxidative activity of catechin, caffeic acid, ferulic acid, and tannic acid in different assays (Maqsood and Benjakul 2010). Tannic acid was found to have higher FRAP values than other antioxidants. The result was similar in other *in vitro* assays, including DPPH and ABTS radical scavenging activities.

The FRAP method is rapid, inexpensive, reproducible, and can be correlated well with other *in-vitro* assays. Theoretically, any compound with a redox potential lesser than that of the redox pair Fe(III)/Fe(II) can reduce $Fe^{3+}$ to $Fe^{2+}$ without having actual antioxidative properties, which could lead to overestimation of FRAP values (Gulcin 2020). In addition, compounds having a maximum absorption at 593 nm could also interfere with the actual measure of FRAP values. The time required for completion of the reaction between the antioxidant and $Fe^{3+}$ can range from a few minutes to several hours. This could result in variation in the analysis time for different antioxidants.

**Table 2.1 DPPH radical scavenging activity and ferric reducing antioxidant power of various phenolic acids, flavonoids, and synthetic antioxidants**

| Reference | Antioxidant | % Inhibition of DPPH | FRAP ($OD_{593}$) |
|---|---|---|---|
| Vaisali et al. 2016b | Ferulic acid | 34 (35 µM) | 0.23 (75 µM) |
| | Caffeic acid | 70 (35 µM) | 1.9 (75 µM) |
| | Sinapic acid | 40 (35 µM) | 0.4 (75 µM) |
| | Catechin | 83 (35 µM) | 1.019 (50 µM) |
| | Quercetin | 93 (35 µM) | 0.894 (50 µM) |
| | Rutin | 78 (35 µM) | 0.636 (50 µM) |
| Kilic et al. 2013, 2014 | EDTA | ND | 96.2 (45 µg/mL) |
| | BHA | 73.9 (30 µg/mL) | 68.1 (45 µg/mL) |
| | BHT | 58.1 (30 µg/mL) | 64.5 (45 µg/mL) |
| | α-tocopherol | -- | 81.2 (45 µg/mL) |
| | Ascorbic acid | 92.1 (30 µg/mL) | 72.3 (45 µg/mL) |
| | *p*-coumaric acid | 55.6 (30 µg/mL) | 78.3 (45 µg/mL) |
| | Ellagic acid | 85.6 (30 µg/mL) | 48.9 (45 µg/mL) |
| Katalinic et al. 2004 | Catechin | 94.4 (0.5 M) | 2 (rel.a.) |
| | Quercetin | 95.3 (0.5 M) | 4.8 (rel.a.) |
| | Ascorbic acid | 91.5 (0.5 M) | 2 (rel.a.) |
| | Trolox | 92.63 (0.5 M) | 2 (rel.a.) |
| | BHT | 49.2 (0.5 M) | 0.2 (rel.a.) |
| He et al. 2018 | Catechin | 32.3 (0.4 M) | 42.5[a] (0.4 M) |
| | Epicatechin | 40.3 (0.4 M) | 42.2[a] (0.4 M) |
| | Epigallocatechin | 53.3 (0.4 M) | 63.8[a] (0.4 M) |
| | Epigallocatechin gallate | 77.2 (0.4 M) | 96.9[a] (0.4 M) |

| | | | |
|---|---|---|---|
| Manigandan et al. 2015 | Taxifolin | 91.19 (0.1 M) | 86.61 (0.1M) |
| | Ascorbic acid | 97.92 (0.1 M) | 93.74 (0.1M) |
| Soobrattee et al. 2005* | Gallic acid | -- | 5.25 |
| | Rosmarinic acid | -- | 6.21 |
| | Chlorogenic acid | -- | 3.22 |
| | Ferulic acid | -- | 1.33 |
| | Quercetin | -- | 7.39 |
| | Myricetin | -- | 4.58 |
| | BHT | -- | 0.04 |
| | BHA | -- | 3.05 |
| | Trolox | -- | 1.91 |
| Bandoniene et al. 2002 | Caffeic acid | 93 (0.5 M) | -- |
| | Ferulic acid | 72 (0.5 M) | -- |
| | Chlorogenic acid | 50 (0.5 M) | -- |
| | Gentisic acid | 95 (0.5 M) | -- |
| | Gallic acid | 94 (0.5 M) | -- |
| | Vanillic acid | 16 (0.5 M) | -- |
| | Rutin | 92 (0.5 M) | -- |
| | Epicatechin | 79 (0.5 M) | -- |
| | Phloridzin | 13 (0.5 M) | -- |
| | Naringin | 12 (0.5 M) | -- |
| Majewska et al. 2011 | Trolox | 82.3 (50 µg/mL) | -- |
| | Quercetin | 79.2 (50 µg/mL) | -- |
| | Rhamnetin | 62.5 (50 µg/mL) | -- |
| | Isorhamnetin | 60.1 (50 µg/mL) | -- |
| | Luteolin | 62.4 (50 µg/mL) | -- |
| Liu et al. 2013a | Kaempferol | 87.06 (8 mM) | -- |
| | Rutin | 81.08 (8 mM) | -- |
| Shen et al. 2019 | *p*-coumaric acid | 6.99 (125 µg/mL) | 0.39 (50 µg/mL) |
| | Trolox | 82.04 (125 µg/mL) | 1.99 (50 µg/mL) |

| | | | |
|---|---|---|---|
| Masek et al. 2016 | Caffeic acid | 28.5 (30 µg/mL) | 30.8% (30 µg/mL) |
| | *p*-coumaric acid | 5.3 (30 µg/mL) | 4.6% (30 µg/mL) |
| Kikuzaki et al. 2002 | Ferulic acid | 27.3 (20 µM) | -- |
| | Methyl ferulate | 18.01 (20 µM) | -- |
| | Butyl ferulate | 20.3 (20 µM) | -- |
| | Octyl ferulate | 23 (20 µM) | -- |
| | BHT | 29.2 (20 µM) | -- |
| | Propyl gallate | 87.1 (20 µM) | -- |
| | Lauryl gallate | 81.6 (20 µM) | -- |
| Nenadis and Tsimidou 2002 | Dihydrocaffeic acid | 94.6 (0.5 M) | -- |
| | Rosmarinic acid | 93.4 (0.5 M) | -- |
| | Sinapic acid | 88.4 (0.5 M) | -- |
| | Trolox | 86 (0.5 M) | -- |
| | TBHQ | 69.6 (0.5 M) | -- |
| Chen and Ho 1997 | Caffeic acid | 51.5 (20 µM) | -- |
| | Ferulic acid | 24.8 (20 µM) | -- |
| | Rosmarinic acid | 85.6 (20 µM) | -- |
| | Chlorogenic acid | 36.3 (20 µM) | -- |

*- FRAP measured as mM Fe (II)/L; rel.a.-relative activity; a- relative percentage of absorbance at 400 µM of EGCG.

#### 2.1.4.3 ABTS radical scavenging activity assay

This assay determines the ability of an antioxidant to scavenge the ABTS (2,2-azinobis-3-ethylbenzthiazoline-6-sulfonic acid) free radical as a measure of the reduction in absorbance at 734 nm. Unlike DPPH, ABTS is not a stable free radical and has to be oxidized from ABTS to $ABTS^{\cdot+}$ by interaction with oxidants prior to the assay. Additionally, $ABTS^{\cdot+}$ radicals exhibit both HAT and SET reactions (Gulcin 2020). This makes it a versatile and reliable tool for analyzing the antioxidant properties of various compounds. The radical, cationic form of ABTS could be generated through different methods, including chemical reaction (using manganese dioxide or potassium persulfate), enzymatic reaction (using metmyoglobin or horseradish peroxidase), or electrochemical generation (Magalhães et al. 2008). The commonly used method is the interaction with potassium persulfate for 16 hours, resulting in the formation of $ABTS^{\cdot+}$ radical as per the following reaction.

$$S_2O_8^{-2} + 3ABTS \rightarrow 2SO_4^{-2} + 3ABTS^{\cdot+}$$

The radicals are generated through the scission of $K_2S_2O_8$ after the electron transfer in the presence of excess ABTS. The reaction between ABTS radical and antioxidant is rapid and, in most cases, was completed within 0.25-0.5 min (Gulcin 2020). The results are expressed in percentage inhibition, $IC_{50}$ as well as Trolox equivalents (TE). Trolox is usually considered the standard, and several fruit and plant extracts have been analyzed for ABTS radical scavenging activity (Pisoschi and Negulescu 2012). In a study by Maqsood and Benjakul, among ferulic acid and caffeic acid, the highest radical scavenging activity was found in ferulic acid (Maqsood and Benjakul 2010). Though caffeic acid has more hydroxyl groups, ferulic acid showed better activity, while caffeic acid performed better in DPPH radical scavenging activity. This could indicate that the role of the structure and the functional groups present in the phenolic compound significantly influence the interaction with ABTS. Nevertheless, the assay serves as a screening tool for identifying the presence of potential natural antioxidants from various plants, herbs, spices, etc. Medicinal and aromatic plant extracts have been analyzed using ABTS scavenging activity by Miliauskas and researchers (Miliauskas et al. 2004). The extracts with high radical scavenging activity were also correlated to have higher total phenolic contents.

ABTS is an effective assay for both lipophilic and hydrophilic compounds and is superior to DPPH radicals due to its higher reactivity. This enables the evaluation of a large variety of antioxidant substances. It can also be used over a wide range of pH depending on the type of antioxidant studied. However, the ABTS radical cation is not naturally found in food or biological systems which represents a lack of biological evidence (Munteanu and Apetrei 2021). Additionally, the time taken by antioxidants to reach the end point might vary for different antioxidants which could lead to underestimation of antioxidative capacity due to reading before the reaction is complete.

### 2.1.4.4 Metal chelating activity

Antioxidants feature diverse mechanisms to delay oxidation across disparate reaction systems. However, it is noteworthy that not all antioxidants exhibit radical scavenging activity. Certain antioxidants possess alternative antioxidative properties, including metal chelating activity and singlet oxygen quenching activity. It is important to consider these diverse mechanisms of action when assessing and selecting antioxidants for a given application. The metal chelating assay measures the ability of a compound to chelate metal ions, including ferrous ($Fe^{2+}$) ions. Oxidation in a lipid matrix is influenced by various minor components present, such as metal ions, moisture, and other surface-active compounds. Metal ions can act as pro-oxidants and can facilitate the production of reactive oxygen species, thereby accelerating oxidation. Among the transition metals, iron is found to have higher reactivity in Fenton reactions and can also decompose lipid hydroperoxides into more reactive peroxyl and alkoxy radicals (Gulcin 2020). Metal-chelating antioxidants can effectively chelate these metal ions before they can initiate Fenton reactions, leading to the loss of the pro-oxidant effect. It also hinders metal-catalyzed oxidation reactions.

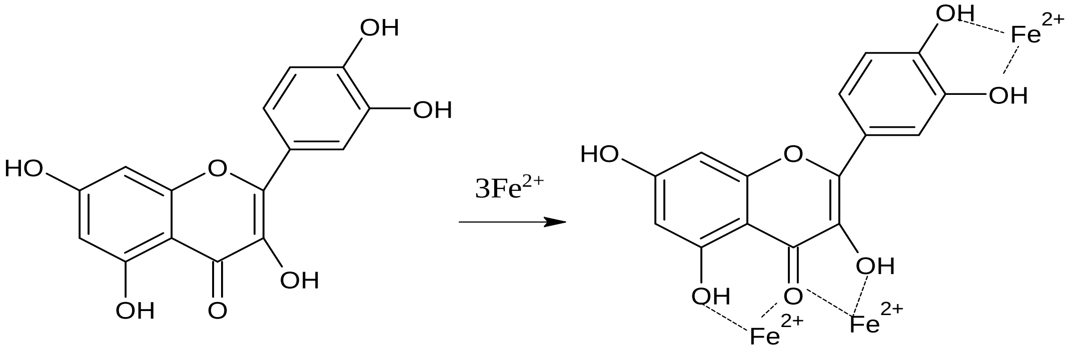
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + OH^{\bullet} \text{ (Fenton reaction)}$$

The assay is carried out by measuring the change in absorbance due to the formation of a complex of $FeCl_2$ with ferrozine. Ferrozine can react with iron ions and form a red-colored complex, which is hindered by the presence of metal-chelating antioxidants. When antioxidants chelate the metal ions, ferrozine can no longer form the colored complex with these ions, resulting in reduced absorbance at 562 nm. This decrease is measured and compared with the blank solution without any antioxidants to assess the metal chelating activity.

$$3\text{ Ferrozine} + Fe(H_2O)_6^{2+} \rightarrow Fe - (\text{Ferrozine})_3^{4-} + 6H_2O$$

The Fe-binding ability of antioxidants could be attributed to the specific functional groups present that chelate the metal ions. For example, the antioxidant taxifolin can chelate $Fe^{2+}$ ions in the 4-oxo, 5-OH groups between the heterocyclic and the A rings, the catechol moiety of the B ring, and the 3-OH groups, 4-oxo group in the heterocyclic ring (Gulcin and Alwasel 2022). This forms a complex with metal ions, sterically hindering the interaction between lipids and metals (Fig. 2.6). Similarly, other natural antioxidants such as resveratrol and curcumin also possess metal-chelating abilities. $Fe^{2+}$ ions can be readily chelated by compounds with the proper function-structure configuration of two or more -OH, -COOH, -SH, $-OCH_3$, -C=O, $-PO_3H_2$, $-NR_2$, -O-, and -S-functional groups (Gulcin and Alwasel 2022). Based on the number of chelation sites present, a single antioxidant can chelate up to 2-3 metal ions (Fig. 2.6). The catechol moiety is the main contributor to metal binding, as shown by the bathochromic shift produced by Cu binding to quercetin, which is more pronounced than the chelating ability of kaempferol (Van Acker et al. 1996). Other compounds, such as EDTA, can also chelate metal ions, though it does not have antioxidative properties. EDTA is a powerful metal chelator, and hence, it is often used as a standard to compare the activities of natural antioxidants and extracts.

The method is widely used to understand the metal chelating ability of antioxidants since the assay uses easily available reagents, with good reproducibility and repeatability. However, the method may not reflect the total amount of phenolic compounds with antioxidative activity since compounds such as EDTA can also chelate metal ions without any antioxidative potential. Hence, the method does not correlate well with other assays, including ABTS, DPPH, and FRAP antioxidant assays.



**Taxifolin** **Taxifolin-$Fe^{2+}$ complex**

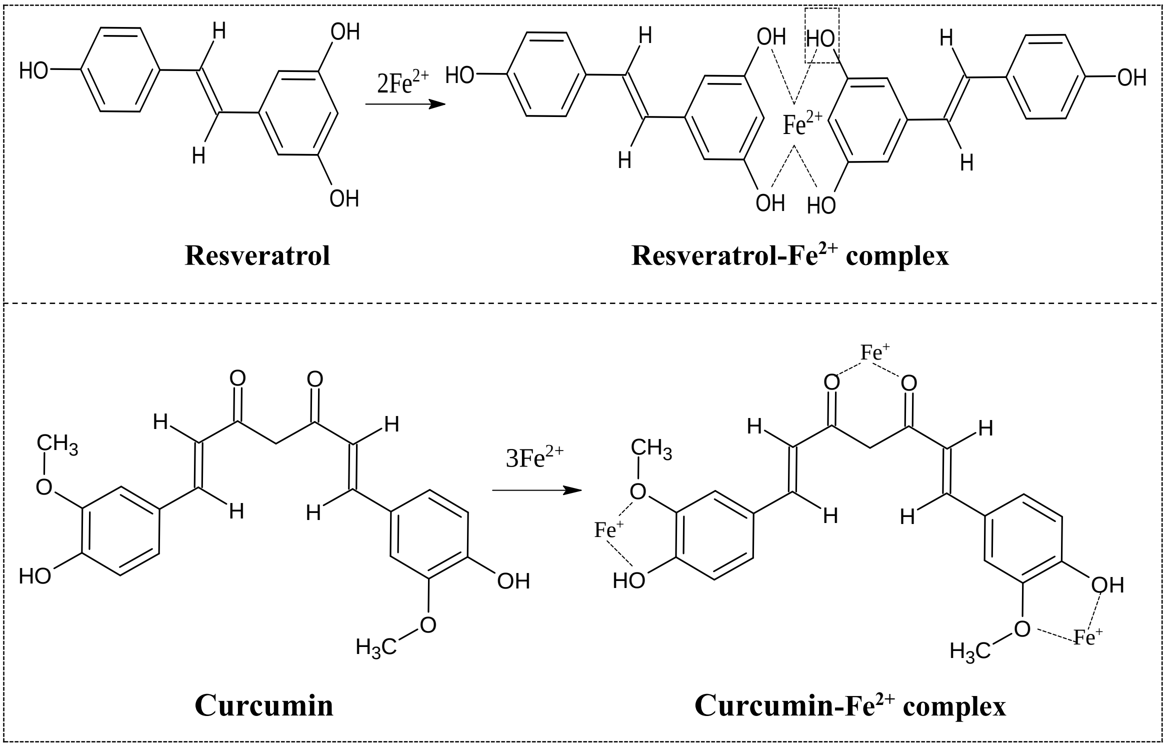


**Fig. 2.6 Metal chelating antioxidants (adapted from Gulcin and Alwasel 2022)**

### 2.1.5 Role of antioxidants in human health

Antioxidants have been an innate part of the human diet for centuries since most fruits and vegetables are known to have antioxidants. There is significant evidence from epidemiological studies that suggest that the consumption of diets that are rich in fruits and vegetables can reduce the likelihood of developing several chronic diseases, including specific cancers, cardiovascular disorders (CVD), and neurodegenerative diseases. These diets are often attributed with beneficial effects due to their high content of phenolics and flavonoids (Del Rio et al. 2013). Phenolic compounds are one of the main classes of secondary metabolites produced in plants and provide color, texture, and flavor (Gulcin 2020). These dietary antioxidants can significantly reduce the deleterious effects of reactive oxygen species in the human body by repairing the damage induced through oxidation, nitration, and halogenation of biomolecules such as lipids, carbohydrates, and nucleic acids (Jideani et al. 2021).

Consumption of food rich in antioxidants is proven to increase the antioxidative capacity of plasma/serum (Zhang et al. 2015). For example, the intake of red wine, strawberries, vitamin C, or spinach was found to increase the total antioxidant capacity of serum in elderly women (Cao et al. 1998a). Another study reported that the consumption of 10 servings of fruit and vegetables for 15 days increased plasma antioxidant activity (Cao et al. 1998b). In addition, several antioxidants such as resveratrol, curcumin, and anthocyanins are found to have anti-inflammatory properties since they can reduce inflammation through inhibition of prostaglandin production and nuclear factor-κB activity, enzyme inhibition, as well as an increase in cytokine production (Costa et al. 2013; Hutchin-Wolfbrandt and Mistry 2011).

Excessive production of oxidants is a significant contributor to the development of cardiovascular diseases. This oxidative damage has a particularly harmful effect on endothelial cells, leading to negative vasodilatory effects. However, the consumption of antioxidants can modify the molecular events that contribute to these negative effects, ultimately improving endothelial function. Therefore, antioxidants play a crucial role in preventing cardiovascular diseases. Several studies have shown that the Mediterranean diet, rich in tomato products, can reduce the risk of cardiovascular disease. This is because lycopene, found in tomatoes, can improve the endothelial function of CVD patients (Gajendragadkar et al. 2014). Additionally, allicin, an antioxidant organosulfur compound found in garlic, has been found to protect the cardiovascular system by inducing vasorelaxation and alleviating cardiac hypertrophy, angiogenesis, platelet aggregation, hyperlipidemia, and hyperglycemia (Chan et al. 2013).

Flavanones, a subgroup of flavonoids present in citrus fruits, were found to have potential anti-obesity activities both *in vitro* and *in vivo* (Girones-Vilaplana et al. 2014). Polyphenols present in grapes are capable of reducing chronic inflammation caused by obesity, helping prevent metabolic diseases. They act as antioxidants by blocking pro-inflammatory cytokines or endotoxin-mediated kinases and transcription factors, exerting their antidiabetic activity (Chuang and McIntosh 2011). Peroxyl radicals and lipid peroxidation are two factors that can lead to mutations in DNA, which are crucial for the initiation of the carcinogenic process. Antioxidant phytochemicals have been shown to protect against DNA damage and can potentially modulate the initiation of carcinogenesis. Recent studies have suggested that making appropriate lifestyle modifications could prevent more than two-thirds of human cancers and that diet contributes to about 35% of human cancer mortality (Sak 2014). Polyphenols, including ellagitannins and epicatechin gallate, has been found to possess anticarcinogenic properties (Barrajon-Catalan et al. 2010; Cordero-Herrera et al. 2013). Studies have suggested that biophenols are toxic to rapidly proliferating cancer cells but not to normal cells (Paredes-López et al. 2010). Additionally, certain polyphenols, such as those found in green tea, milk thistle, and grape seeds, can protect the skin from the harmful effects of UV radiation, including the risk of skin cancer (Nichols and Katiyar 2010). These protective properties are mainly attributed to four mechanisms: reducing UV radiation-induced inflammation, preventing oxidative stress, preventing DNA damage, and suppressing immune responses.

Although numerous studies have been conducted both *in vitro* and *in vivo*, the precise role of various classes of antioxidants in human health is still not completely understood. These studies have been limited in various aspects, leading to an incomplete understanding of antioxidants. Nonetheless, it is evident that the consumption of antioxidants through food is crucial for overall well-being.

### 2.1.6 Esterification of antioxidants

Oxidation in fish oil is a major recurrent problem in the pharmaceutical, food, and nutraceutical sectors. The use of antioxidants as additives to combat the oxidation of lipids is the commonly used method. Several synthetic antioxidants are utilized for this purpose. However, there is a rising concern about the use of synthetic antioxidants because of their potential toxicity and carcinogenic nature (Figueroa-Espinoza and Villeneuve 2005). In recent years, natural antioxidants have been exploited to provide better oxidative stability. Phenolic compounds found in plants have the ability to retard oxidation of unsaturated fats present in fish oils. For efficient inhibition of lipid oxidation, an antioxidant must be able to accumulate in the oil interfaces where oxidation occurs (Figueroa-Espinoza and Villeneuve 2005). However, the relatively low solubility of the natural antioxidants limits their application in oil-based foods and processing. Multifunctional amphiphilic antioxidants are often prepared to overcome solubility issues (Figueroa-Espinoza and Villeneuve 2005).

Phenolic acids are esterified with fatty acyl donors by chemical or enzymatic methods. The natural antioxidants may be modified (i) to improve their stability (chemical/heat)

(ii) to alter hydrophilic-lipophilic balance (HLB) (iii) to improve miscibility with lipids (iv) to improve their bioavailability in nutraceutical products (Ferreira-Dias et al. 2013). The esters are formed by the reaction between the COOH groups with fatty alcohols or the phenolic OH group with fatty acids. The esterification with the carboxyl group preserves the phenol hydroxyl groups that determine the antioxidant activity (Cruz Figueroa-Espinoza et al. 2013).

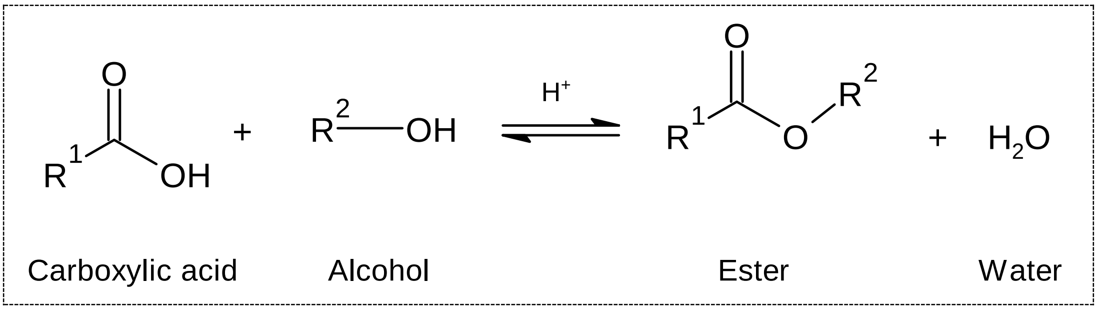


**Fig. 2.7 Esterification reaction**

### 2.1.6.1 Enzymatic esterification

Enzymes such as lipases, tannin acyl hydrolases, feruloyl esterases, and cutinases are employed for esterification in either solvent-based or solvent-free conditions. Acetone, butanol, cyclohexane, diethyl ether, tert-butanol, and tert-amyl alcohol are some of the commonly used solvents. In solvent-free conditions, the fatty alcohol is used in excess, acting as both the reactant and solvent (Cruz Figueroa-Espinoza et al. 2013). Lipases have broad substrate acceptance and stability, do not need cofactors, and are able to catalyze synthesis reactions in non-aqueous media. They can catalyze reactions that involve carboxyl groups, such as esterification, transesterification, and aminolysis (Ferreira-Dias et al. 2013). They are produced from different sources, and their structure-activity relationships are studied extensively. Lipases from *Candida antarctica, Thermomyces lanuginosus, Aspergillus niger, Candida rugosa,* and *Mucor miehei* are the most commonly used commercial products. These are used in food, nutraceuticals, fine chemicals, cleaning, agrochemicals, and energy as biocatalysts because of their selectivity and stability (Ferreira-Dias et al. 2013).

Lipase B from *Candida antarctica* (CALB) is the most widely studied and is often applied in its immobilized form in several reactions. It was commercialized by

Novozymes as Novozyme 435, an immobilized CALB using a macroporous acrylic polymer resin *via* interfacial activation. The structure of the CALB is completely resolved and has an active site made of a Ser-His-Asp catalytic triad with a small lid. The enzyme has the ability to adsorb on hydrophobic surfaces and does not form aggregates, making its handling easier (Ortiz et al. 2019). The enzyme has been successfully used in esterification reactions of various organic molecules under solvent-free and solvent-based conditions with good yields (Kirk and Christensen 2002).

Lipase obtained from the fungus *Thermomyces lanuginosus* is available in various immobilized forms (using different support materials and immobilization methods). It is a basophilic, thermally stable lipase that finds application majorly in the food industry and in other industries including biodiesel and fine chemicals production. Its active site is made of Ser-His-Asp catalytic triad, similar to that of the CALB lipase. The lipase has been characterized by its high stability and activity, which enables its usage in most reaction media, from solvent-free reactions to biphasic solvents (Domínguez de María et al. 2009). The enzyme has been used to produce ethyl butyrate under solvent free conditions by Paludo and others (Paludo et al. 2015). The yield was optimized using Central Composite Design with temperature and substrate molar ratio as the variables. Maximal yield (61%) of ethyl butyrate was obtained at 30°C with 1:1 molar ratio and 35% (by substrate mass) of the enzyme loading. Recently, a series of hydrophobic and amphiphilic esters were synthesized using *Thermomyces lanuginosus* lipase in immobilized forms using n-hexane as the solvent (Roque et al. 2024). All esters were obtained with more than 60% yield using the commercial immobilized lipase indicating its superior activity and stability.

The nature of the substrate affects the activity of the enzyme used. The structure-activity relationship between the substrate, enzyme, and solvent was studied by Guyot et al. (1997). In esterification reactions of cinnamic acid derivatives, the presence of a double bond on the side chain conjugated with the cycle and of a para hydroxyl group inhibits the enzyme lipase. The presence of two hydroxyl (OH) groups conjugated to the benzene cycle and a double bond in the side chain interferes with the esterification and results in trace amounts of ester. For example, esterification of 2,5-dihydroxybenzoic and 3,4-dihydroxybenzoic acids with octanol produced only a 3% yield, even after 50 days.

Buisman et al. (1998) studied the esterification of cinnamic and benzoic acid derivatives with fatty alcohols of different carbon chain lengths. Esterification of hydroxy and methoxy substituted benzoic acid derivatives such as gentisic, gallic, vanillic, and syringic acid gave only trace amounts (<2%) after one week of reaction. This further confirms the inhibiting effects of electron-donating groups conjugated to the carboxylic group in these derivatives. There may also be an impact because of the steric hindrance on the esterification reaction rate. Therefore, the conversion of phenolic acid into ester is influenced by the structure of the substrate, type/nature of acyl donor, solvents used, and water content in reaction media. The lipophilisation may result in enhanced antioxidant efficiency depending on the nature of esterification.

The optimization of process parameters and solvent polarity in the lipase-mediated synthesis of rutin fatty ester was studied by Vaisali et al. (2017). Process parameters, including enzyme loading, molar ratio, reaction temperature, the effect of solvent, initial water activity, and addition of molecular sieves, were optimized in the study. Synthesis with acetone showed higher reaction rates (52.14%) compared to tert-butanol (13.02%) in the production of rutin fatty ester. The optimal process parameters were enzyme load: 10 mg/mL and 15 mg/mL, 1:5 and 1:4 molar ratio of rutin: fatty acid, temperature of 55ºC and 60ºC and water activity of 0.07 and 0.23 for acetone and tert-butanol respectively. The addition of 150 mg/mL of molecular sieves was optimal for the tert-butanol system, whereas no addition of molecular sieves was found to be optimal in acetone-based ester synthesis. These process parameters affect the formation of ester significantly, and thus, optimization is required to achieve higher conversion rates in different reaction media.

Since the efficiency of antioxidants depends majorly on the structure, the number and position of acylation have to be taken into consideration. The enzyme used for esterification should be a potent tool for acylation with appreciable yields. The increase in chain length of the acyl donor was observed to reduce the conversion yield. This may be because of the impact of longer chain length on the active site of the enzyme. Antioxidant capacity was enhanced with an increase in acyl chain length up to 8 carbon atoms in octanol with rosmarinic acid (Laguerre et al. 2010). When the chain length was increased further, the antioxidant capacity was reduced. This ‘cut-off effect’ is due to the nonlinear behavior of the antioxidant, which implies that the determination of critical chain length may be helpful in producing a lipophilic molecule with optimal antioxidant activity. The following table summarises the research on enzymatic esterification of various antioxidants, including the acyl donors, reaction conditions, and yield obtained.

**Table 2.2 Enzymatic Lipophilisation of antioxidants with different acyl donors**

| **Antioxidant** | **Acyl donor** | **Enzyme** | **Reaction conditions** | **Yield of ester and application** | **Reference** |
|---|---|---|---|---|---|
| Rutin | Decanoic acid | *Candida antarctica* lipase immobilized on acrylic resin | 55ºC and 60ºC for 96 hours at 150 rpm in a molar ratio of 1:5 and 1:4 with 10 and 15 mg/mL of immobilized lipase in acetone and tert-butanol, respectively | 60.74% and 65.73% in acetone and tert-butanol solvent systems for use in n-3 PUFA rich fish oils | Vaisali et al. 2017 |
| Epicatechin | Free fatty acids (oleic acid, palmitic acid) in crude camellia seed oil | Novozyme 435 (Immobilized lipase B from *Candida antarctica*) | 50ºC for 36 hours with 4Å molecular sieves and 10 mg enzyme | 95.5% and used in crude camellia seed oil | Chen et al. 2017 |
| Quercetin | Ferulic acid | *Rhizopus oryzae* lipase | 45ºC for 96 hours at 200 rpm with cyclo-octane as solvent and 750IU enzyme | 93.2% and synthesis was scaled up for industrial application | Kumar et al. 2016 |
| Cinnamic acid | Ethanol | Lipozyme TLIM | 40ºC for 24 hours at 170 rpm with iso- | 89.2%. Enzyme catalysed method | Wang et al. 2016 |

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| | | (Immobilised enzyme from *Thermomyces lanuginosus*) | octane as solvent and molar ratio of 1:3 | explored to provide food-safe and environment friendly method to synthesis benzyl cinnamate | |
| Dihydromyricetin | Vinyl acetate | Novozyme 435 | 35ºC for 48 hours in acetonitrile with 1:25 molar ratio | 86.6±0.33% | Li et al. 2015 |
| | | Lipozyme TLIM | | 60.9±0.25% | |
| | | Lipozyme RMIM (Immobilised lipase from *Mucor miehei*) | | 59.5±0.17%<br>Esterified dihydromyricetin for application in nutraceutical and functional foods, | |
| Vanillic acid | DHA | Novozyme 435 | 1:4 molar ratio of vanillic acid and DHA with lesser than 0.1 water activity | 54% ester for functional food ingredient | Roby et al. 2015 |
| Dihydrocaffeic acid | C4-C18 straight chain fatty alcohol | CALB (Lipase B from *C.antarctica*) | 60ºC for 5-7 days at 300 rpm with 1:3 molar ratio and 100 mg enzyme | 95% for hexanol | Yang et al. 2012 |
| | | | | 56% for 1-butanol | |
| | | | | 44% for octa-decanol | |
| Rutin | C4-18 fatty acids | CALB | 60ºC for 168 hours at 250 rpm with 1:5 molar ratio in 2-methylbutan-2-ol | 27-62% for C4-18 esters with >50% conversion for short and medium chain fatty acids intended for | Viskupicova et al. 2010 |

| | | | | | |
|---|---|---|---|---|---|
| | | | | application in lipophilic food matrices. | |
| Rutin | Palmitic and lauric acid | Novozyme 435 | 50ºC for 144 hours at 200 rpm with 1:4 molar ratio and 20 g/L enzyme in acetone | 70-77% after 96 hours. Antioxidative activity of esters compared through different assays. | Lue et al. 2010 |
| Cinnamic acid | Triolein | Novozyme 435 | 55ºC for 18 days at 150 rpm with 1:4.5 molar ratio in hexane-butanone solvent | 42%. Esterification was done to synthesise phenolic lipids with specific nutritional properties | Karbourne et al. 2005 |
| Rutin<br>Naringin | C8-12 fatty acids | Novozyme 435 | 45ºC with 1:2 molar ratio with 50mg enzyme | 45-50%. Study done to improve the hydrophilic/hydrophobic balance of flavonoids. | Kontogianni et al. 2003 |
| Dihydrocaffeic acid | Trilinolein (TLA) and Trilinolenin (TLNA) | Novozyme 435 | 55ºC at 150 rpm with 1:2 molar ratio with 25 mg enzyme | 66% for TLA after 5 days; 62% for TLNA after 12 days. The study deals with the biosynthesis of phenolic lipids. | Sabally et al. 2006 |

The use of the enzymatic method for esterification is considered highly selective; fewer by-products are formed, and it requires fewer purification steps (Hills 2003). The disadvantages may include longer reaction times, higher catalyst costs, and lower conversion yields because of the inhibition of enzymes. However, the use of immobilized enzymes might help in reducing the cost of the catalyst. The immobilized enzymes can be reused after recovery (Figueroa-Espinoza and Villeneuve 2005). The influence of reaction conditions, such as the nature of the substrate, solvents, product, pH, and water activity on conversion, can be minimized through optimization. Another strategy called chemo-enzymatic esterification may also be employed sometimes. This includes a two-step solvent-free condition wherein the phenolic acid is converted first into methyl ester through a chemical method and followed by an enzyme-based transesterification (Figueroa-Espinoza et al. 2013).

### 2.1.6.2 Chemical esterification

The successful esterification of phenolic acids using enzymes depends on various factors, including the structure of the phenolic compound, type, and length of acyl donor used, reaction solvent, pH, and optimal temperature. Since esterification is a reversible reaction, the water released during the reaction plays a significant role. Excess water could revert the reaction to ester hydrolysis, leading to loss of yield of lipophilic counterparts. The removal of water released during the reaction can be challenging in certain media, leading to lower yields when utilizing the enzymatic method. Several structural aspects of the phenolic acid tend to inhibit the esterification reaction partially or completely (Figueroa-Espinoza et al. 2013). Phenolic acids with catechol moiety wherein one of the hydroxyl groups is in *para*- position with regard to the side chain having the carboxylic acid, such as caffeic and protocatechuic acids, only give trace yields of ester using lipase as the catalyst after several days of reaction due to enzyme inhibition (Guyot et al. 1997). This prolonged reaction time and low yields render the enzymatic esterification method unsuitable for the lipophilisation of some phenolic acids.

Chemical esterification involves the use of acidic catalysts such as hydrochloric and sulfuric acids. The chemical method is faster, simpler, and provides reliable yields (Chalas et al. 2001). Because of these reasons, conventional acids are still preferred industrially for several reactions, including esterification, hydration, and hydrolysis (Baghernejad 2011). However, conventional acids could cause high toxicity, corrosion, and difficulty in product and catalyst separation, and purification might become tedious. Since these are used as homogeneous catalysts, the amount of acid catalyst required to achieve the desired yield could be higher, leading to increased waste generation.

Examples include *p*-toluenesulfonic acid (PTSA), trifluoromethanesulfonic acid, and boric acid. PTSA has been successfully used to esterify phenolic acids such as caffeic and dihydrocaffeic acid with good yields (Wang et al. 2013; Wei and Jingnan 2018).

PTSA could be a potential replacement for lipase-catalyzed esterification reactions and is characterized by the mildness of the reaction, easy availability, inexpensive, and good functional group tolerance, which might help improve yields of phenolic esters (Wei and Jingnan 2018). The solid acid catalyst can be used even at higher temperatures without the formation of any toxic by-products (Baghernejad 2011). The homogeneous catalyst was efficient in the synthesis of valeric acid ester of butanol with a conversion of 93.5%, while the heterogeneous catalysts had lower conversion percentages (Khudsange and Wasewar 2018). The authors have attributed this superior yield to the homogeneity of the reaction and the strong acid concentration of PTSA.

In recent years, heterogeneous catalysts, such as sulfonic resins, have emerged as a substitute for strong acids in the esterification of several phenolic acids. This type of catalyst is characterized by mild reaction conditions and good functional group tolerance, which allows the formation of esters in good yields even in the presence of some amount of water. The advantages of using heterogeneous catalysts include low cost, different ranges of acid/base strengths, reusability, environmental compatibility, and ease of handling (Gujarathi et al. 2013). Since the sulfonic resins could be easily removed from the reaction mixture by filtration, the recovery and re-activation of the catalyst becomes much easier. This makes them economically more viable than homogeneous catalysts. Macroporous exchange resins, including Pirolite, Relite, and Amberlyst, have the ability to efficiently catalyze esterification and transesterification reactions and replace homogeneous chemical catalysts. Amberlyst-15 is one such acidic cation-exchangeable resin that has high catalytic activity in esterification reactions. It is made of a macro reticular polystyrene base with strongly acidic sulfonic groups (Pal 2015). These heterogeneous catalysts are easily separated from the reaction mixture, leading to reduced purification steps, and are also thermally stable up to 120°C (Liu et al. 2013b). These chemical catalysts enable shorter reaction times and higher ester yield by overcoming structural inhibitions present in the enzymatic method. Amberlyst-15 is a potent and selective acid catalyst used for esterification and transesterification reactions (Melfi et al. 2020). The use of Amberlyst-15 for esterification reactions has been reported throughout the literature (Le et al. 2021; Mateos et al. 2008; Pacheco et al. 2014; Sun et al. 2009) with good yields and no side reactions.

**Table 2.3 Esterification reactions catalyzed by homogeneous and heterogeneous chemical catalysts**

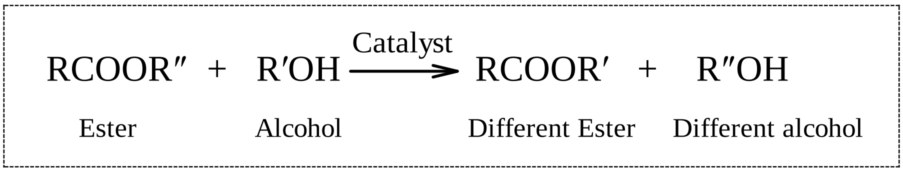
| Antioxidant | Acyl donor | Catalyst and type | Reaction conditions | Yield of ester | Reference |
|---|---|---|---|---|---|
| Caffeic acid | Methanol | *p*-toluenesulfonic acid (*p*-TSA) (homogeneous catalyst) | 65°C, molar ratio of 1:20, catalyst mass ratio 8% for 4 hours | 84.0% | Wang et al. 2013 |
| Caffeic acid,<br>Ferulic acid<br>Sinapic acid | Octanol | Sulfuric acid (homogeneous catalyst) | 0.5 mM phenolic acids with 6 mM octanol, 3Å molecular sieves(40 mg/mL) at 100°C for 2-3 hours | 83% octyl sinapate<br>57% octyl caffeate<br>68% octyl ferulate | Szydłowska-Czerniak et al. 2018 |
| Ferulic acid, coumaric acid, caffeic acid | C1-C20 | Amberlite IR-120H (heterogeneous catalyst) and sulphuric acid (homogeneous catalyst) | 5% w/w and 5% v/v of solid and liquid acid catalyst with varying molar ratios of phenolic acid with alcohols for 120 hours | >90% | Sørensen et al. 2014 |
| Dihydrocaffeic acid | Hexanol | *p*-TSA (homogeneous catalyst) | 1:30 molar ratio without molecular sieves at 80°C for 2 hours with 1 mol% *p*-TSA | >90% | Wei and Jingnan 2018 |

| | | | | | |
|---|---|---|---|---|---|
| Caffeic acid | Dodecanol | Ionic liquid ([Hnmp]$HSO_4$) (homogeneous catalyst) | 1:10.2 molar ratio, 9.8% catalyst dosage at 87°C for 118mins | 94.67± 1.32% | Liu et al. 2022 |
| Caffeic acid | Propanol | Acidic ionic liquid 1-butylsulfonic-3-methylimidazolium tosylate (homogeneous catalyst) | 1:20 molar ratio, 40% ionic liquid at 90°C | 98.7±0.8% | Liu and Han 2015 |
| Resveratrol | C3-C22 acyl chlorides | Pyridine (homogeneous catalyst) | Resveratrol in ethyl acetate with addition of acyl chlorides at 50°C | Varying yields of mono-, di- and tri-esters | Oh and Shahidi 2017 |
| Sinapic acid | Methanol, Ethanol, n-propanol, butanol | Sulphuric acid (homogeneous catalyst) | 1 g sinapic acid in 75 mL of the alcohol stirred at room temperature for 5 days | 79-94% | Gaspar et al. 2010 |
| Rosmarinic acid | C1,C4,C8, C12, C16, C18, | Amberlite IR-120H (heterogeneous catalyst) | Varying molar ratio of alcohols with 5% w/w catalyst stirred at 250 rpm at 55-70°C in the presence of 40 mg/mL 3Å molecular sieves. | Satisfactory yields | Panya et al. 2012 |

| | | | | | |
|---|---|---|---|---|---|
| Rosmarinic acid | Methanol to eicosanol | Amberlite IR-120H (heterogeneous catalyst) | Varying molar ratio of alcohols with 5% w/w—total weight of both substrates previously dried at 110 °C for 48 h. stirred at 250 rpm at 55-70°C in the presence of 40 mg/mL molecular sieves | 98.5% (C1)<br>99.3% (C4)<br>99.5% (C8)<br>4.4% (C12)<br>81.6% (C16)<br>99.0% (C18–20) | Lecomte et al. 2010 |
| Protocatechuic acid | C1-C18 | Sulphuric acid (homogeneous catalyst) | Molar ratio of 1:3 (acid: alcohol) with 4% v/v of catalyst at 45-65°C stirred at 250 rpm | 59–95% | Grajeda-Iglesias et al. 2016 |
| Protocatechuic acid | Methanol, ethanol, propanol | Sulphuric acid (homogeneous catalyst) | 1 g phenolic acid in 75mL of alcohol with 2 mL of acid catalyst for 5 days | 71-81% | Reis et al. 2010 |
| Acetic acid | n-octanol | Amberlyst-15 (heterogeneous catalyst) | 1:1 molar ratio, 500 rpm, 10% catalyst loading | 58.2% | Liu et al. 2013b |
| Cinnamic acid | Phenols | Amberlyst-15 (heterogeneous catalyst) | 1:1 molar ratio with 0.2 g catalyst at 130°C | Moderate to high yields | Le et al. 2021 |
| Oleic, stearic, lauric, levulinic acid | Methanol, ethanol | Amberlyst-15 (heterogeneous catalyst) | 1:9 molar ratio with 20% catalyst at 110°C in supercritical $CO_2$ | 50-90% | Melfi et al. 2020 |

| | | | | | |
|---|---|---|---|---|---|
| Nonanoic acid | 1-propanol | Amberlyst-15 (heterogeneous catalyst) | 1:10 molar ratio with 8% catalyst loading at 90°C | Satisfactory yields | Sharma et al. 2014 |

Notably, certain classes of phenolic antioxidants could not be esterified using the enzymatic method, irrespective of the reaction conditions studied. In such cases, the methyl ester of the compound is first synthesized, from which higher esters are produced using a trans-esterification reaction (Fig. 2.8). This enables higher yield and shorter reaction times than direct esterification in a solvent-free system. This is achieved through better interaction between methyl ester and fatty alcohols than the non-esterified precursor and immediate removal of methanol released during transesterification through evaporation (Figueroa-Espinoza et al. 2013).



$$RCOOR'' + R'OH \xrightarrow{\text{Catalyst}} RCOOR' + R''OH$$

**Fig. 2.8 Transesterification reaction**

### 2.1.6.3 Applications of esters of natural antioxidants

Lipophilisation of phenolic acids is done to alter the solubility and hydrophilic-lipophilic balance (HLB) for use in oils and emulsions. Several esters of phenolic acids, such as gallic acid, caffeic acid, rutin, and rosmarinate esters, are studied for their antioxidative activity in different oils (Aruoma et al. 1993; Cumming and Marshall 2021; Laguerre et al. 2010; Viskupicova et al. 2010). Lipophilised epicatechin derivatives were studied by Chen et al. (2017). The oxidative stability of camellia seed oil was better in the presence of epicatechin oleate and epicatechin palmitate. The esterification was done with the seed oil rich in free fatty acids and epicatechin with *Candida antarctica* lipase B as the catalyst. The acid value was reduced from 3.7 to 2.5 mg KOH/g through esterification. Ramadan (2012) investigated the antiradical characterization of quercetin-enriched lecithin in sunflower oil during accelerated oxidation for 15 days at 60ºC. The oxidative stability was better with quercetin-enriched lecithin when compared with lecithin or quercetin alone.

Oxidative stability of refined sardine oil containing trace water in the presence of rutin and its lipophilic ester was studied for 20 days of storage (Vaisali et al. 2018). The extent of oxidation was analyzed by determining the peroxide value, *p*-anisidine value, conjugated diene, and TBARS values. The rutin fatty ester of decanoic acid was found to reduce primary (50%) and secondary oxidation (42.46%) efficiently when compared to the hydrophilic rutin compound (20.6 and 20.43%). Epigallocatechin gallate (EGCG) esters of stearic acid, eicosapentaenoic acid, and docosahexaenoic acid were prepared, and their antioxidative efficiency was analyzed in bulk oil (Zhong and Shahidi 2011). The derivatives of EGCG were found to inhibit stripped corn oil oxidation better than the parent EGCG compound.

Lipophilised esters of octadecanol and phenolic extracts of *Sorbus aucuparia* were used in refined rapeseed oil (Aladedunye et al. 2015). The storage stability of the oil was studied at accelerated oxidation at 65ºC for 7 days. The presence of lipophilised extract resulted in 43% decrease in peroxide value and thus exhibiting significantly higher storage stability. Stearate, palmitate, oleate, linoleate, linolenate esters of rutin increased the induction time for oxidation in sunflower oil during rancimat test (Viskupicova et al. 2010). This selective modification aids in maintaining the antioxidant capacity while simultaneously increasing lipophilicity and solubility in fats, which enhances the storage stability. Hydroxy, methoxy, alkyl ester derivatives of *p*-hydroxybenzoic acids were employed in fish oil to investigate the oxidation rate and antioxidant activity (Farhoosh et al. 2016). Efficient protection against oxidative deterioration was offered by gallic acid, ethyl protocatechuate, protocatechuic acid, vanillic acid, and syringic acid. The presence of the OH group enhances the antioxidative activity significantly more than the $OCH_3$ group.

Cumming and Marshall (2021) have studied the esterification of ethyl caffeate with glycerol to produce glyceryl caffeate. The ester produced was used during the storage stability study in bulk tuna oil. The activity was compared with α-tocopherol and was found to be superior when using rancimat test. The oxidative stability of propyl esters of phenolic acids was evaluated by Silva et al. (2001). The antioxidative capacity was observed to increase in the order of propyl ferulate, propyl isoferulate, α-tocopherol, propyl caffeate, propyl hydrocaffeate, and propyl gallate in refined sunflower oil. A dose-dependent increase in antioxidative activity was observed for propyl gallate, propyl hydrocaffeate, and propyl caffeate.

**Table 2.4 Esters of various natural antioxidants in bulk oil and emulsion systems**

| **Antioxidant / Ester** | **Oil / Emulsion system** | **Result** | **Reference** |
|---|---|---|---|
| Rosmarinic acid and C4, C8, C12, C18 and C20 esters | O/W emulsion of stripped soybean oil | C4 and C8 esters showed better oxidative stability than other esters | Lee et al. 2013 |
| Rutin and C4-C18 fatty acyl esters | Sunflower oil | Rutin esters of stearate, palmitate, oleate, linoleate and linolenate were better in Rancimat test. | Viskupicova et al. 2010 |
| Caffeic acid and C1-C16 alkyl ester | O/W emulsion of Olive oil | Cut-off effect observed with C8 ester showing maximum potential | Costa et al. 2015 |
| Hydroxytyrosol and C1-C16 esters | O/W emulsion of olive oil | Cut-off effect observed with C8 ester showing maximum potential | Almeida et al. 2016 |
| Sinapic acid esters of hexanol and palmitoyl chloride | Flaxseed oil | Palmitoyl sinapate efficiently reduced oxidation | Arslan et al. 2024 |
| Gallic acid and C1-C18 esters | DHA algae oil | Octyl gallate showed better potential than other esters | Shen et al. 2021 |
| Sinapic acid esters of C4, C8 and C12 | O/W emulsion of echium oil | C12 ester was better than C4 ester in | da Silveira et al. 2020 |

| | | | |
|---|---|---|---|
| | | reducing secondary oxidation product formation | |
| Resveratrol and esters C3-C22 | Stripped corn oil and emulsions | Resveratrol showed best stability in O/W emulsion while esters C6, C8, C10, C12 and C16 was better in bulk oil | Oh and Shahidi 2018 |
| Caffeic acid and C2, C4 and C6 esters | Sunflower oil | C4 ester showed better antioxidative potential than other esters and caffeic acid | Lu et al. 2022 |
| Hydroxytyrosol and ester derivatives | Fish oil-in-water emulsions | Hydroxytyrosol octanoate exhibited maximum efficiency | Lucas et al. 2010 |
| Octyl esters of sinapic, ferulic, and caffeic acid | Refined rapeseed oil | Octyl caffeate was better than other octyl esters in prolonging induction time | Szydłowska-Czerniak et al. 2018 |
| Hydroxytyrosol and esters of acetate, butyrate, octanoate and laurate | Cod liver oil and O/W emulsions of cod liver oil | Hydroxytyrosol was better in bulk oil, while octanoate ester was efficient in emulsions | Medina et al. 2009 |
| Hydroxy, methoxy and alkyl derivatives of p-hydroxybenzoic acid | Fish oil | Methyl gallate was the most effective among the esters studied | Farhoosh et al. 2016 |
| Propyl esters of caffeic, ferulic, isoferulic and hydrocaffeic acids | Refined sunflower oil | Propyl hydrocaffeate was better than propyl caffeate in Rancimat test | Silva et al. 2001 |

| | | | |
|---|---|---|---|
| Acetylferulic paeonol ester and ferulic paeonol ester | Fish oil | Acetylferulic paeonol ester was better in reducing oxidation at 60°C than synthetic antioxidant BHT | Yu et al. 2021 |
| Chlorogenic acid and C4, C8 and C12 ester | Menhaden Fish oil-in-water emulsions | Except dodecyl ester, other esters and chlorogenic acid were better in reducing oxidation | Sasaki et al. 2010 |
| Dihydrocaffeic acid and its octyl and oleyl esters | Fish oil-enriched emulsions | Octyl dihydrocaffeate was better than oleyl dihydrocaffeate in reducing oxidation | Sørensen et al. 2012a |
| Acetyl ferulic7-hydroxyisoflavoneester (AHE) | Fish oil | AHE retarded oxidation better than ferulic acid and TBHQ | Wang et al. 2023 |
| Triacetylferulic resveratrol ester (TRE) | Fish oil | TRE shoed better antioxidative potential than resveratrol, TBHQ and ferulic acid | Xue et al. 2023 |
| Hydroxytyrosol and its esters of decanoic, lauric, myristic, palmitic, stearic, oleic and eicosapentaenoic acids | Anchovy bulk oil and O/W emulsions | Hydroxytyrosol eicosapentaenoate showed the best oxidative stability in bulk oil as well as O/W emulsion | Akanbi and Barrow 2018 |
| Rutin ester of decanoic acid | Sardine fish oil | Rutin ester exhibited better potential than rutin in reducing | Vaisali et al. 2018 |

|  |  |  |  |
|---|---|---|---|
|  |  | primary and secondary oxidation products. |  |
| Rutin and C12, C16 esters; Dihydrocaffeic acid and C8, C18:1 esters | Fish oil-enriched milk emulsions | Rutin laurate and both the dihydrocaffeate esters were better performing than unmodified phenolics. | Sørensen et al. 2012b |

### 2.1.7 3,4-dihydroxyphenylacetic acid (3,4-DHPA)

Phenolic acids can be classified into five subclasses based on their structure, among which hydroxyphenylacetic acid represents a series of hydroxylated phenylacetic compounds. 3,4-dihdyroxyphenylacetic acid belongs to the hydroxyphenylacetic acid subgroup having two hydroxyl groups located at the 3- and 4- positions (Fig. 2.9). It has a catechol moiety, which is essential for exhibiting antioxidative properties. In addition to antioxidative potential, several natural phenolic acids, including 3,4-DHPA, are reported to have anti-inflammatory, anti-cancer, and cardioprotective properties (Afnan et al. 2022; Sova and Saso 2020). 3,4-DHPA can efficiently reduce lipid peroxidation and potentially replace synthetic antioxidants such as BHT and BHA in edible oils (Li et al. 2019).

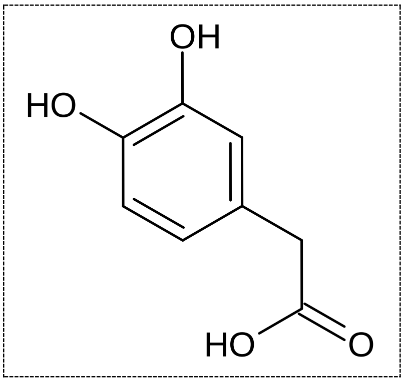


**Fig. 2.9 Structure of 3,4-DHPA**

The wastewater from olive mills is typically disposed of as waste, despite containing a substantial amount of phenolic compounds that render it a cost-effective source of natural antioxidants. However, due to the high concentration of these compounds, microbial degradation is limited. Fki and colleagues successfully extracted ethyl acetate from olive mill wastewater to recover and detoxify products (Fki et al. 2005). They employed a continuous counter-current extraction unit to achieve this. 3,4-DHPA that was extracted from this process was assessed in oil oxidation assays (refined husk and olive) and was found to exhibit superior antioxidant activity compared to hydroxytyrosol, which is a powerful antioxidant.

Among the different phenolics, with numerous health benefits, 3,4-DHPA is a rarely studied phenolic antioxidant having a structure analogous to caffeic acid and hydroxytyrosol. It has excellent antioxidative properties with an $IC_{50}$ value of 6 mg/L in the DPPH scavenging assay which is better than the synthetic antioxidant, Butylated hydroxytoluene (BHT) with $IC_{50}$ of 8.32 mg/L (Trabelsi et al. 2015). It is an ortho-dihydroxylated compound that has not been exploited for use as an antioxidant in lipid systems due to its very low solubility (Pagoni and Vassiliou 2018).

Though 3,4-DHPA has an excellent antioxidative profile, it has been less explored for use in oil and related food products to control oxidation. The hydrophilic nature of phenolic acid could render their usage in hydrophobic media such as oils limited since higher concentrations could lead to insolubility. Esterification with lipophilic donors might enhance the solubility and hydrophobicity of 3,4-DHPA. Enzyme-catalyzed synthesis of phenolic lipids by transesterification of flaxseed oil with 3,4-dihydroxyphenylacetic acid in the solvent-free medium was done by Sorour et al. (2012). The effect of parameters such as water activity, concentration for the enzyme, and agitation speed was optimized. The radical scavenging activity of the synthesized lipid was 7.2-fold lower than the parent compound but half that of α-tocopherol. In a similar study by Aziz et al. (2012), phenolic lipids are prepared from krill oil and 3,4-DHPA in a solvent-free medium. Through enzymatic transesterification, a conversion yield of 56.79% was obtained after 24 hours with 3,4-DHPA and phospholipids present in krill oil as substrates.

The studies available investigated the use of 3,4-DHPA in synthesizing phenolipids with unsaturated fatty acids. However, the potential of esterification of 3,4-DHPA with fatty alcohols to be used in retarding oxidation in bulk oils and emulsions has not been explored. In the scope of this context, the current study aims to synthesize esters of 3,4-

DHPA using enzymatic and chemical catalysts to demonstrate their effectiveness in retarding the oxidation in n-3 PUFA-containing lipid system. Additionally, an attempt has been made to correlate the effect of acyl chain length on the antioxidative potential of 3,4-DHPA in both *in vitro* assays and oxidative stability studies in bulk oil systems. To the best of our knowledge, this is the first-ever work to report the synthesis and application of lipophilic esters of 3,4-DHPA for extending the oxidative stability of n-3 PUFA-rich lipid matrices.

## 2.2 FISH OIL

Fish oil is complex in nature, containing a diverse group of fatty acids ranging from saturated to polyunsaturated (Stansby 1969). It is known as the main dietary source of essential long-chain polyunsaturated fatty acids (PUFAs). n-3 and n-6 PUFAs, including α-linolenic acid, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and linoleic acid, are essential fatty acids since they are not synthesized in the human body (Saini and Keum 2018). These essential fatty acids are to be obtained through diet or supplementation with PUFA-rich foods. The consumption of fish and other n-3 PUFA-rich foods is highly recommended owing to their various health benefits. As per the US FDA, an intake of at least 8 ounces of seafood is recommended to achieve daily intake of 250 mg/day of EPA and DHA. Globally, from 1961 to 2016, the fish supply grew at an annual rate of 3.2%, exceeding the world population growth rate of 1.6% (Coppola et al. 2021). Consequently, between 1961 and 2017, the global per capita fish consumption increased from 9.0 kg to 20.5 kg, with an estimated increase to 20.3 kg in 2016 (Coppola et al. 2021). Global annual fish oil production consistently ranged between 1 and 1.25 million tonnes for several years (Pike and Jackson 2010).

### 2.2.1 Sources of fish oil

Fish oil is commercially marketed in three forms: purified fish oil, concentrates as triglycerides and ethyl esters. The content of n-3 PUFA in these products ranges from 20-60% in purified fish oils, while the concentrated fish oil esters may contain up to 80% of PUFA (Mozuraityte et al. 2016). India is the world's second-largest producer of fish in both total fish production and aquaculture, according to Suyani et al. (2020). The Indian coastline is comprised of nine maritime states, five of which are on the west coast (Gujarat, Maharashtra, Goa, Karnataka, and Kerala) and four on the east coast (Tamil Nadu, Andhra Pradesh, Odisha, and West Bengal) and is bordered by the Arabian Sea on the west and the Bay of Bengal on the east. The state of Karnataka has a coastline of about 320 km along the southwest coast, which includes 23 fish meal and fish oil plants (Ponnusamy et al. 2012). The species used for these fish meal plants basically include Indian oil sardine (95-97%), followed by threadfin breams, carangids, flatfish, lesser sardine, and silver bellies.

There is a vast difference in the composition of fish oils based on the species, age, diet, geographical location, maturity, and parts used for the extraction. Based on the lipid content, fish can be categorized into lean fish, medium fatty fish, and fatty fish. Lean fish such as cod, haddock, sole, largehead hairtail, and saithe contain less than 2.5% fat, while medium fatty fishes, including hake, sea bass, and ocean perch, have about 2.5-6% fat. Fatty fishes may contain 8 to 25% fat, and examples include sardine, herring, anchovy, mackerel, tuna, and salmon (Gil and Gil 2015).

#### 2.2.1.1 Indian oil sardine

*Sardinella longiceps*, commonly referred to as the Indian oil sardine, is a small pelagic fish that inhabits coastal shelf waters at depths ranging from 20 to 200 meters. The species is primarily distributed along the northeast, southeast, southwest, and northwest coasts of India, as well as in the Gulf of Oman and the Gulf of Aden. It is an economically significant fishery resource, contributing approximately 10% of the total fisheries of India. In addition to its food value, *Sardinella longiceps* is also utilized as a raw material for the manufacture of fish meal. Ecologically, this species is of great importance as it occupies a crucial position in the trophic network as a planktivore that is preyed upon by larger predators.

**Table 2.5 Contribution of major pelagic fish landings in India (adapted from CMFRI 2023)**

| Groups | Catch (lakh tonnes) 2022 | Catch (lakh tonnes) 2023 |
|---|---|---|
| Indian oil sardine | 2.51 | 2.45 |
| Indian mackerel | 3.28 | 3.43 |
| Ribbon fishes | 2.27 | 2.70 |
| Cephalopods | 2.06 | 2.13 |
| Non-penaeid prawns | 1.47 | 2.11 |

Indian oil sardine is rich in n-3 PUFAs, particularly DHA and EPA, with a low level of linolenic acid (<2%) and a higher proportion of n-3 PUFAs than n-6 PUFAs. It is a high-fat fish with muscle lipid content greater than 8%. Lipid storage sites are located in subcutaneous tissues, muscle tissue, belly flab, liver, mesenteric tissue, and the head (Sukumaran et al. 2023). The lipid content was comparatively higher (approximately 5.9 folds) than the Indian mackerel (Bahurmiz et al. 2017). In the state of Karnataka, fish oil is available as Refined oil (Grade A) with less than 1% free fatty acid (Rs 85/Kg), Centrifuged oil (Grade B) with less than 3% free fatty acid and (Rs 52/Kg), and Non centrifuged oil (Grade C) with less than 4% free fatty acid (Rs 45/Kg).

**Table 2.6 Composition of fatty acids as % total fatty acids of fish flesh (Adapted from Bahurmiz et al. 2017)**

| Fatty acid | Indian Oil sardine | Indian mackerel |
|---|---|---|
| 12:0 (lauric) | <0.1 | <0.1 |
| 14:0 (myristic) | 7.0 ± 0.08 | 3.3 ± 0.03 |
| 16:0 (palmitic) | 25.5 ± 0.34 | 23.1 ± 0.29 |
| 18:0 (stearic) | 4.9 ± 0.04 | 10.6 ± 0.07 |
| **Total saturates** | 37.5 ± 0.46 | 36.7 ± 0.38 |
| 16:1n-7 (palmitoleic) | 6.7 ± 0.02 | 3.9 ± 0.01 |
| 18:1n-9 (oleic) | 12.8 ± 0.22 | 7.2 ± 0.10 |
| 18:1n-7 (vaccenic) | ND | 2.3 ± 0.06 |

|  |  |  |
|---|---|---|
| 20:1n-9 (eicosenoic) | 1.6 ± 0.02 | 0.8 ± 0.04 |
| 20:1n-11 (gadoleic) | 2.4 ± 0.04 | 0.1 ± 0.07 |
| **Total monoenes** | 23.4 ± 0.13 | 14.3 ± 0.08 |
| 18:2n-6 (linoleic) | 1.4 ± 0.02 | 1.7 ± 0.02 |
| 18:3n-6 (gamma linolenic) | 0.3 ± 0.00 | 0.3 ± 0.00 |
| 20:3n-6 (Dihomo- γ -linolenic) | 0.2 ± 0.00 | 0.2 ± 0.04 |
| 20:4n-6 (arachidonic) | 0.9 ± 0.00 | 2.0 ± 0.04 |
| 22:5n-6 (Docosapentaenoic) | 0.3 ± 0.05 | 0.4 ± 0.02 |
| **Total n-6 PUFAs** | 3.0 ± 0.03 | 4.5 ± 0.13 |
| 18:3n-3 (α-linolenic) | 0.6 ± 0.01 | 1.1 ± 0.01 |
| 18:4n-3 (Stearidonic) | 2.0 ± 0.05 | 0.7 ± 0.00 |
| 20:3n-3 (eicosatrienoic) | 0.1 ± 0.00 | 0.2 ± 0.00 |
| 20:4n-3 (Eicosatetraenoic) | 0.5 ± 0.01 | 0.3 ± 0.00 |
| 20:5n-3 (Eicosapentaenoic) | 8.3 ± 0.14 | 4.8 ± 0.02 |
| 22:5n-3 (Docosapentaenoic) | 1.1 ± 0.06 | 1.1 ± 0.02 |
| 22:6n-3 (Docosahexaenoic) | 14.3 ± 0.53 | 24.7 ± 0.31 |
| **Total n-3 PUFAs** | 26.9 ± 0.41 | 32.8 ± 0.32 |
| **Total PUFA** | 29.9 ± 0.43 | 37.4 ± 0.20 |
| **n-3/n-6** | 8.8 ± 0.06 | 7.3 ± 0.27 |

Recently, the whole genome of *Sardinella longiceps* was characterized, and the core genes (Elovl 1a, 1b, Elovl 2, Elovl 4a, 4b, Elovl 8a, 8b, and Fads 2) responsible for n-3 PUFA biosynthesis were identified. This knowledge about the whole genome might help improve the measures for the conservation and sustainable utilization of the Indian oil sardine efficiently (Sukumaran et al. 2023).

### 2.2.1.2 Other species

Large pelagic fishes of several genera and species are widely distributed and generally have a high market value (Rajesh et al. 2020). South-West coast of India, comprising the coastal regions of Karnataka and Kerala, contributed to 37% of total marine fish production in 2023 (CMFRI 2023). Fisheries on the southwest coast of India are dominated by small pelagic fishes, including sardines, whitebait, and mackerels (Morgan 2006). Pelagic fishes account for 55% (1.93 million tonnes) of total fish production in terms of quantity in 2023 (CMFRI 2023). Pelagic fishes such as Indian mackerel, Indian oil sardine, ribbonfish, anchovies, Thryssa sp., carangids, Bombay duck, seer fishes, tunnies, etc., are found predominantly in the state of Karnataka. The southwest coast contributes around 23.07% of the demersal catch in India (Zacharia et al. 2019). Demersal fishes such as bullseyes, threadfin breams, croakers, lizard fishes, pomfrets, triggerfish, elasmobranchs, and catfishes are found.

**Table 2.7 Contribution of major demersal fish landings in India (adapted from CMFRI 2023)**

| Groups | Catch (lakh tonnes) 2022 | Catch (lakh tonnes) 2023 |
|---|---|---|
| Threadfin breams | 2.01 | 2.07 |
| Other perches | 1.35 | 0.58 |
| Croakers | 0.91 | 1.03 |
| Lizard fishes | 0.72 | 0.61 |
| Catfishes | 0.61 | 0.62 |
| Silver bellies | 0.55 | 0.44 |
| Rock cods | 0.51 | 0.69 |

### 2.2.1.3 Fish oil extraction

Fish oil is manufactured from whole fish, body parts including liver, trimming from edible fish, and other fish by-products (Bimbo 2020). About 51% of fish oil is obtained from fish by-products (Muralakar et al. 2023). Most pelagic fishes are rich in body oil and are majorly caught for the production of oil. Demersal fishes contain less body oil, but higher lipid content is observed in parts such as the liver. Thus, oil from the liver of lean fishes, including cod and haddock, is extracted as fish liver oils. The methods used to produce fish oil depend on the type of raw material. This includes wet rendering, hydrolysis, silage production, and dry rendering. Hydraulic pressing is a batch process used in the industrial extraction of fish oil. The oil is obtained by pressing a mass of moderately cooked oil-bearing fish. In general, wet rendering involves cooking to coagulate protein and release moisture and oil, followed by pressing, separating the oil from water to recover crude oil, and drying the residual protein (obtained as a fish meal).

Another method to retrieve oil from fish is solvent extraction or leaching, wherein soluble constituents present either as solids or liquids are removed using a solvent (Sahena et al. 2009). The effectiveness of solvent extraction is influenced by the polarity of the lipids present compared to the solvent's polarity. Polar lipids (glycolipids or phospholipids) dissolve more readily in polar solvents (alcohols) than in nonpolar solvents (hexane). Conversely, nonpolar lipids (triacylglycerols and triglycerides) dissolve more easily in nonpolar solvents than in polar ones. The disadvantage might be the denaturation of proteins and loss of functional properties when solvents are used for extraction. Alternative techniques include enzymatic extraction (alcalase, neutrase) and supercritical fluid extraction fractionation which avoids harsh reaction conditions (Sahena et al. 2009).

Fish oil obtained through dry rendering and solvent extraction methods is darker in color, requiring additional refining steps. For extraction of fish oil from the liver, it is first subjected to grinding and cooking using low-pressure steam. Then, the oil is separated and refined in several steps. Concentrating the PUFA content is usually done using solvent fractionation, winterization, and molecular distillation. Using these methods, EPA, and DHA concentrations of up to 30% could be achieved. For higher concentrations (65 to 80%), hydrolysis or esterification is combined with supercritical fluid extraction, urea complexation, and molecular distillation (Sahena et al. 2009). The fish oil produced is used in pharmaceuticals, aquaculture, and animal feeds. Fish oil is exported from India to countries such as Saudi Arabia, Ethiopia, Chile, and Bangladesh etc., according to the export data from the Department of Commerce, India. The market for marine oils has been evolving since the 1990s as an increase in demand is observed for long chain PUFA-rich oils because of their beneficial health effects.

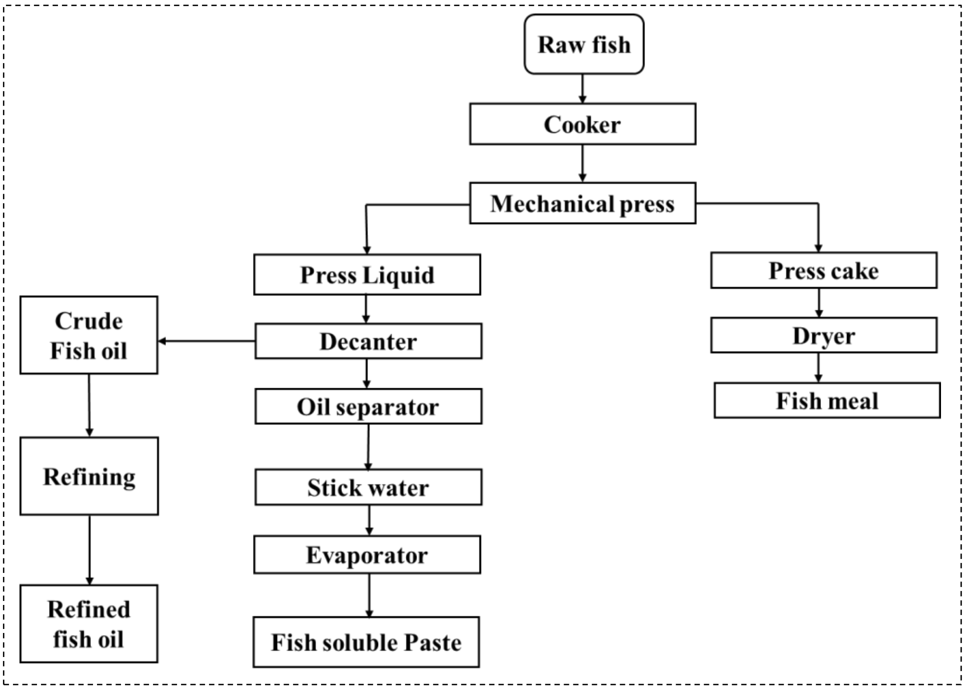


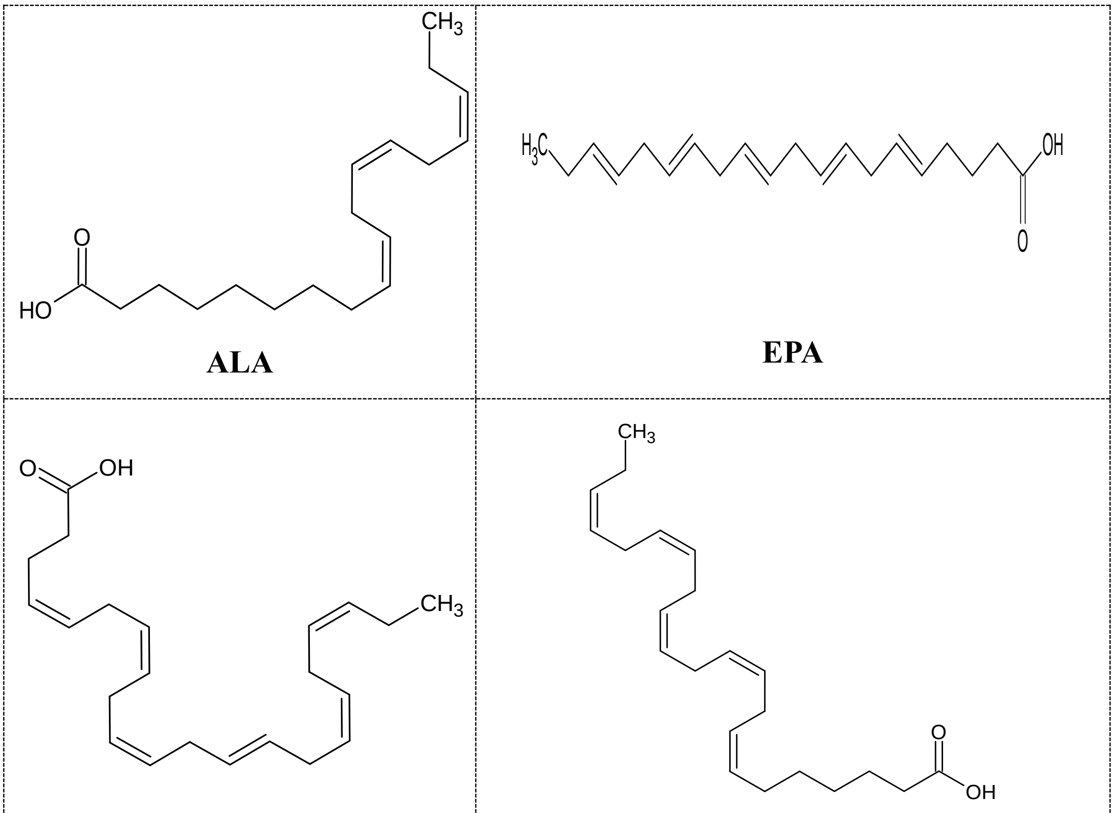
**Fig. 2.10 Conventional Fish Oil extraction method**

### 2.2.2 Composition of fish oil

Fish oils are different in composition from vegetable oils in that they have a large quantity of unsaturated fatty acids (Moffat and McGill 1993). The majority of long-chain fatty acids are found in the form of triacylglycerol (TAG), followed by phospholipids (PL), diacylglycerols, cholesterol esters, and fat-soluble vitamin esters (Saini and Keum 2018). Triacylglycerol consists of three fatty acids esterified to a glycerol backbone, whereas phospholipids have two fatty acids esterified to a glycerol backbone and a phosphorous group linked to the headgroup (Burri et al. 2012). The high content of TAG serves as a temperature and mechanical isolator. Fish generally may contain up to 1-1.5% PLs and 10-15% TAGs.

#### 2.2.2.1 Long-chain PUFAs

Fish oil consists of long-chain fatty acids of carbon chain length C18-24 in higher amounts than other oils and fats. Fatty acids with 5 to 6 double bonds make up to 15-30% of total fatty acids present in fish oil. PUFAs are generally derived from unicellular phytoplanktons and seaweeds, which then accumulate in fish (Sahena 2009). Vegetable oils contain less than 1% of such fatty acids. In addition to LC-PUFAs, fish oil contains considerable quantities of saturated and monounsaturated fatty acids and monoenes (Stansby 1969). Polyunsaturated fatty acids are categorized into n-3 and n-6 based on the position of double bonds from the methyl end of the carbon chain (Sahena et al. 2009). PUFAs of fish oil with chain length 18-22 are majorly of n-3 type while the proportion of n-6 fatty acids is very small (<10%). Zhang et al. (2020) studied the fatty acid composition of some commercial fish species from the Pearl River Estuary, China. The fatty acid profiles of a total of 22 marine fishes were analyzed. PUFAs comprised of 10.3 to 28.4% of total fatty acids in all studied species. The predominant fatty acids present were EPA, DHA, DPA, and Eicosatrienoic acid, while n-6 PUFAs were of low levels (0.93-8.31%). In another study, the nutritional profile of 10 edible fish species of the Lakshadweep Sea was investigated (Dhaneesh et al. 2012). Composition analysis showed that the PUFA content was about 30.35-35.11% of the total fatty acids present. The n-3 PUFA content ranged from 13.05 to 21.14%, while n-6 PUFAs ranged from 6.8 to 9.82% of total fatty acids.



**ALA**

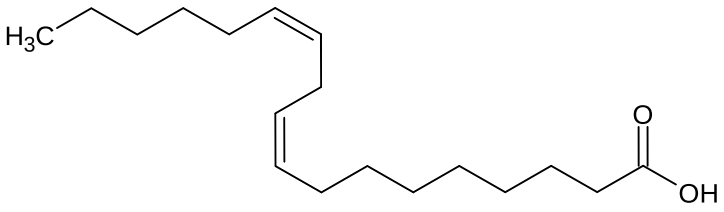
**EPA**

**DHA**

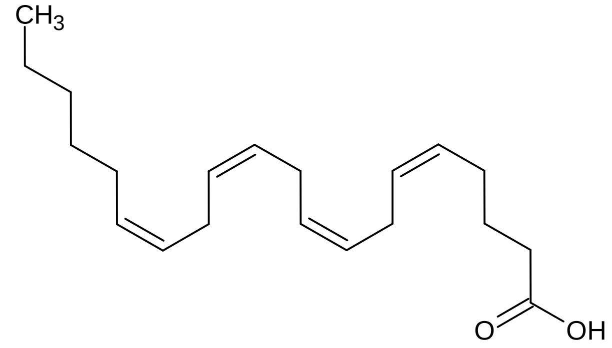
**DPA**

**Fig. 2.11 Structure of n-3 PUFAs**

There is a considerable variation in the amount of fatty acids present in fish oil based on the diet, age, maturity, climatic conditions, and the type of species. Total lipid content and fatty acid profiles of ribbon fish (*Lepturacanthus savala*) samples collected during winter, summer, and post-monsoon periods were studied (Ramesh et al. 2015). Ribbonfish belongs to the category of lean and low-fat fish with 2-4% fat. Arachidonic acid, EPA, DPA, and DHA were the principal unsaturated fatty acids present. It was found to have high levels of n-3 PUFA with 12.37-43.14% and had a favorable n-3/n-6 PUFA ratio of 1.79-5.04. The nutritional characteristics of the flesh of Indian mackerel and oil sardines were analyzed by Bahurmiz et al. (2017). PUFA was present in Indian mackerel at 37.4%, followed by saturated and monounsaturated fatty acids (36.7% and 14.3% respectively). The major PUFA present in Indian mackerel was of n-3 category, of which DHA and EPA were the most abundant.



**Linoleic acid**



**Arachidonic acid**

**Fig. 2.12 Structure of n-6 PUFAs**

The oil from the liver of triggerfish, Balistes Spp., was extracted, and the composition was studied by Thankappan and Xavier (2011). Triggerfish contains 7-8% liver and 65% liver oil. The triggerfish liver oil was found to have significant levels of polyunsaturated (32.63%), monounsaturated (23.84%), and saturated (42.62%) fatty acids. These levels are on par with fish oil from sardines and mackerels (Table 2.8). EPA and DHA contributed to 73% of the total PUFA present in the triggerfish liver oil.

**Table 2.8 Fatty acid composition of fish oil in comparison to triggerfish liver oil (Thankappan and Xavier, 2011)**

| Fatty acids | Sardine oil | Mackerel oil | Triggerfish liver oil |
|---|---|---|---|
| Saturated | 40.2 | 37.8 | 42.62 |
| Monounsaturated | 28.2 | 27.5 | 23.84 |
| Polyunsaturated | 29.7 | 32.3 | 32.63 |

### 2.2.2.2 Phospholipids and other miscellaneous components

Phospholipids present in fish oils of different species include lecithin and cephalin (Stansby 1969). These make up to 75% of the total phospholipids present. The principal unsaturated fatty acids found in lecithin and cephalin are C20:5 (EPA) and C22:6 (DHA). Alkylglycerols are present in shark liver oil, and this represents the ether-linked lipids in fish oil. It is also found in dogfish flesh and liver oil. Fish oil also contains sterols, vitamins, and pigments. Sterols are found in low quantities in about 100 mg/100 g of wet-weight of fish.

Apart from phospholipids, fish liver oils often contain higher amounts of hydrocarbons, including squalene, pristane, and zamene. Herring body oil was also reported to have 0.05% hydrocarbons (Stansby 1969). Certain species of fish have high proportions of wax esters in their body and liver oils. These wax esters are formed when a fatty acid with low unsaturation is esterified to a fatty alcohol with a 16 or 18-carbon chain length. Other minor components of fish oil include vitamins and pigments. Vitamins A and D are present in varying amounts in the liver and body oils of fish. Some fish, such as halibut, have high levels of vitamin A, up to 700,000 units per gram of liver oil. Certain tuna liver oils contain up to 250,000 units of vitamin D per gram, while many other body and liver oils have values usually under 100 units per gram (Stansby 1969).

### 2.2.3 Refining of fish oil

Refining involves the removal of undesirable compounds from crude fish oil with minimal elimination of valuable nutrients in order to maintain the quality and shelf life of fish oil. As a result, knowing the composition of the crude fish oil is essential for designing an efficient refining process. Apart from triglycerides containing long-chain PUFAs, compounds such as unsaponifiable matter, wax, pigments, heavy metals, moisture, tocopherols, and oxidation products (primary and secondary oxidation products) are also present. Generally, refining of fish oil includes degumming, deacidification, and bleaching, which are crucial to remove impurities such as phospholipids, free fatty acids, and minor components (Vaisali et al. 2015). The challenging part of refining fish oil is the removal of undesirable components while retaining the n-3 PUFA content. Additionally, fish oil is more prone to oxidation than any other edible oils due to the presence of PUFAs. Hence, refining is essential to slow down lipid oxidation and to maintain the nutritional profile of fish oil.

**Table 2.9 Approaches for refining of fish oil**

| Process | Conventional approach | Green technology |
|---|---|---|
| Degumming | Acids such as phosphoric acid, oxalic acid, and lactic acid, along with water treatment, are used to remove phospholipids.<br>Soft degumming using chelating agent (EDTA) with an emulsifying agent. | Use of enzymes (phospholipases) and membrane separation techniques to remove phospholipids |
| Deacidification | Alkali hydrolysis using NaOH, followed by precipitation of free fatty acid as soap stock. | Re-esterification using enzymes and solvent extraction methods |
| Bleaching | Using solid adsorbents such as activated charcoal to remove pigments and lipid oxidation products | Ultrasound-assisted bleaching and supercritical fluid extraction to remove persistent organic pollutants |

| | | |
|---|---|---|
| | | (POPs). Short-path distillation (SPD) with short time and high vacuum at higher temperatures. |
| Deodorization | Steam distillation at high temperatures can induce several side reactions including polymerization, oxidation, and isomerization. | SPD, liquid-liquid extraction, and nanofiltration as alternatives. Better efficiency in reducing volatiles and reduced degradation of PUFAs |
| Enrichment | Urea complexation to remove saturated and mono-unsaturated fatty acids. Lower efficiency in reducing MUFA contents | Crystallization using differences in melting point and enzyme-catalyzed ethanolysis of all non-n-3 PUFAs |

#### 2.2.3.1 Degumming

The presence of phospholipids and other mucilaginous compounds is first removed from crude fish oil using degumming. Phospholipids can accelerate oxidation since they are more prone to oxidation than acylglycerols (Vaisali et al. 2015). Degumming can be done through chemical, enzymatic, and membrane-based methods. Chemical treatment is generally preferred in industries since they use cheap chemicals and easier disposability (De Greyt 2012). The process includes water degumming followed by acid treatment. The initial water treatment removes the hydratable phospholipids, while acid treatment works to remove the non-hydratable phospholipids. However, there is a considerable loss of acylglycerols in this method, which is undesirable. Rather, a single-step degumming of fish oil can be done using acids such as orthophosphoric acid, lactic acid, and acetic acid (Charanya et al. 2017). The method involves the addition of the acids in the required concentration, which essentially reduces the pH of the oil. At low pH, the hydratable and non-hydratable phospholipids become hydrophilic and form sludge, which can be easily removed through centrifugation. The strategy works well for fish oil, such as sardine oil, as optimized by Charanya et al. (2017). They studied the degumming of sardine oil using these acids and analyzed the phospholipid content before and after degumming. Orthophosphoric acid at 5% w/w was found to reduce phospholipid content efficiently in sardine oil. In addition, the degumming process also reduced the metal ion contents including copper, iron and mercury. Metal ions tend to bind to phospholipids at acidic pH and get precipitated along with the phospholipids. Alternatively, membrane degumming involves the removal of phospholipid through a membrane of suitable size using hydraulic pressure. The method is energy efficient as it requires fewer steps and does not involve the use of chemicals (Vaisali et al. 2015). Enzymatic degumming using phospholipase and acyltransferase was done in fish liver oils by Lamas (2022). Phospholipases are hydrolytic enzymes that can hydrolyze the ester bonds of phospholipids (Marsol-Vall et al. 2022). A comparison was made between chemical and enzymatic degumming, wherein the enzymatic method was better, and the nutritional profile of the oils was also maintained in the enzymatic method. The enzymes are also used in immobilized form to improve reusability and to reduce the cost involved (Li et al. 2016).

#### 2.2.3.2 Deacidification

The deacidification step removes the free fatty acid content, which is responsible for rancidity and undesirable flavor. The presence of a higher amount of unsaturated fatty acids in fish oil leads to the release of free fatty acids, increasing the susceptibility to oxidative damage. Thus, deacidification is a crucial step in the refining of fish oils. The conventional method of deacidification includes the use of alkali to precipitate free fatty acids as soap stocks, which are then removed by centrifugation or water wash. The oil produced is of acceptable quality, but the method results in higher oil loss and difficult-to-treat wastewater (De Greyt 2012). Hence, alternatives for alkali treatment, including physical and biological deacidification, re-esterification, and solvent extraction, are continuously sought.

Physical deacidification involves heating the oil to the desired temperature under vacuum to remove free fatty acids. The method is dependent on the volatility of different components of the fish oil (Vaisali et al. 2015). It is energy-consuming but gives better yields than alkali treatment. Steam distillation used in industries could result in cyclization, isomerization, and polymerization reactions of unsaturated fatty acids at higher temperatures. This ultimately alters the physicochemical and organoleptic properties of fish oil, leading to reduced acceptability. In the biological method, microorganisms are used to utilize free fatty acids without secretion of extracellular lipase. Species such as *Pseudomonas* isolated from soil capable of utilizing free fatty acids are employed. Regardless of the ambient reaction conditions, the difficulties in removing the microorganism from oil, designing and maintaining pure cultures, and the scalability of the process render it unsuitable for large-scale applications.

Re-esterification of free fatty acids is carried out to convert them into neutral glycerides through esterification. The excess free fatty acids react with glycerol in high temperatures (200-280°C) in the presence of a catalyst with the release of water. Chemical catalysts, including zinc dust, zinc chloride, and 0.5% of naphthalein-beta-sulphonic acid, are used for esterification reactions. Enzymatic catalysts are employed since they are comparatively gentler in nature than chemical catalysts. Commercial lipases can also be employed for deacidification. This approach is adopted to ensure that the process is carried out efficiently while maintaining a suitable level of safety. Wang et al. (2012) have utilized lipase to deacidify tuna oil. The acid value was reduced from 10.2 to 0.4 mg KOH/g with an increase in PUFA content from 32 to 82%. However, the cost and reusability of enzymatic catalysts pose difficulties when employed on a large scale. Laomin and researchers developed a packed bed reactor with immobilized lipase for the re-esterification of free fatty acids (Laomin et al. 2012). Other heterogeneous chemical catalysts, such as ion-exchange resins, are also employed for the esterification reaction.

The solvent extraction method is effective on a large scale and is based on the difference in the solubility of free fatty acids and triglycerides. The method avoids the loss of oil resulting due to soap formation in alkali treatment (Charanya et al. 2017). For fish oil rich in EPA and DHA, short-chain alcohols such as methanol, ethanol, propanol, and butanol are efficient in reducing free fatty acid contents. The process involves liquid-liquid extraction in different solvent-to-oil ratios with these short-chain alcohols, and the repetition of the step depends on the acidity of the oil. Methanol was able to remove free fatty acids from fish oil effectively in a 1:1 ratio when compared to other short-chain alcohols (Charanya et al. 2017). As the length of the carbon chain increases, the hydrophobicity increases, leading to incomplete separation of solvents from the oil phase. Additionally, the solvent-to-oil ratio should be optimal since higher concentrations can hydrolyze triglycerides also (Charanya et al. 2017).

#### 2.2.3.3 Bleaching

Bleaching is done to remove impurities such as pigments, lipid oxidation products, and phospholipids and soaps that remain after degumming and deacidification. Additionally, fish oil often contains several organic pollutants such as polychlorinated biphenyls (PCBs), and dioxins. Improvement of fish oil quality and removal of these persistent pollutants is achieved through bleaching using a solid adsorbent. The fish oil is treated with solid adsorbents such as activated charcoal or bleaching earth at a temperature of about 60-90°C under vacuum. Efficient bleaching requires optimization of temperature, amount of adsorbent to be used, and contact time. A temperature of 80°C was found to be efficient for bleaching sardine oil using granulated activated charcoal (Charanya et al. 2017). Further increase in temperature led to increased free fatty acid content, probably due to hydrolysis of glycerides. At higher temperatures, the viscosity of the oil reduces which facilitates the attachment of impurities onto the solid adsorbent. This adsorption is due to the presence of a higher surface area and acidity of the solid adsorbents such as clays (Vaisali et al. 2015). Chakraborty and Joseph (2015) used a combination of activated charcoal and Fuller's earth to bleach sardine fish oil. After bleaching, lipid oxidation products were reduced, and the color of the oil also improved along with an enhanced PUFA content. In some cases, ultrasound-assisted bleaching was attempted using activated bleaching earth in canola oil (Icyer and Durak 2018). The use of ultrasound did not significantly improve the refined oil quality, but it reduced the contact time by about 50% compared to the conventional bleaching method.

### 2.2.4 Human Health and n-3 PUFAs

In recent years, PUFA-rich foods have been gaining interest since there are a number of indications of a positive health impact on human consumption. These long-chain fatty acids are noted for their anti-inflammatory, anticoagulant, antithrombotic, and antiarrhythmic properties and can improve the lipid profile. Several studies indicate the role of polyunsaturated fatty acids in the prevention and treatment of cardiovascular diseases, rheumatoid arthritis, cancer, diabetes, asthma, and others. The American Heart Association recommends consumption of at least 2 servings of fatty fish per week because of their cardiovascular benefits. The requirements for total fat and fatty acids as recommended by WHO/FAO in the percentage of energy (%E) are as follows: Total Fats: 20-35%E as acceptable macronutrient distribution range (AMDR); SFA: 10%E as upper-level AMDR; Total PUFA: 6-11%E as LA, ALA, EPA and DHA while the adequate intake is set as 2.5-3.5%E; MUFA: by difference. The recommended daily intake of essential fatty acids EPA and DHA is 0.25-2 g/day, and ALA intake should be obtained at a minimum of 0.5% E. Fish oil is an excellent source of not only PUFA but also a good source of protein, B vitamins, and minerals such as potassium, phosphorous and selenium (Gebauer et al. 2006). Vitamins A and D are present in varying amounts in fish body and liver oils.

**Table 2.10 Recommended intake of n-3 PUFAs**

| Recommending organisation | Suggested intake |
|---|---|
| World Health Organisation (WHO) | Daily intake of 0.25 to 0.5 grams of EPA, DHA combined |
| American Heart Association (AHA) | Fatty fish intake for at least two times a week for healthy individuals (approximately 0.5 g EPA, DHA/ day). For patients with coronary heart disease, an intake of 1g EPA, DHA/day is recommended, preferably from oily fish. |
| European Food Safety Authority (EFSA) | Intake of 250 mg of EPA and DHA is advised to maintain general heart health. An additional 100-200 mg DHA is recommended for pregnant women. |
| Global Organization for EPA and DHA Omega-3s (GOED) | Minimum intake of 500 mg of EPA and DHA per day for healthy individuals. |

| | |
|---|---|
| U.S. Department of Agriculture (USDA) | Recommends consumption of 8 ounces of seafood (fish and shellfish) equivalent to 250 mg of EPA and DHA per day. |
| Food and Agriculture Organisation (FAO) of the UN | Minimum of 250 mg of EPA, DHA per day for adult males and non-pregnant females |

In several cardiovascular diseases (CVD), including myocardial infarction, stroke, congenital heart disorders, and coronary heart disease, the effect of consumption of n-3 PUFA has been studied (Mozaffarian et al. 2016). The consumption of n-3 PUFA can improve the plasma lipid profile (Sahena et al. 2009), and EPA and DHA can reduce blood pressure (Mori 2006). The beneficial effects of n-3 PUFAs in preventing the development of atherosclerosis are believed to be due to their anti-inflammatory effects on platelets and endothelial cells. Platelets, through their interaction with the vascular endothelium, play a major role in the development of atherosclerosis. A study conducted by Mori and others (1997) revealed that consuming n-3-PUFAs (3 to 4 g/day) for three weeks significantly reduced platelet aggregation induced by collagen and platelet-activating factor (PAF), regardless of whether the n-3 PUFAs were ingested as daily fish meals or fish oil capsules. In a systemic review analysis conducted by Schwab et al. (2014), the effect of n-3s on cardiovascular health was studied by analyzing articles published between 2000 and 2012. The study found convincing evidence for the reduction of fasting serum or plasma total and LDL cholesterol levels when saturated fat was partially replaced with polyunsaturated or monounsaturated fats. Despite several positive findings, recent studies in patients with existing CVD have failed to replicate these results. These studies could be limited because of the small sample size, low dosage of EPA and DHA, and a shorter duration of follow-up (Calder 2018).

Several biological activities of EPA and DHA may influence the viability and proliferation of tumor cells. It is possible that DHA will encourage apoptosis in tumor cells through oxidative stress. According to Calder (2018), EPA and DHA have the ability to directly affect cancer cells, tumor microenvironment, and the host's reaction to tumors through these actions. According to several studies, these fatty acids may reduce the risk of breast and colorectal cancers. Supplements with EPA and DHA have been shown to enhance appetite, energy intake, body weight, and quality of life in individuals with lung cancer. Clinical research has demonstrated that individuals with non-small cell lung cancer can maintain their body weight and muscle mass after chemotherapy by consuming 2.2 g of EPA and DHA per day (Murphy et al. 2011).

New research suggests that taking supplements of n-3 PUFA may help individuals with neurodegenerative diseases like Parkinson's (PD) and Alzheimer's (AD). Even though there is no conclusive evidence, it is recommended that individuals with PD or AD take n-3 PUFA supplements to improve their symptoms or slow down cognitive and physical decline. Studies show that diets high in n-3 PUFA may help reduce the risk of dementia, Alzheimer's, or cognitive decline. n-3 PUFAs can help maintain cognitive function by supporting the preservation of neuronal function and cell membrane integrity in the brain. Research has shown that AD patients have lower levels of DHA in their blood than people who are not cognitively impaired (Chew et al. 2015; Tully et al. 2003).

Numerous epidemiological studies have demonstrated the link between n-3 PUFAs and both mother and child health during pregnancy. Prenatal growth patterns, visual acuity, neurological and cognitive development, autism spectrum disorders, attention deficit hyperactivity disorder (ADHD), learning disorders, atopic dermatitis, allergies, respiratory disorders, length of gestation, preterm birth, birth weight, peripartum depression, gestational hypertension/preeclampsia, and postnatal growth patterns are all impacted by n-3 PUFAs (Newberry et al. 2016). Increased n-3 PUFAs in the mother's diet may help with the outcomes of childhood allergy illness, according to a new meta-analysis based on clinical data (Best et al. 2016). Furthermore, a higher dietary intake of very long-chain n-3 fatty acids may be associated with a decreased risk of psychological and psychiatric problems, according to some epidemiological studies (Calder 2018). Very long-chain n-3 fatty acids may help with childhood development and adult psychiatric and psychological illnesses, including depression, according to intervention studies. The consumption of n-3 PUFAs has been associated with several health benefits, prompting numerous regulatory standards to recommend adequate intake of these fatty acids through food. Given their importance, it is imperative to ensure that the recommended levels are met to safeguard against any potential health risks that may arise from inadequate consumption.

### 2.2.5 Oxidation of fish oil

Marine fish oils are highly susceptible to oxidation during storage because of the presence of double bonds in the polyunsaturated fatty acids. This leads to the oxidation of fish oil, resulting in the formation of products that deteriorate the nutritional quality and produce undesirable flavor and taste. Degradation of fish oil quality during storage and transport remains a problem in food and nutraceutical industries due to the production of oxidation products. When exposed to oxygen, light, and heat, unsaturated fatty acids present in fats and oils undergo oxidation (Ismail et al. 2016). The development of rancidity and off-flavors and odors is a barrier to using PUFA-rich fish oil. Moreover, the oxidation products could be toxic to humans, which causes health concerns among consumers. Bis-allylic carbons are carbons present between two double-bonded carbon atoms. These carbons present in n-3 and n-6 PUFAs have low activation energy for loss of hydrogen and free radical formation, which makes them more prone to oxidation. For example, EPA, DHA, ALA, AA, and LA have 4, 5, 2, 3, and 1 bis-allylic carbon(s), respectively (Fig. 2.12).

### 2.2.6 Mechanisms of oxidation

Oxidation proceeds through a free radical chain reaction which is described in three stages including initiation, propagation and termination processes (Hsieh and Kinsella 1989). The catalysts of oxidation include light, heat, enzymes, metals, metallo-proteins, and microorganisms that leads to production of oxidation products through autoxidation, photo oxidation, thermal or enzymatic oxidation (Shahidi and Zhong 2010). Thus, various intrinsic and extrinsic parameter could initiate, and accelerate oxidation of n-3 rich oils during storage and transport.

The various stages of oxidation are demonstrated in Fig. 2.13. During initiation phase, unsaturated lipid molecules lose a hydrogen atom to produce free radical which then reacts with oxygen leading to the formation of peroxyl radicals. The induction phase predominantly consists of generation and monomolecular decomposition of hydroperoxides (Wang et al. 2024). The peroxyl radical thus formed act as chain carriers and attacks a new lipid/fatty acid molecule. Formation of hydro peroxides during initiation phase varies in enzymatic, photo and autoxidation pathways of lipid oxidation (Chaijan et al. 2017). The presence of high temperature accelerates the oxidation during thermal oxidation. The polyunsaturated fatty acids present in different forms including simple alkyl esters, free fatty acids, acylglycerols and phospholipids are the primary reactant affected by oxidation (Shahidi and Zhong 2010). Once the hydroperoxide concentration reaches a critical micelle concentration ($CMC_L$), the stage of oxidation transits from initiation to propagation. During the propagation stage, the rate of production of lipid hydroperoxides increases sharply, attaining its maximum value ($R_{max}$) in the middle of the phase ($P_m$), before decreasing to negligible levels at the endpoint ($P_e$), where the highest concentration of LOOH ($LOOH_{max}$) is attained. Subsequently, the decomposition of LOOHs becomes significant from Pm onwards. Finally, during the termination phase, LOOH molecules are decomposed at a higher rate than their production, leading to a decrease in their overall concentration (Farhoosh 2018).

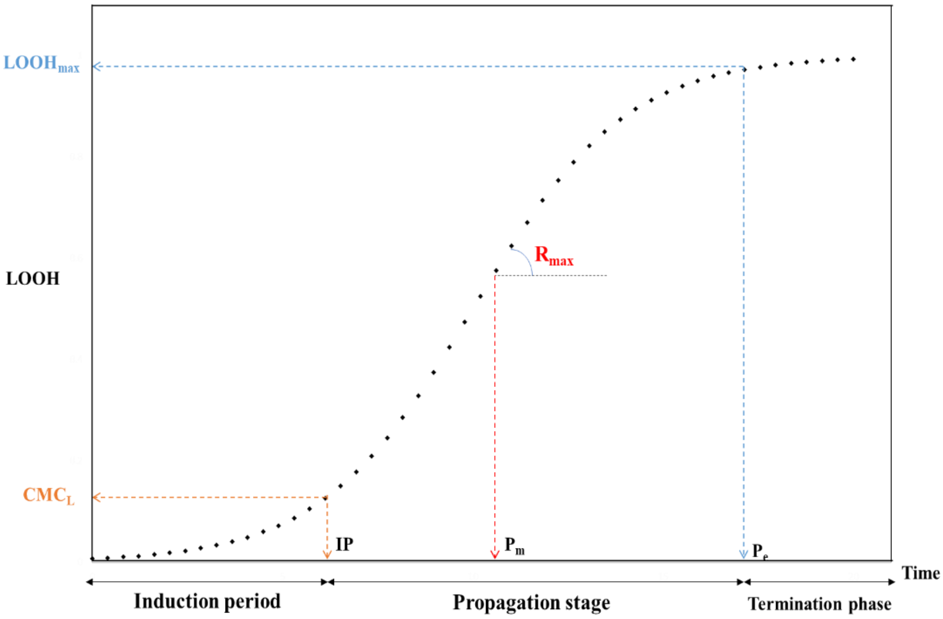


Fig. 2.13 Different stages of oxidation (adapted from Wang et al. 2024)

### 2.2.6.1. Autoxidation

In autoxidation, triplet oxygen reacts with fats and oils in radical form. Since the double bonds of fatty acids exist in singlet state, the triplet oxygen must be activated to the singlet oxygen state for the initiation of oxidation reaction. This activation of oxygen is accelerated by the presence of heat, light, and transition metals. Hydrogen is abstracted from the unsaturated fatty acid (RH) resulting in the formation of lipid free radical ($R^{\bullet}$). The hydrogen molecule attached to the bis allylic carbon has a very low bond dissociation energy making it simpler for its removal from n-3 PUFA (Choe and Min 2005). The oxidative stability of PUFA is inversely proportional to the number of bis allylic positions present in them. Based on the number of bis allylic positions, PUFAs in fish oil are susceptible to oxidation in the order LA<ALA<AA<EPA<DHA (Miyashita 2014). The removal of hydrogen from PUFA is the first and rate limiting step in the initiation of oxidation reaction. The rate of oxidation during initiation phase is slow because of the slow formation of free radicals prior to hydro peroxide accumulation and possible interaction with endogenous antioxidants (Barden and Decker 2013).

The formed lipid free radical reacts with molecular oxygen to form peroxyl radical ($ROO^{\bullet}$). The peroxyl radical also acts as an initiator for the abstraction of hydrogen from lipid molecule to form lipid hydro peroxides (ROOH) and lipid free radicals ($R^{\bullet}$). The lipid free radical reacts again with molecular oxygen to form new peroxyl radical and the reaction progresses in the propagation phase (Chaijan et al. 2017). The lipid free radicals generated during initiation and propagation phases are stabilised by rearrangement of methylene interrupted double bonds in PUFA (Barriuso et al. 2013). Conjugated dienes and trienes thus formed are used as an indicator for the measurement of oxidation (Shahidi and Miraliakbari 2005).

**Initiation:** $RH \rightarrow R^{\bullet} + H^{\bullet}$

**Propagation:** $R^{\bullet} + {}^{3}O_2 \rightarrow ROO^{\bullet}$

$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$

**Termination:** $ROO^{\bullet} + R^{\bullet} \rightarrow ROOR$

$R^{\bullet} + R^{\bullet} \rightarrow RR$

The hydro peroxides formed during propagation phase decomposes to give rise to new alkoxyl, hydroxyl and peroxyl radicals which further serves as initiators for oxidation (Chaijan et al. 2017). The decomposition may be mediated by the presence of metals or due to the interaction between two hydro peroxides. These primary oxidation products are unstable and break down into secondary oxidation products such as aldehydes, ketones, alcohols, hydrocarbons, and volatile organic acids.

In termination phase, non-radical products are formed by the reaction between radicals or reaction with other non-radical compounds. For example, an antioxidant (AH) donates a hydrogen atom to the radical species resulting in the neutralization of the lipid radical. This produces a new radical from the antioxidant ($A^\bullet$) which is less reactive when compared to the lipid radical. Thus, at the end of termination reaction, a stable or a less reactive product is formed. The reaction between peroxy, alkoxyl and/or alkyl radicals is represented below.

$$RO^\bullet + RO^\bullet \rightarrow ROOR$$

$$RO^\bullet + R^\bullet \rightarrow ROR$$

$$ROO^\bullet + ROO^\bullet \rightarrow ROOR + O_2$$

$$2RO^\bullet + 2ROO^\bullet \rightarrow 2ROOR + O_2$$

$$ROO^\bullet + AH \rightarrow ROOH + A^\bullet$$

$$RO^\bullet + AH \rightarrow ROH + A^\bullet$$

$$R^\bullet + AH \rightarrow RH + A^\bullet$$

The formation of volatile products in the oxidation of fish, echium, linseed and soybean oil triacylglycerols at high temperatures was studied by Shibata et al. (2015). Higher levels of propenal and propanal were detected in fish oil while only a small amount of acrolein (2-propenal) was found in soybean oil oxidation. Products such as aldehydes and vinyl ketones are responsible for the development of unpleasant fishy odour and rancid off-flavours even when they are present in very low threshold levels (Miyashita 2019). The hydro peroxides formed upon oxidation of EPA and DHA are very easily decomposed into secondary volatile oxidation products when compared to the oxidation of other polyunsaturated fats. Hence there is development of undesirable taste and odour in fish oil rich in EPA and DHA even in low levels of hydro peroxides.

### 2.2.6.2. Photo-oxidation

The initiation of lipid oxidation can also proceed through photo-oxidation. Hydro peroxide molecules are formed in the presence of photo sensitizers (Sen) such as chlorophyll, riboflavin, myoglobin, pheophytins and heavy metals (Shahidi and Zhong 2010). These photosensitizers get activated to the excited singlet state ($^1$Sen*) by absorbing energy from light. The excited state of photosensitizers is highly unstable and thus returns to the ground state by reacting either with double bonds in lipid substrates or with triplet state oxygen. The reaction with lipid substrates is called type I photo-oxidation where hydro peroxides are produced without the formation of the alkyl radical (Wsowicz et al. 2004). The reaction with triplet oxygen results in the formation of singlet oxygen ($^1O_2$) thereby initiating oxidation process (Type II photo-oxidation).

**Type I**

$$\text{Sen} \xrightarrow{h\upsilon} {}^1\text{Sen}^*$$

$$ {}^1\text{Sen}^* + \text{RH} \longrightarrow \bullet\text{SenH} + \text{R}^\bullet \xrightarrow{O_2} \text{ROOH} + \text{Sen or}$$

$$ {}^1\text{Sen}^* + \text{RH} \longrightarrow (\text{Sen}^{\bullet-} + \text{RH}^{\bullet+}) \text{ or } (\text{Sen}^{\bullet+} + \text{RH}^{\bullet-}) \xrightarrow{O_2} \text{ROOH} + \text{Sen}$$

**Type II**

$$\text{Sen} \xrightarrow{h\upsilon} {}^1\text{Sen}^*$$

$$ {}^1\text{Sen}^* + O_2 \longrightarrow \text{Sen} + {}^1O_2$$

$$ {}^1O_2 + \text{RH} \longrightarrow \text{ROOH}$$

Singlet oxygen reacts readily (almost 1500 times faster) in lipid oxidation than the triplet oxygen state. The reaction rate of linoleic acid with singlet and triplet oxygen is 1.3x10^5 /M/s and 89/M/s respectively (Rawls and Van Santen 1970). After the formation of hydro peroxide, the autoxidation proceeds through the other phases leading to the production of oxidation products.

### 2.2.6.3 Enzymatic oxidation

Enzyme mediated mechanism of lipid oxidation take place in the presence of enzymes such as lipoxygenase, cyclooxygenase in a non-radical mechanism (Niki 2004). Lipoxygenase is an iron bound enzyme and catalyses the oxidation of EPA, DHA, linoleic, linolenic, and arachidonic acid rich oils. Iron must be present in ferrous form in the active site of lipoxygenase enzyme for its activity (Ghnimi et al. 2017). The concentration of lipoxygenase found plays an important role in the initiation of oxidation reaction (Schilstra et al. 1993). There is a lag phase in the initiation of enzymatic oxidation which is inversely proportional to the concentration of the enzyme. Hydrogen atom from the methylene group of polyunsaturated fatty acid is abstracted by the active site of the enzyme resulting in the formation of a conjugated diene system. This in turn reacts with oxygen to produce lipid peroxy radical. The peroxy radical removes hydrogen from another unsaturated fatty acid molecule resulting in the release of hydro peroxides (Chaijan et al. 2017; Wsowicz et al. 2004).

## 2.2.7 Factors affecting oxidation of fish oil

### 2.2.7.1 Nature of fatty acids

Lipid oxidation is a complex reaction leading to the production of undesired off-flavours and taste in fish oils. The rate of lipid oxidation and oxidative stability varies due to several intrinsic and extrinsic factors (Charanyaa et al. 2019). The degree of unsaturation of oil and its distribution, presence of antioxidant and pro-oxidant compounds, environmental factors such as light, temperature and oxygen play a major role in the oxidation of lipids. Oil containing long chain unsaturated fatty acids are more susceptible to oxidation because of the presence of double bonds. Higher degree of unsaturation makes the fish oil more prone to oxidation. The bond dissociation energies of hydrogen-carbon in allylic and bis-allylic methylene positions in fatty acids with double bonds are lower (Erickson 1999). Thus, these double bonds increase the susceptibility to oxidative degradation. Furthermore, the bis-allylic methylene hydrogen is easily abstractable than the allylic positions (Richards 2005). Therefore, the oxidation rate of fatty acids based on the methylene bridge index (MBI- mean of bis-allylic methylene positions) is in the order stearic<oleic<linoleic<linolenic acid.

Glyceryl esters of fatty acids are oxidized faster than their free forms. Phospholipids present also increases the susceptibility of oxidation in fish oils. This enhanced reactivity of phospholipid in oxidation is due to the higher proportions of PUFA present in membranes. The arrangement of lipids in phospholipid membrane makes the catalytic sites in close proximity for oxidative enzymes (Erickson 1999).

### 2.2.7.2 Presence of pro-oxidants and antioxidants

The presence of certain compounds may either aggravate or retard oxidative degradation of fish oils. Compounds that initiate facilitate or accelerate lipid oxidation are called pro-oxidants while those that retard the oxidation process are called antioxidants. The antioxidants maybe a native constituent of fish oil or added as an additive. These antioxidants act as free radical acceptors, metal chelators, quenchers of singlet oxygen and reactive oxygen species. Compounds that are naturally occurring in crude fish oil including tocopherols, carotenoids, amino acids and peptides, ascorbic acid have antioxidative effect. However, they usually do not provide adequate protection against oxidation during storage since they are removed by refining.

Fish oil may contain pro-oxidants such as transition metals, free fatty acids, photosensitizers, enzymes and heme proteins. Transition metals including iron, copper ions facilitate/promote lipid oxidation reactions. These metal ions are present in crude fish oil (Jacobsen 2010). Refining of fish oil including degumming, deacidification, bleaching and deodorization are done to remove impurities that affect the storage stability of PUFA rich fish oils. Trace levels of transition metals may be present after refining. These metals are also part of proteins such as haemoglobin, myoglobin in crude fish oils. They may also be contaminated during processing for production equipment. The presence of small amount of iron (nanomolar concentration) is sufficient to increase the susceptibility to oxidation (Mozuraityte et al. 2016). Iron acts as a promoter for lipid oxidation in the presence of hydro peroxides. The metal ions catalyse the decomposition of hydro peroxides into alkoxyl and peroxyl radical. The resulting products initiate the chain reaction of autoxidation.

$$\mathrm{Me}^{n+} + \mathrm{ROOH} \longrightarrow \mathrm{RO}^{\bullet} + \mathrm{Me}^{n+} + \mathrm{OH}^{-}$$
$$\mathrm{Me}^{(n+1)} + \mathrm{ROOH} \longrightarrow \mathrm{ROO}^{\bullet} + \mathrm{Me}^{n+} + \mathrm{H}^{+}$$

The transition metals are also involved in breaking down unsaturated lipids directly but since it is a slow reaction, it is considered less important in promoting lipid oxidation (Reische 1998). The presence of ferric ions in crude sardine oil was found to exert strong pro-oxidant activity during storage for a period of five weeks (Charanyaa et al. 2019).

Free fatty acids (FFAs) in marine oils can have pro-oxidative effect for the oxidation of the oil. Aubourg (2001) observed the effect of free fatty acids on the oxidation of commercial cod liver oil. The effect of FFA content and type were studied. The presence of short chain-length fatty acids and longer chain-length fatty acids has a pro-oxidative influence at 30ºC. The pro-oxidant activity was increasing with increase in the degree of unsaturation in the order stearic<oleic<linoleic<linolenic acids. The enhanced oxidative effect of FFA may be due to the catalytic effect of the carbonyl groups in the decomposition of peroxides (Miyashita and Takagi 1986). The surface tension of oil reduces in the presence of FFAs and enhances the transfer of oxygen into oil leading to oxidative deterioration (Kittipongpittaya et al. 2014). Since the amount of FFAs present affects the quality and stability of fish oil, it plays a vital role in the determination of overall oil quality and price (Vaisali et al. 2015).

Photo-oxidation is triggered by the presence of photo-sensitizers including porphyrins and riboflavins. Crude fish oil may contain pigments and other photo-sensitizers like chlorophyll obtained during extraction from planktivorous fishes such as anchovies, sardines and mackerels (Indrasena and Barrow 2010). Several enzymes are also capable of causing oxidative degradation of lipids. Active catalysts are generated by enzyme systems that reduce iron in the membranes in the presence of long chain PUFAs (Erickson 1999). Lipoxygenase enzyme forms a complex with substrates that can initiate oxidation (pigments including chlorophyll and carotenoids). It can also directly act on PUFAs when the lipids are bound to membranes of lipoproteins (Min and Ahn 2005). Therefore, presence of several of these endogenous components leads to the reduced stability and quality of PUFA rich fish oils.

### 2.2.7.3 Storage Conditions

Apart from the influence of the fatty acid profile and minor components, a major impact on oxidative stability is caused by the storage conditions. Prolonged storage of fish oils exposes it to factors such as temperature, air, sunlight, and moisture which affect the quality of the oil. During processing of oil, high temperatures used may enhance lipid oxidation rate by causing breakdown of hydro peroxides into free radicals (Johnson and Decker 2015). Several studies have demonstrated that when oil is exposed to lower temperature for a long time, more secondary oxidation products are formed. But when higher temperature is used for a short period of time, the degradation is comparatively lesser. Therefore, the duration of exposure to high temperature also affects the storage stability.

Storage temperature of oil can also influence the production of lipid oxidation products. The storage stability of crude fish oils at 4ºC and -18ºC was studied by Boran and others for a period of 150 days (Boran et al. 2006). Quality parameters such as peroxide value (PV), iodine value, ester value, acid value (AV), saponification value (USM) and thiobarbituric acid (TBARS) values were analysed. Oil stored at 4ºC exceeded the PV, AV and USM acceptable limits for human consumption within 90 days while oil stored at -18ºC did not exceed the limits within 150 days of storage. Thus, by reducing the temperature of storage, the shelf life was increased by two-fold.

The presence of oxygen results in rancidity of oil and leads to the production of undesirable flavour and odours. The primary determinant of the extent of oxidation is oxygen present in bulk oil in either dissolved form or in the headspace. Although non-dissolved oxygen has low surface area, they can be transferred from the headspace reservoir into oil by diffusion or mechanical agitation. Dissolved oxygen in lipids facilitates the production of volatile oxidation products and generation of free radicals. Min and Wen analysed the formation of volatile compounds in oils in the presence of 2-8 ppm of dissolved oxygen during storage (Min and Wen 1983). The study found that rate of dissolved oxygen disappearance in soybean oil was three-fold faster in 8.5 ppm compared to the 2.5 ppm oxygen. Thus, the dissolved oxygen content should be lower for higher oxidative stability.

The formation of hydro peroxides can be controlled by removing the oxygen in headspace by purging an inert gas such as nitrogen. Masella et al. (2010) studied the effect of stripping of olive oil to remove dissolved oxygen on the stability. The removal was effective in reducing the formation of hydro peroxides when compared to the non-stripped olive oil. The effects of independent and combined factors such as light, moisture and temperature were studied in sardine oil (Charanyaa et al. 2019). Moisture in combination with light was highly detrimental to oil quality during storage for a period of five weeks. Thus, refined oil has to be stored after removal of air in dark conditions to prevent oxidative damage.

#### 2.2.7.4 Role of physical structures

The understanding of lipid oxidation has been evolving for the past century from the free radical mechanism in the 1940s to the polar paradox theory in the 1990s and then on to the physical structures such as association colloids between 2009-2015. According to the polar paradox theory, polar antioxidants work better in the bulk oil while non-polar antioxidants work better in emulsions. However, several contradictions to the polar paradox theory have been reported which led to the proposal of other theories including the cut off effect. Further in recent years, the team led by Decker and McClements have suggested that the oxidation in a bulk oil system occurs primarily at the oil-water interface, especially at the reverse micelles structures formed in the bulk oil. This led to a shift from viewing bulk oil oxidation as a simple chemical reaction to investigating the impact of multiple physicochemical and structural factors, including the relative positioning and interaction between various constituents (Wang et al. 2024).

Bulk oil contains various minor components that have both hydrophobic and hydrophilic properties, such as mono- and diacylglycerols (MAGs and DAGs), phospholipids (PLs), sterols, free fatty acids (FFA), and polar amphiphilic products resulting from lipid oxidation, such as lipid hydroperoxides, aldehydes, ketones, and epoxides (Chaiyasit et al. 2007). Due to the presence of a small amount of water in oil, these surface-active components tend to associate in the lipid medium, forming association colloids like reverse micelles and lamellar structures. Reverse micelles are characterised to be dynamic, nanoscopic, roughly spherical aggregates consisting of a hydrophilic core surrounded by a monolayer of surface-active molecules (Chaiyasit et al. 2007). The polar head groups of these molecules extend into the water core, while the aliphatic chains extend into the lipid medium. Reverse micelles act as effective nano-reactors, facilitating increased interactions between lipid- and water-soluble components, and can significantly alter oxidation reaction rates (Ghosh and Tiwary 2001). Compounds with low hydrophilic-lipophilic balances (HLB) can form reverse micelles in bulk oils. Examples include free fatty acids (HLB ≈1.0), diacylglycerols (HLB ≈1.8) and monoacylglycerols (HLB ≈3.4-3.8) (McClements 2004). The simultaneous presence of these minor components in oils including n-3 PUFA rich fish oil makes these reverse micelles compositionally and structurally complex.

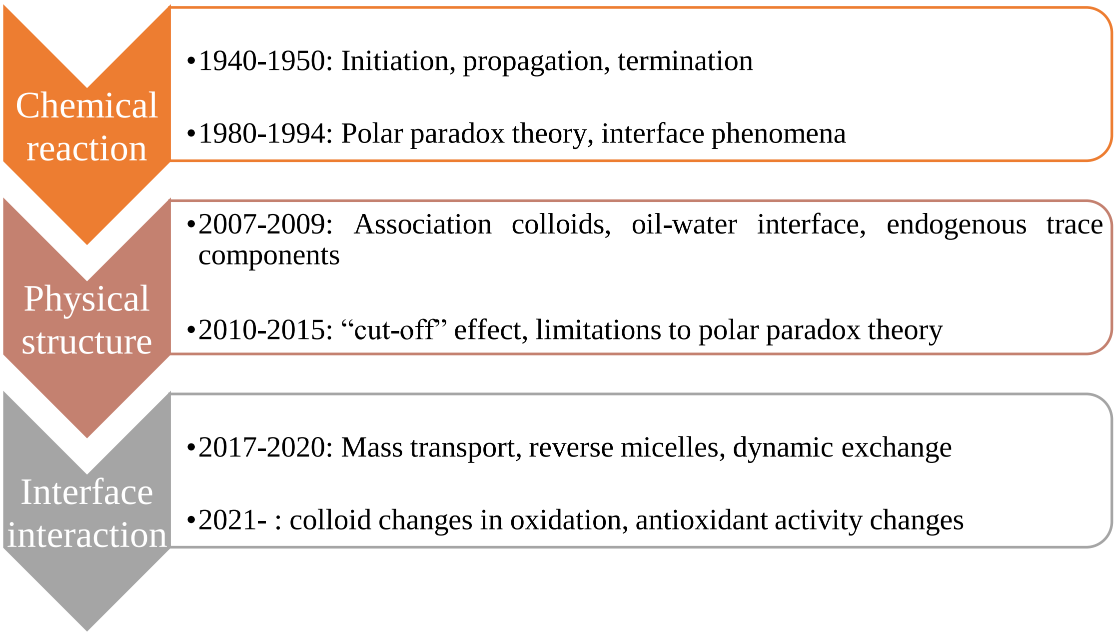


**Fig. 2.14 Evolution of theories on lipid oxidation (adapted from Wang et al. 2024)**

The formation of these association colloids in bulk oil varies depending on the composition of the surrounding oil, and its structure and composition changes according to the oxidative state of the oil. The amphiphilic minor components are not removed completely during refining, and are capable of assembling into nanometric systems in non-polar media at concentrations above their critical micelle concentration (CMC). Several researchers have studied the impact of these minor amphiphilic components on oxidation in bulk and emulsified oil systems (Bąkowska et al. 2022; Chen et al. 2011; Cui et al. 2014; Yoo et al. 2024). Most of the papers have reported that phospholipids have greater influence on the formation of association colloids than the presence of FFAs, DAGs and sterols. When FFAs alone were present, there was no formation of reverse micelles even in concentrations of up to 5 wt% of bulk oil (Kittipongpittaya et al. 2014). Additionally, the CMC of phospholipids (lecithin) for the formation of reverse micelles varied in the presence of other components including oleic acid, since it can act as a cosurfactant and can either increase or decrease the CMC. Higher concentrations of oleic acid can bring a change in the attractive/repulsive forces among the phospholipid molecules leading to an increase in CMC (Wang et al. 2024). Since oleic acid can act as cosurfactant, it can expand the core of the reverse micelles resulting in increased accommodation of water molecules. It is evident that the stability and characteristics of reverse micelles are greatly influenced by the microenvironment surrounding them.

The presence of phospholipids and water are the primary reactants for the formation of association colloids. Water concentration in oil varies during prolonged storage since it can either be absorbed from the environment or lost from the oil, and it is usually found in the form of association colloids as they are insoluble in oil (Chaiyasit et al. 2007). These association colloids are further stabilised by the presence of surface-active agents in the bulk oils. Additionally, water is released as a by-product during oxidation reactions involving hydroperoxide decomposition (Budilarto and Kamal-Eldin 2015). In the absence of adequate amount of water, association colloids are not formed even in the presence of phospholipids. For example, stigmasterol did not form reverse micelles with 400ppm of water but it did form association colloids in the presence of 1000 ppm of water which is above its CMC (Bąkowska et al. 2022; Kittipongpittaya et al. 2016). Additionally, the CMC of phospholipid-based reverse micelles tends to rise as the water content increases, as noted by Wang et al. in 2024. This phenomenon can be attributed to the impact of water content on the size and quantity of associated colloids formed, as well as the activity of reactants at the water-oil interface and within the reverse micelle core (Chaiyasit et al. 2007). It has been observed that the small amount of water present in reverse micelles is highly immobilized in their interior core, and its physicochemical properties differ from those of bulk water (Chowdhary and Ladanyi 2009). As the percentage of water content in the system increases, the mobility of water within the hydrophobic core also increases, eventually reaching the mobility of bulk water (Mora et al. 2021). However, due to the intricate composition of bulk oil, the behavior of water molecules in association colloids in edible oils has not yet been completely presented in the literature.

During the various phases of oxidation, the colloidal structures could differ in structure and number owing to the free radical chain reaction leading to the release of oxidation products which are amphiphilic. At the end of the initiation phase, due to the release of water and LOOH, there was an increase in the size of the reverse micelles owing to the migration of water into the hydrophilic core (Keramat et al. 2021; Toorani et al. 2020). The hydroperoxides formed can migrate to the water-oil interface and react with water soluble metal ions (Chaiyasit et al. 2007; Chen et al. 2012). Furthermore, the presence of antioxidants having surface activity can alter the composition and organisation of these reverse micelles. This further affects the efficiency of antioxidants added to combat lipid oxidation in such cases.

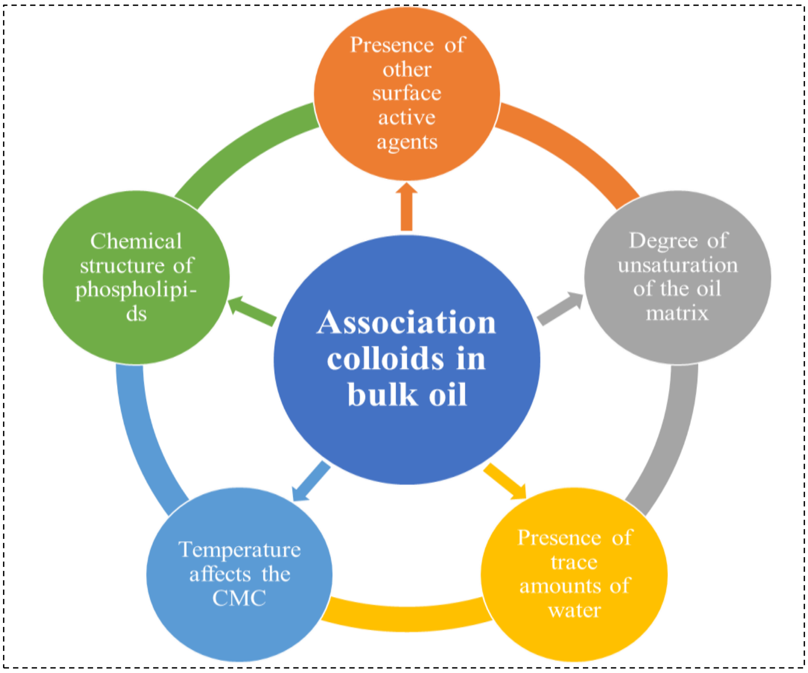


**Fig. 2.15 Factors affecting association colloids in bulk oil (adapted from Wang et al. 2024)**

In food systems, the efficiency of antioxidants depends not only on their chemical reactivity but also on various physicochemical phenomena such as interfacial processes, relative localizations/orientations, and interactions between the participating components. Hence, an antioxidant could exert pro-oxidative (Velasco et al. 2023) or anti-oxidative (Chen et al. 2011) effects depending on its localization in the different interfaces of bulk oil containing physical structures. Antioxidants capable of accumulating at the site of oxidation at the interfaces might be more efficient than other antioxidants. Due to the formation of reverse micelles, the number of microreactors of oxidation increases which could bring the antioxidants in close proximity to these microreactors of oxidation (Wang et al. 2024). Measurement of surface tension and interfacial tension has been done to comprehend the accumulation of antioxidants either at the oil-air interface or oil-water interface respectively. Chaiyasit and others have reported that the surface tension does not change in the presence of various antioxidants in a hexadecane model system (Chaiyasit et al. 2007). However, the interfacial tension decreased in the presence of increasing concentrations of antioxidants, indicating their accumulation at the water-oil interface rather than the oil-air interface.

Other than the two primary reactants, factors such as temperature, the composition of phospholipid present, and degree of unsaturation adversely affect the formation and stabilization of these physical structures in bulk oils. For example, CMC of phospholipids increases with the increase in unsaturation but decreases with increase in temperature (Wang et al. 2024). Hence, it is difficult to generalize the understanding of a complex microenvironment since several factors are involved. Therefore, further studies are necessary for various edible oils to expand our knowledge of these association colloids and their impact on oxidation in a bulk oil system.

### 2.2.8 Products of fish oil oxidation

Flavor of food is one of the important sensory attributes that affect consumer behavior. The desirable flavor may be lost and secondary undesirable flavors might develop during processing and storage. This often happens with foods containing lipids, leading to loss of sensory properties (Eriksson 1978). Unsaturated FAs undergo oxidation, and hydroperoxides are formed. These hydroperoxides are odorless initially. However, they are highly unstable and break down into volatile products such as aldehydes, ketones, alcohols, and hydrocarbons (Grosch 1982). The degradation products cause a major impact on the sensory properties of fat-containing foods even in low concentrations. This is due to the lower odor threshold of several oxidation products in oil, which is as low as 0.0015 μg/L and 0.0001μg/L for aliphatic aldehydes 2,6-trans, cis-nonadienal and 1-octene-3-one respectively.

Since PUFAs are the primary site of oxidation, there may be deterioration of essential n-3, n-6 FAs, and nutritional value of fish oils. Several researchers have studied the effect of intake of lipid oxidation products in animal and human clinical studies. Supplementation with fish oils rich in PUFAs was provided, and its effect on oxidative stress was analyzed. No significant impact on oxidative stress based on the measurement of oxidation markers in blood and urine was reported in this study (Ottestad et al. 2012). Studies on the the use of oxidized PUFAs are still emerging, and there is no conclusive evidence pointing toward adverse health effects. The Global Organisation for EPA and DHA omega-3s (GOED), Food and Agriculture Organization of the United Nations: World Health Organization (FAO/WHO) has done series of tests to monitor the quality of PUFA rich oils. Acceptable levels of quality parameters such as peroxide value, *p*-anisidine value, and TOTOX values were established.

**Table 2.11 Standard for fish oils intended for human consumption (Codex Alimentarius Commission, 2017)**

| Parameters | Range |
|---|---|
| Acid value | ≤ 3 mg KOH/g |
| Peroxide value (PV) | ≤ 5 milli equivalent of active oxygen/kg oil |
| *p*-anisidine value (PAV) | ≤ 20 |
| Total oxidation value (TOTOX) | ≤ 26 |

### 2.2.9 Methods to measure oxidation of fish oil

The lipid oxidation products are analysed to measure the level of oxidation of oil. This includes primary oxidation products such as changes in fatty acids, formation of lipid hydro peroxides, conjugated dienes/trienes and secondary oxidation products such as carbonyl, aldehydes, volatiles and malondialdehyde (Ross and Smith 2006). Peroxide value (PV) analyses the amount of primary oxidation product peroxide present in oil. It is a robust method and is used in wide variety of oils. Peroxides are the initial product formed during oxidation. They are highly unstable and break down into several oxidation products. Therefore, its concentration decreases with time and a low value of PV does not necessarily mean that the oil is of good quality (Shahidi and Wanasundara 2002).

Peroxide value may thus indicate early or advanced stage of oxidation and the results have to be correlated with other methods for quantification including conjugated diene (CD) value. In CD value determination, the conjugates are initially extracted with a small amount of organic solvent and measured in spectrophotometer (Shahidi and Wanasundara 2002). The values of peroxides and conjugated dienes may provide information about oxidation in early phases. Since the primary oxidation products decompose rapidly, it is essential to measure secondary oxidation products. This is done through *p*-anisidine value (*p*-AV) which is a colorimetric method that measures the presence of 2-alkenals and 2,4 alkadienals (Kiokias et al. 2008). The reaction between *p*-anisidine amine group and the aldehyde carbonyl group produces a Schiff base and is measured at 350 nm. The *p*-AV shows good correlation with other indicators such as PV, TBARs etc.

Other methods, such as thiobarbituric acid reactive substances (TBARs) that measure the level of malondialdehyde (MDA), which is a decomposition product of lipid peroxides, are also available. MDA has a low odour threshold of about 2-2.5 mg MDA/kg and is considered as a marker for oxidation (Jones and Roper 2017). To obtain reliable information on the state of oxidation of oil, a combination of primary and secondary lipid oxidation measurement is performed. TOTOX value is the expression of total oxidation based on the values of *p*-anisidine and peroxide values. In addition to these methods, chromatographic methods such as high-performance liquid and gas chromatography combined with mass spectrometry are also utilized for the quantification of oxidation products.

### 2.2.10 Methods to control oxidation

Fish oils rich in long-chain PUFAs have been used in the pharmaceutical and food industries because of their cardioprotective, anti-inflammatory, and anti-cancer properties (Shahidi and Miraliakbari 2005). However, due to oxidation, qualitative and sensory properties are affected. Better handling and improved storage conditions may help in preventing exposure to extreme environmental factors. Oxidation rate increases with increase in temperature. A 10ºC rise in the temperature causes a 50% decrease in the shelf life i.e. doubles the oxidation rate (Jacobsen et al. 2010). Storage of fish oils should be done at low temperature to slow the oxidation process. Dark conditions are to be maintained to limit the photo-oxidation reaction triggered by photosensitizers.

The major reactant for oxidation is the presence of dissolved oxygen. To reduce the dissolved oxygen concentration, purging with inert gas such as nitrogen is the commonly used method. In a study by Sionek et al. (2013), nitrogen purging was used to remove oxygen in refined rapeseed oil. This removal of oxygen in headspace enhanced the oxidative stability of the oil by reducing the formation of hydro peroxides by 22%. To displace oxygen, the oil storage containers can also be blanketed with inert gas such as nitrogen. Soft gelatin encapsulation is used to protect the marine oils from exposure to oxygen, light and humidity. Encapsulation technique involves the packing of the oil into miniature sealed capsules which protects the core for harsh conditions. However, the physical properties of the packing material along with storage conditions may influence the oxidative stability of oil.

The commonly used method used for delaying oxidation is the addition of antioxidants. The use of antioxidants has been found to be effective, convenient and economical when compared to other techniques (Shahidi and Zhong 2010). An antioxidant can be defined as a compound that significantly delays or inhibits the oxidation of oxidizable substrate when present in comparatively low concentrations (Halliwell et al. 1997). Antioxidants can increase the shelf life of food products by retarding lipid peroxidation through different mechanisms. This may include scavenging of free radicals, chelation of metal ions, quenching singlet oxygen to prevent the formation of peroxides, breaking the autoxidative chain reaction, and reducing localized oxygen concentration (Nawar 1996). The chemical potency of an antioxidant and its solubility determine its efficiency in oil systems. Antioxidants are used as food additives because of their ability to prolong shelf life of food products without adverse changes in the nutritional and sensory aspects.

# CHAPTER 3

## SYNTHESIS AND CHARACTERIZATION OF 3,4-DIHYDROXYPHENYLACETIC ACID ESTERS

# CHAPTER 3

3,4-dihydroxyphenylacetic acid (3,4-DHPA) is a phenolic antioxidant with excellent antioxidative capacity in aqueous media. However, the hydrophilic nature of 3,4-DHPA might result in solubility issues in non-polar systems, limiting its usage in the majority of food products. To improve the hydrophobicity of 3,4-DHPA, esterification with fatty alcohols using different catalysts was attempted. Lipases (triacylglycerol ester hydrolases E.C.3.1.1.3) are used in various pharmaceutical, food, and detergency reactions (Hasan et al. 2006). These enzymes are capable of catalyzing the esterification and transesterification reactions between carboxylic acid and alcohols, resulting in the formation of esters. Compared to conventional chemical catalysts, enzyme-based catalysis is characterized by improved selectivity, substrate specificity, and mild reaction conditions (Villeneuve 2007).

## 3.1 Enzymatic synthesis of esters

The first section of this chapter presents an investigation into the enzymatic esterification of 3,4-DHPA using a range of acyl donors with differing chain lengths. It is widely accepted that an increase in the chain length of the phenolic acid can enhance its lipophilicity. However, when the chain length surpasses a critical limit, the heightened lipophilicity may impact its location in the oxidation sites due to steric hindrance (Cruz Figueroa-Espinoza et al. 2013). This “cut-off” phenomenon has been observed with various phenolic esters in different lipid media, and the critical chain length varies depending on the system studied. Therefore, it is imperative to maintain an optimal chain length to preserve the antioxidative activity of 3,4-DHPA in fish oil. Additionally, the lipophilisation process using lipase is influenced by several factors, including the nature of the solvent employed, water activity, the nature of the substrate, and the reaction time. It is crucial to consider these factors to ensure the successful implementation of the lipophilisation process using lipase. The current study aims to optimize the enzymatic esterification method for the synthesis of 3,4-DHPA lipophilic esters by considering all these relevant parameters.

## 3.2 Chemical synthesis of esters

In the second section of this chapter, the chemical catalyst-based approach of esterification was explored to achieve higher ester yields. Chemical catalysts are characterized by high yields, wide applicability, and better compatibility with other functional groups. The chemical method of esterification offers a significant advantage in that the catalysts utilized are not impeded by the release of water, which is formed as a by-product during the esterification process. As such, there is no requirement to remove the water through molecular sieves, which can further enhance the yield of the ester by promoting the esterification reaction over hydrolysis.

The optimization of the reaction parameters for efficient conversion of 3,4-DHPA to its lipophilic esters using homogeneous and heterogeneous chemical catalysts in suitable reaction media was explored. *p*-toluene sulfonic (PTSA) acid was utilized as homogeneous catalysts in solvent-free and solvent-based conditions. PTSA has been successfully applied for esterification of several carboxylic acids with alcohols of varying chain lengths (Khudsange and Wasewar 2018; Kopchev and Bayryamov 2022; Wang et al. 2013; Wei and Jingnan 2018). Compared to other homogeneous catalysts, PTSA is highly soluble in hydrophobic solvents, cost-effective, and easily available. Hence, it was chosen for the esterification of 3,4-DHPA, and its further optimization was done for parameters such as molar ratio, reaction time, and catalyst loading.

However, using a homogeneous catalyst might pose difficulties in catalyst removal and require tedious purification steps to obtain esters of high purity. To improve these aspects, the use of a heterogeneous catalyst such as Amberlyst-15 was proposed. Cation exchange resins, including Amberlyst-15, are environmentally friendly and thermally stable, making esterification a green process (Liu et al. 2013b). Several ion exchange resins were successfully applied in solvent-free conditions for the synthesis of chlorogenic acid ester of octanol with high efficiency and purity (Pappalardo et al. 2023). Hence, these types of catalysts can efficiently lipophilise 3,4-DHPA, and the purification steps are also minimal.

### 3.3 Characterization of esters of 3,4-DHPA

The synthesized esters, after partial purification, were subjected to structural characterization using MS and NMR analysis. It is essential to analyze the purity of the lipophilic esters when they are intended to be used in storage stability studies. These analytical techniques help identify the structure of the synthesized ester and the location at which the acyl donor has been attached. Mass Spectrometry (MS) analysis is instrumental in estimating the molecular weight of various esters and the presence of other minor impurities and reaction intermediates could also be determined. The $^{1}H$ and $^{13}C$ NMR studies offer valuable insights into the arrangement of hydrogen atoms in esters, as well as information about the structure of the carbon backbone. This helps in determining the complete structure of lipophilized esters, which is crucial for ensuring their purity and composition. Based on the elucidated structure, the log P values, a measure of the lipophilicity of the esters, were also calculated using the Molinspiration Cheminformatics Tool.

Further, the antioxidative potential of the synthesized esters was determined using various *in vitro* assays. Antioxidants employ diverse mechanisms to retard oxidation in lipid systems. These *in vitro* assays measure the ability of the antioxidants to scavenge free radicals, chelate metal ions, and quench singlet oxygen. Among these mechanisms, free radical scavenging ability is commonly analyzed for phenolic acids, including 3,4-DHPA. Phenolic hydroxyl groups are majorly responsible for the radical scavenging ability wherein they donate hydrogen atoms to the lipid free radicals, resulting in the formation of antioxidant radicals, which are highly stable compared to free radicals. Since the phenolic acid has been lipophilized using acyl donors of different chain lengths, the study of its antioxidative activity is important to estimate its influence on the hydrogen-donating ability of the resulting ester. Though a number of *in vitro* studies based on various principles are available, three assays, including DPPH and ABTS radical scavenging activity and ferric-reducing power assays, were utilized in the current study since these are rapid, sensitive, and widely recognized.

## 3.4 MATERIALS AND METHODS

### 3.4.1 Materials

3,4-dihydroxyphenylacetic acid (>98.0%, GC) was procured from Tokyo Chemical Industry Ltd., India. Lipase from *Candida antarctica* and *Thermomyces lanuginosus* immobilized on Immobead-150 having an activity of 2000 U/g and 3000 U/g, respectively, and Lipase acrylic resin (≥5000 U/g, recombinant, expressed in *Aspergillus niger*) were from Sigma Aldrich, India. *p*-toluenesulfonic acid (PTSA), sodium acetate, ferric chloride, potassium persulfate, and glacial acetic acid were used in analytical grade and were from Loba Chemie, India. Amberlyst-15 dry ($H^+$ form, with sulfonic groups) was of industrial grade, strongly acidic catalyst manufactured by S.D. Fine-Chem Ltd. India. Methanol, ethanol, propanol, butanol, pentanol, hexanol, octanol, decanol, dodecanol, tert-butanol, hexane, cyclohexane, 2-butanone, and tert-amyl alcohol were of analytical grade and were procured from Merck, India. Molecular sieves of size 3Å were obtained from Molychem, India. 2,2-diphenyl-1-picrylhydrazyl (DPPH, 95% pure) and BHT (99% pure) were obtained from Sisco Research Laboratories, India. TBHQ, TPTZ (2,4,6-tripyridyl-s-triazine), and ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic) acid) were of analytical grade and were procured from Merck, India. Solvents used for HPLC were of HPLC grade and obtained from Merck, India.

### 3.4.2 Enzymatic esterification reactions

#### 3.4.2.1 Drying of reagents

All reagents used in enzymatic esterification should be dried using molecular sieves. Esterification catalyzed by lipase is a reversible reaction wherein acid and alcohol react to form an ester in the absence of water. When water is present in excess, the reverse reaction is favored, which leads to the hydrolysis of the ester into acid and alcohol. Thus, the solvents and alcohol donors used in the reaction, except for the enzyme lipase, were dried using 150 mg/mL of 3Å molecular sieves (heat activated) before addition.

### 3.4.2.2 Synthesis of esters using solvent-free method

Solvent-free esterification reactions were initially tried using a range of alcohols wherein the acyl donor itself will act as the solvent. The method described by Guyot et al. (1997) was followed with few modifications. 100 mg of 3,4-DHPA was dissolved in 7.5 mL of various alcohols, including butanol, hexanol, octanol, and decanol. To this mixture, 2.5% (w/w) of immobilized lipase enzyme from *Candida antarctica* (CALB) and *Thermomyces lanuginosus* (TLL) was added. To remove the water produced during the reaction, molecular sieves (150 mg/mL) are added at different intervals. The reaction mixture was incubated at 55°C in orbital shakers at 150 rpm until the formation of esters. Samples were withdrawn every 24 hours and were analyzed in HPLC to find the conversion of the phenolic acid to ester.

### 3.4.2.3 Synthesis of esters using solvent-based method

For the synthesis of esters of 3,4-DHPA, a single solvent system comprising solvents such as tert-butanol, tert-amyl alcohol, n-hexane, and cyclohexane was employed according to the method described by Vaisali et al. (2017). The molar ratio of phenolic acid to alcohol was kept at 1:4 with a total reaction volume of 7.5 mL. Immobilized lipase of varying concentrations (CALB and TLL) was added to the reaction mixture to initiate esterification. The vials were incubated at 55°C in orbital shakers at 150 rpm until the esters were formed. Periodically, samples were analyzed using HPLC to confirm the formation of esters.

### 3.4.2.4 Synthesis of esters using binary solvent-based method

Binary solvent systems made of n-hexane/tert-butanol and n-hexane/2-butanone were tried with methanol, ethanol, propanol, butanol, and hexanol as acyl donors. The binary solvents were taken in a proportion of 75:25 and 85:15 for esterification reactions. The phenolic acid: acyl donor ratio was varied from 1:8 to 1:100 with 2.5-5% (w/w) of immobilized lipase CALB. The reaction was incubated at 55°C in orbital shakers at 150 rpm until the expected esters were formed. The conversion of phenolic acid to ester was monitored using HPLC every 24 hours.

### 3.4.3 Chemical esterification reactions

#### 3.4.3.1. Synthesis of ester using homogeneous catalyst

The synthesis of phenolic ester was carried out using the chemical method in shake flasks, according to Wei and Jingnan (2018), with few modifications. In the chemical method, *p*-toluenesulfonic acid (PTSA) was used as the homogeneous catalyst at 1 mol %, 1:30 molar ratio of 3,4-DHPA to alcohol in solvent-free conditions. The flasks were kept at 150 rpm at 60ºC in an orbital shaker for ester synthesis. The reactions were monitored by HPLC and the percent conversion of ester was calculated. Based on the initial studies, the synthesis parameters, such as the molar ratio of the antioxidant to the fatty alcohol group and the percentage of catalyst loading, were optimized to achieve the maximum yield of the ester.

After the esterification process, the reaction mixture was washed three times consecutively with equal volumes of water. Centrifugation was done at 7000 rpm for 10 minutes, and the top phase was removed. The subsequent water phases were pooled, and liquid-liquid extraction using ethyl acetate was performed in a 2:1 (v/v) ratio. The residual catalyst was removed by washing it with saturated sodium carbonate and brine solution, and, consequently, drying it using $MgSO_4$. Ethyl acetate was then removed by evaporation, and the ester was stored. The ester was then analyzed in HPLC, MS, and NMR for purity.

#### 3.4.3.2 Synthesis of esters using heterogeneous catalyst

For the esterification of 3,4-DHPA with methanol, a binary solvent system consisting of n-hexane and 2-butanone in the ratio 75:25 was utilized according to the method described by Sabally et al. (2005). 3,4-DHPA to methanol was used in the molar ratio of 1:60 in the presence of 15% (w/w) of Amberlyst-15 as the catalyst. The flasks were shaken at 150 rpm for 48 hours at 55°C. The reaction was monitored using HPLC, and the methyl ester that was obtained was then used for the *trans*-esterification reaction. The excess reaction solvents were evaporated at 60°C in a rotary evaporator, and the methyl ester was obtained as a semi-solid product. Methyl ester was then reacted with butanol and hexanol through a transesterification reaction using similar solvent conditions to that of esterification in a 1:1 molar ratio of methyl ester to alcohol. The butyl and hexyl esters thus obtained were partially purified and were stored for further use.

### 3.4.4 Characterization of 3,4-DHPA esters

#### 3.4.4.1 Analysis of esters using MS and NMR

The molecular characterization of the produced ester was done using Mass and NMR spectrometry. The esters were analyzed in MS using Xevo QToF, Waters, USA system in the negative mode of ionization. Samples were prepared in 1 mg/mL concentration in MS grade methanol and filtered through a 0.22 µm filter prior to analysis. The esters were analyzed up to the m/z range of 1200. $^1$H NMR and $^{13}$C NMR spectra were obtained on a Bruker Avance Neo 400 MHz NMR spectrometer (Bruker, Switzerland) using DMSO as the solvent for all esters.

#### 3.4.4.2 Calculation of miLog P values

The effectiveness of an antioxidant in a given media depends on its distribution and where it can exert its activity. Esters are amphiphilic molecules that have different polarities, and hence, their distribution varies based on the acyl chain length. Log P values are indicators of the solubility of a compound in hydrophobic media. Hence, the lipophilicity of the esters was calculated as miLog P values using the molecular properties calculator available from Molinspiration Cheminformatics.

#### 3.4.4.3 DPPH radical scavenging activity

The radical scavenging activity of 3,4-DHPA and the esters was evaluated by the reaction with a stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). The method described by Maqsood and Benjakul (2010) was followed with a few modifications. 2 mL of 0.1 mM DPPH was added to 2 mL of 3,4-DHPA and its esters in increasing concentration in methanol. The solution was mixed well and incubated in the dark for 30 minutes. Absorbance was measured at 517 nm in a UV-visible spectrophotometer. DPPH solution without the antioxidant was used as the control. Radical scavenging activity was calculated as %DPPH scavenging activity = $[(A_{control} - A_{test})/A_{control}] * 100$.

### 3.4.4.4 Ferric Reducing Antioxidant Power (FRAP)

The ability of antioxidants to reduce ferric to ferrous form was measured by the reaction with TPTZ based on the method described by Benzie and Strain (1996) with few modifications. 0.3 M acetate buffer, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solutions were prepared. A working solution with 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3$ solution was prepared fresh before use. The working solution was incubated at 37ºC for 30 minutes in a water bath. 3.5 mL of this FRAP reagent was added to 0.5 mL of 3,4-DHPA and the esters in different concentrations. The solution was mixed well and incubated in the dark for 20 minutes. Absorbance was measured at 593 nm in a UV-visible spectrophotometer. The increase in the absorbance value corresponds to the increase in ferric-reducing antioxidant power.

### 3.4.4.5 ABTS radical scavenging activity

The radical scavenging ability of 3,4-DHPA esters was evaluated using ABTS free radicals using a spectrophotometric method described by Maqsood and Benjakul (2010). 7.4 mM ABTS and 2.6 mM potassium persulphate solutions were mixed in equal quantities and allowed to react for 12-16 hours at room temperature in the dark for the synthesis of the free radical. Then, it was diluted with 50 mL methanol to obtain a working solution with an absorbance of 0.7 ± 0.02 units at 734 nm. 150 µL of 3,4-DHPA and lipophilic esters at different concentrations was reacted with 2850 µL of the ABTS working solution at room temperature in the dark. The absorbance was measured at 734 nm after 7 mins of incubation, and the percentage radical scavenging activity was calculated according to the formula %ABTS scavenging activity = $[(A_{control} - A_{test})/A_{control}] * 100$ where $A_{control}$ was the absorbance of the ABTS radical in methanol without the antioxidant.

### 3.4.5 Analytical Methods

The chromatography analysis was done using an HPLC unit (Prominence Modular HPLC, Shimadzu Analytical, India) with RP-C18 column of dimensions 4.6x250 mm with 5 µm particle size. The method described by Martínez-Hu´elamo et al. was followed with some modifications (Martínez-Hu´elamo et al. 2015). The mobile phases used were water (A) and acetonitrile (B) containing 0.1% formic acid in the gradient system as follows: 0 min (95% A, 5% B), 2 min (75% A, 25% B), 2.5 min (10% A, 90% B), 2.65–2.8 min (0% A, 100% B), 2.9 min (95% A, 5% B). 10 μL of the reaction mixture was withdrawn periodically and the volume was made up to 1 mL with HPLC grade methanol. The sample was then filtered using a 0.2 μm syringe filter prior to injection. The flow rate used was 1 mL/min, and the sample volume was 25 μL. Quantification was done at 238 nm. Percentage conversion is calculated as the area of the ester peak divided by the combined area of the ester peak and the antioxidant peak multiplied by 100.

### 3.4.6 Statistical analysis

All *in vitro* studies were done in triplicates and were reported as mean values with standard deviation. The obtained data mean was compared through analysis of variance (ANOVA) using OriginPro, OriginLab Corporation, Northampton, MA, USA, and significance was determined for $p < 0.05$.

## 3.5 RESULTS AND DISCUSSION

The hydrophobic alcoholic group was attached to the hydrophilic phenolic acid, 3,4-dihydroxyphenylacetic acid, through enzymatic and chemical esterification techniques. The esterification was carried out in varying conditions, and the samples were analyzed periodically in HPLC for the quantification of the ester produced. Several parameters influence the esterification reaction of phenolic acid with alcohols, and hence, all of them were varied to obtain the desired product. The parameters include the molar ratio of the phenolic acid to the alcohol, type of catalyst and catalyst loading, presence of molecular sieves, presence of organic solvents, and time of reaction.

### 3.5.1 Enzymatic esterification reactions

#### 3.5.1.1 Synthesis of esters using solvent-free method

Phenolic hydroxyl groups are associated with their ability to scavenge free radicals and, hence, are important in several food and pharmaceutical products. In a given system, the efficiency of phenolic acids as antioxidants is dependent on their heat stability, structure, pH sensitivity, and volatility (Figueroa Espinoza and Villeneuve 2005).

Moreover, the solubility of the antioxidant with respect to the site of oxidation is of utmost importance. Hence, the synthesis of such amphiphilic molecules that retain their original antioxidative capacity has been attempted widely in recent years (Pappalardo et al. 2023; Vaisali et al. 2017; Yang et al. 2012). Although several phenolic acids are esterified, 3,4-DHPA has not yet been lipophilized with multiple acyl donors of varying chain lengths.

For enzymatic esterification, lipase in immobilized form was added to catalyze the formation of phenolic ester. Lipases from different sources, such as *Candida antarctica* (CALB) and *Thermomyces lanuginosus* (TLL), immobilized on Immobead-150, were employed in the reaction mixture at 2.5% (w/w). CALB lipase is the most stable commercially available enzyme, and it is widely applied to various reactions. Immobilization of lipase is highly preferred since it improves reusability and is cost-effective. Further, the immobilization reduces the sensitivity of the enzyme to harsh reaction conditions (Ortiz et al. 2019). Lipases are highly specific in nature, and hence, lipases from two different sources were considered for esterification (Table 3.1).

**Table 3.1 Enzymatic esterification trials carried out under varying reaction conditions and respective yields**

| Phenolic acid | Alcohol | Solvents | Reaction condition | Yield of ester |
|---|---|---|---|---|
| 3,4-DHPA (200 mg) | Decanol, dodecanol | -- | 15 mL reaction mixture with CALB and TLL in 2.5% w/w with and without molecular sieves addition | Maximum of 4.2% (decyl ester) after 120 hours with CALB |
| 3,4-DHPA | Decanol | Tert-butanol | 1:4 molar ratio with 75 mg of CALB and TLL | No detectable yield |
| 3,4-DHPA 100 mg) | Butanol | -- | 7.5 mL reaction mixture with 2.5% w/w CALB, TLL with | No detectable yield |

|  |  |  |  |  |
|---|---|---|---|---|
|  |  |  | and without molecular sieves addition |  |
| 3,4-DHPA (200 mg) | Octanol | -- | 15 mL reaction mixture with CALB and TLL in 2.5% w/w with 300 mg of molecular sieves added after 72 hours | After 16 days, 39-43% conversion was observed with CALB and 9-20% conversion after 16 days with TLL but the yield was not repeatable and consistent |
| 3,4-DHPA | Octanol | Tert-butanol, tert-amyl alcohol | 1:4 molar ratio with 2.5% w/w for a final reaction volume of 15 mL | CALB showed 4-10% conversion after 5 days. Tert-butanol faced severe evaporation |
| 3,4-DHPA (100 mg) | Hexanol | -- | 7.5 mL reaction volume with 2.5% w/w CALB, TLL | No detectable yield |
| 3,4-DHPA | Octanol | Tert-butanol | 1:1, 1:2, 1:4, 1:6, 1:8 and 1:10 molar ratios with 5% w/w CALB, TLL with no molecular sieves addition | 1:8 and 1:10 ratios showed increasing conversion after 7-11 days. Ester was not stable. |

| | | | | |
|---|---|---|---|---|
| 3,4-DHPA | Octanol | -- | 1:50 and 1:100 molar ratios with 5% w/w CALB, TLL | Around 80% conversion with CALB but not consistent and stable |
| 3,4-DHPA | Hexanol | Tert-amyl alcohol | 1:4 molar ratio with 2.5% w/w CALB, TLL | After day 4, an ester peak with 51% conversion was found, and further on days 5, 6, and 7, 49, 56 and 59% conversion were observed with CALB |
| 3,4-DHPA | Hexanol | n-hexane, cyclohexane | 1:4 molar ratio with 2.5% w/w CALB, TLL | No detectable yields and severe evaporative loss |
| 3,4-DHPA | Hexanol | n-hexane:2-butanone | 1:4 molar ratio with 2.5% w/w CALB, TLL in binary solvent ratio of 85:15 and 75:25 | After 48 hours, 41% conversion was observed with 75:25 while 85:15 had evaporative loss |
| 3,4-DHPA | Methanol | n-hexane:2-butanone | 1:4 molar ratio with 2.5% w/w CALB, in the binary solvent ratio of 75:25 | Around 60% conversion after 24 hours followed by a maximum yield of 73% after 3 days of reaction |

Initially, solvent-free conditions were utilized for the esterification with acyl donors such as butanol, hexanol, octanol, decanol, and dodecanol using the method described by Guyot et al. (1997). The lipase-catalyzed reaction between carboxylic acid and alcohol is reversible, and the equilibrium is shifted towards esterification in the presence of an excess of one of the two reactants. In this case, the acyl donor was used in excess, which also acts as the solvent for the reaction. The reaction was continued for a period of 10-16 days at 55°C. To remove water produced during esterification, molecular sieves of size 3Å (150 mg/mL) were added. When butanol was used as an acyl donor, no ester peak was observed in the HPLC analysis of periodical samples. The reaction was extended until 16 days, but esterification was not successful with either CALB or TLL enzymes. Esterification of n-butanol with caffeic acid under similar conditions also did not yield any ester, as observed by Guyot et al. (1997). The antioxidants, 3,4-DHPA, and caffeic acid share a similar structure, with the only discernible difference in the unsaturated side chain of caffeic acid. It has been suggested that this unsaturated side chain is the reason for the enzyme inhibition during esterification. However, despite not having a directly conjugated, unsaturated carboxylic acid chain, 3,4-DHPA still hinders the enzymatic esterification process.

With octanol as the reactant with 3,4-DHPA, only a 39-43% conversion was observed after 16 days with the CALB enzyme, while the TLL enzyme showed only 9-20% conversion. Pentanol ester was found with 84% conversion after 3 days of reaction in the presence of molecular sieves. Hexanol showed a 55% conversion on day 4, but further extension of the reaction reduced the yield of the ester due to the shift in reaction to hydrolysis. Decanol resulted in only 4.2% conversion after 5 days of reaction. In all solvent-free reactions, the yield was inconsistent and required very long reaction times. When the same reaction conditions were repeated, the same yield was not observed with any of the acyl donors. Though excess alcohol is required to shift the reaction to esterification, it can cause enzyme deactivation beyond a certain concentration of the anhydrous alcohol (Guldhe et al. 2015). An increase in alcohol concentration tends to block the nucleophilic site of the enzyme, which is involved in the acylation reaction, leading to reduced enzyme reactivity (Khan et al. 2021). Also, the presence of alcohol enhances the contact between lipase and alcohol, causing water layer displacement, which ultimately changes the native conformation of the enzyme (Staudt et al. 2021). The reactivity and the inhibitory effect are highly pronounced with primary alcohols rather than secondary and tertiary alcohols due to the small size of primary alcohol moieties. Secondary and tertiary alcohols are bulkier and have higher steric hindrance, which lowers the inhibitory effect on the enzyme (Abdul Rahman et al. 2011). All the acyl donors used in the study are primary alcohols, which may explain the reduced ester yields under solvent-free conditions.

#### 3.5.1.2 Synthesis of esters using solvent-based method

A solvent-based system was then employed using tert-butanol and tert-amyl alcohol based on the studies by Vaisali et al. (2017). The phenolic acid and acyl donors were dissolved in a ratio of 1:4, and the reaction volume was made to 5 mL. The solvents were added to enhance the solubility of the reactants, which will, in turn, provide sufficient interaction between the reactants and the catalytic enzyme to improve esterification. It influences the medium hydrophobicity and, hence, might affect the catalysis of the reaction. The solvent utilized should be able to dissolve both the hydrophilic phenolic acid and the relatively hydrophobic acyl donors without affecting the activity of the lipase enzyme. Molecular sieves were also added in 150 mg/mL concentration to remove the water released during the reaction.

The molar ratio of the phenolic acid to the acyl donor was then increased to improve the esterification. It was varied from 1:2 to 1:10 in a tert-butanol-based system of 5 mL volume in the presence of lipases (CALB and TLL) for octanol. Increasing the molar ratio (1:8 and 1:10) brought about a slight increase in the conversion using CALB immobilized lipase, but the product was not stable. Since esterification is a reversible reaction, the product formed might trigger the hydrolysis of the same in the presence of water. In the course of the esterification reaction, the by-product released is water, which may shift the equilibrium towards hydrolysis, meaning that the ester is being hydrolyzed to produce acid and alcohol. To shift the equilibrium to esterification, the introduction of molecular sieves has been utilized. However, if the formation of water is much faster than the absorption in molecular sieves in a relatively hydrophobic reaction medium, then there is an accumulation of water inside the immobilized catalyst, thereby reducing the enzymatic activity (Martins et al. 2013). Esterification of

3,4-DHPA with hexanol was attempted in the presence of tert-amyl alcohol. After day 4, an ester peak with 51% conversion was found, and further on days 5, 6, and 7, 49, 56 and 59% conversion were observed, respectively. Butanol and propanol were reacted with the phenolic acid using tert-butanol as the solvent for a reaction period of 10 days but did not produce any ester. Esterification of ferulic acid with 1-octanol using tert-butanol as the solvent in the presence of CALB lipase yielded only 14% of esters after 13 days at 60°C in a study reported by Compton and others (Compton et al. 2000). Additionally, esterification of hexanol with 3,4-DHPA was tried in solvents like n-hexane and cyclohexane, but no ester peaks were detected, probably due to evaporative loss. This suggests that the enzymatic esterification of some phenolic acids might be more complex to design.

The amount of catalyst used was also varied to improve the conversion of the phenolic acid to the acyl ester. CALB and TLL were employed at 2.5–10 % (w/w) in the reaction mixture containing octanol as the acyl donor, and it was monitored using HPLC. Increasing the catalyst loading should result in better conversion percentage but the maximum percentage obtained was 20% for 7.5% w/w catalyst loading. Further increase led to reduced yield and conversion. Vaisali and others (2017) have studied the effect of enzyme loading on the esterification of rutin with decanoic acid. They obtained higher conversion when the catalyst loading was increased up to 10 mg/mL, and when increased further, the conversion was found to reduce to 40%. They have attributed this to the overcrowding of the catalytic sites of the enzyme which reduces the interaction between the reactants and the enzyme. However, in this study, even after 10 days of the reaction in the presence of higher enzyme loading there was little to no evidence of esterification. The enzymatic activity of the immobilized lipase has been found to be influenced by several factors such as water activity, temperature, pH, presence of organic solvents, and agitation (Ortiz et al. 2019). The leaching of the enzyme from the immobilized support has been observed in the presence of high temperature and organic solvents, which weakens the enzyme-support interaction and eventually makes the enzyme inactivated (Chen et al. 2007; Rueda et al. 2015; Saunders and Brask 2010). Moreover, in the presence of a polar solvent, the enzyme might lose its stability when exposed to high temperatures (Figueroa-Espinoza and Villeneuve 2005). This loss of activity is due to the partial unfolding of the enzyme and several other covalent alterations in the primary structure of the enzyme (Ahern and Klibanov 1985). Both of these phenomena require the presence of water to occur and eventually lead to the change in the active conformation of the lipase. It is possible that the low yield of the ester during enzymatic lipophilisation was due to these several factors combined.

In addition, the TLL enzyme shows better activity in the presence of moderate amounts of detergents like CTAB, which can prevent dimer formation, producing a monomeric enzyme, which is found to be more active (Fernandez-Lafuente 2010). However, the presence of detergents might also act as inhibitors of lipase and could also affect the efficacy of the phenolic antioxidants. They can form a protective layer around the antioxidant thereby reducing its interaction with the lipase enzyme.

#### 3.5.1.3 Synthesis of esters using binary solvent-based method

The use of a single solvent might not be efficient in solubilizing both the hydrophilic phenolic acid and the hydrophobic alcohol groups and result in reduced contact between the reactants. Hence, a binary solvent system comprising both polar and non-polar solvents was designed for esterification. n-hexane (log P 3.9)/tert-butanol (log P 0.5) solvent systems in 75:25 and 85:15 volumetric ratios were utilized for esterification with hexanol. After 48 hours of reaction, the hexyl ester peak was observed in HPLC with 41% conversion in an 85:15 solvent ratio using CALB lipase. The immobilized lipase from *Thermomyces lanuginosus*, having an activity of 3000 U/g, was not successful in esterifying 3,4-DHPA even under binary solvent-based synthesis. This suggests that the CALB lipase has a wide substrate specificity making it a versatile choice for enzymatic esterification reactions.

Sabally et al. (2005) reported the esterification of dihydrocaffeic acid with linoleyl alcohol using Lipozyme IM20 and Novozyme 435 in a binary solvent system comprised of n-hexane and 2-butanone. Novozyme 435 was an efficient catalyst than the Lipozyme IM20 for the esterification. Though the structure of dihydrocaffeic acid is similar to 3,4-DHPA, the presence of an additional -$CH_3$ chain in the former antioxidant enables the enzymatic esterification with fatty alcohols. Meanwhile, 3,4-DHPA with a shorter side chain could face steric hindrance leading to reduced yield of esters. This could explain the reduced efficiency of esterification with longer chain alcohol (hexanol) compared to shorter chain alcohols (methanol) and their corresponding reaction time also increases due to this complexity.

When the binary solvent system comprising n-hexane and 2-butanone was employed for esterification with methanol, ethanol, propanol, butanol, and hexanol, varying yields of ester were obtained. Methyl ester was formed with a conversion of 73% after 3 days while butyl ester was found with 80% conversion in the solvent ratio of 75:25. Hexyl ester was formed with relatively lower yields of about 35% after 11 days of reaction. However, when the experiments were repeated under the same conditions, no peak was found. Such inconsistency in ester formation was experienced throughout the enzymatic method which could be attributed to the reversible nature of the reaction and the constant variation in water content of the system. Additionally, the support material and technique used for the immobilization of lipase also play a vital role in the efficiency of esterification (Ortiz et al. 2019).

#### 3.5.1.4 Influence of immobilization techniques and support material on esterification

In this study, lipase from *Candida antarctica* immobilized on two different support materials was utilized for esterification. Immobilized enzymes make catalyst recovery, reuse, and purification of products easier than free enzymes. Nonetheless, the support material used for immobilization and the type of immobilization technique employed plays a major role in the accessibility of the lipase active site to the substrates of interest. Covalent bonding is considered an irreversible immobilization technique, meaning that the lipase, once attached to the solid support, will not be able to escape into the solution even at high temperatures and in the presence of co-solvents. On the contrary, interfacial activation-based immobilization, as employed in the popular Novozym 435 (Fig. 3.1), is reversible and can lead to enzyme instability and leaching in unfavorable conditions (Mohamad et al. 2015). Even though Novozym 435 is widely used in various esterification reactions, there are a number of difficulties faced in the process (Ortiz et al. 2019). Enzyme leaching, the solubility of support material in organic media, support material mechanical fragility, and retention of hydrophilic compounds are the major concerns regarding the esterifications catalyzed by Novozym 435. Thermal inactivation in the presence of certain solvents has been observed (Saunders and Brask 2010). This leads to a reduction in enzyme activity, thereby reducing the esterification efficiency.

In both solvent-free and solvent-based reactions, we observed a significant settling of the support material after four days of esterification at 55°C as white precipitates (Fig. 3.2a). Here, we employed a high stirring speed of about 150 rpm in orbital shakers to avoid mass transfer limitations. This also heavily impacted the stability of the support material, and the results agree with the study by Garcia-Galan et al. (2011) and Santos et al. (2015). Lipase acrylic resin (equivalent to Novozym 435 obtained from Sigma Aldrich, India) utilizes the same support material and the immobilization technique. Moreover, lipase immobilized using interfacial activation can retain hydrophilic by-products, including water, and form a water layer inside the catalyst, causing enzyme deactivation (Séverac et al. 2011). All these factors contributed to the very poor performance of the enzyme immobilized on the acrylic resin in solvent-based conditions.

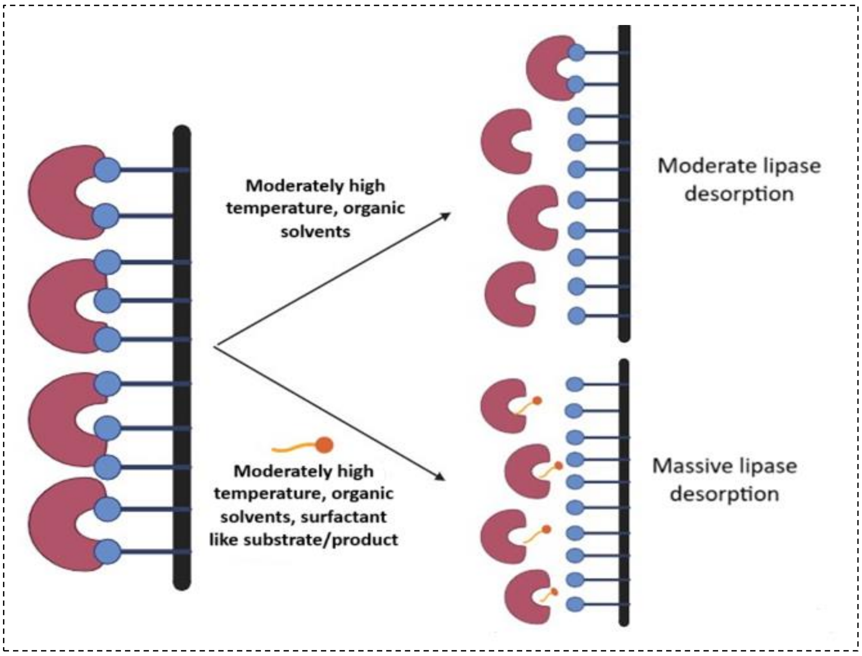


**Fig. 3.1 Desorption of immobilized lipase under unfavorable conditions (adapted from Ortiz et al. 2019)**

Similar conditions were employed for the esterification of 3,4-DHPA with acyl donors using lipase immobilized on Immobead-150 obtained from *Candida antarctica*. Despite the use of solvents and high temperatures, there were no observed white precipitates, indicating the mechanical stability of the support material (Fig. 3.2b). Though the lipase activity was only 2000 U/g in Immobead-150, the yields obtained were better than the lipase acrylic resin (≥5000 U/g). Further increases in the carbon chain length did not produce efficient yields, suggesting the importance of the length of the acyl donor in the success of esterification. Acyl donors with fewer carbons were easily esterified in lesser reaction times when compared to alcohols with longer and bulkier carbon chain lengths. The efficiency of esterification also decreases considerably. For example, for the formation of methyl ester, it took only 48 hours of reaction (results not shown) to reach maximum yield, while the esterification proceeded much slower for higher alcohols such as butanol, hexanol, and octanol. The acyl donors decanol and dodecanol, being bulky, were not able to access the active site of the enzyme lipase. Pleiss et al. (1998) have explained in detail how the source of the lipase affects the active site conformations and, subsequently, the carbon chain length specificity. Lipase obtained from *Candida antarctica* has a funnel-shaped hydrophobic scissile binding site, and its length and extent of hydrophobicity determine the acyl chain length specificity. When the acyl chain is longer, the steric hindrance around the binding site increases which subsequently reduces the formation of ester.

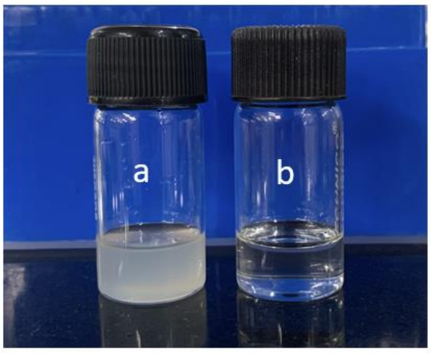


**Fig. 3.2 Solvent layer after stirring for 5 days at 150 rpm at 55°C (a) Lipase immobilized on macroporous acrylic resin (b) Lipase immobilized on Immobead-150**

Though in terms of stability, lipase immobilized on Immobead-150 was better than the lipase acrylic resin, the efficiency of esterification was still not satisfactory and consistent. This indicates the influence of the substrate (phenolic acid) used for esterification on the efficiency of esterification and the role of water released in shifting the equilibrium towards hydrolysis (Arzola-Rodríguez et al. 2022). There is a paucity of literature on the successful enzymatic esterification of 3,4-DHPA. Generally, the enzymatic method involves disadvantages such as (i) high cost of enzymes (ii) mass transfer limitations leading to lower conversions (Pourzolfaghar et al. 2016). In addition, this reduced conversion could be attributed to the inhibition of the lipase enzyme, which is influenced by the structural characteristics of the phenolic acid, the acyl donor used, its molar ratio, and the nature of the solvent employed. In spite of our best efforts to improvise, there was no effective esterification of the phenylacetic acid using lipase enzyme and hence the method was not continued for further studies.

### 3.5.2 Chemical esterification reactions

#### 3.5.2.1 Synthesis of ester using homogeneous catalyst

Liquid chemical catalysts, including sulphuric acid, have been conventionally used for esterification reactions, resulting in good yields of the ester. The yield of ester was higher, but the process involved the use of harsh conditions, which increased the toxicity and warrants tedious purification steps, making them environmentally unsuitable. The use of solid catalysts is reported to be advantageous since they are less corrosive in nature and are relatively less polluting (Baghernejad 2011). *p*-toluenesulfonic acid is a widely used solid catalyst in various organic reactions in chemical industries, which involve the utilization of an acid catalyst. It is further considered green, reusable, inexpensive, and efficient in several organic transformation reactions. It has been claimed to have better catalytic activity than conventional sulphuric acid (Shanmugam et al. 2004). Therefore, this study has attempted to use PTSA as a catalyst for the esterification of 3,4-DHPA with medium-chain alcohols to improve the lipophilicity of the compound.

When an acid catalyst is present, carboxylic acid becomes unstable and is easily attacked by alcohol, which donates a proton and initiates the nucleophilic attack. By altering the reaction conditions, such as temperature, or by employing an excessive amount of alcohol, a catalyst's activity can be further increased (Khan et al. 2021). Accordingly, PTSA was added in mol % to the reaction between acyl donor and 3,4-

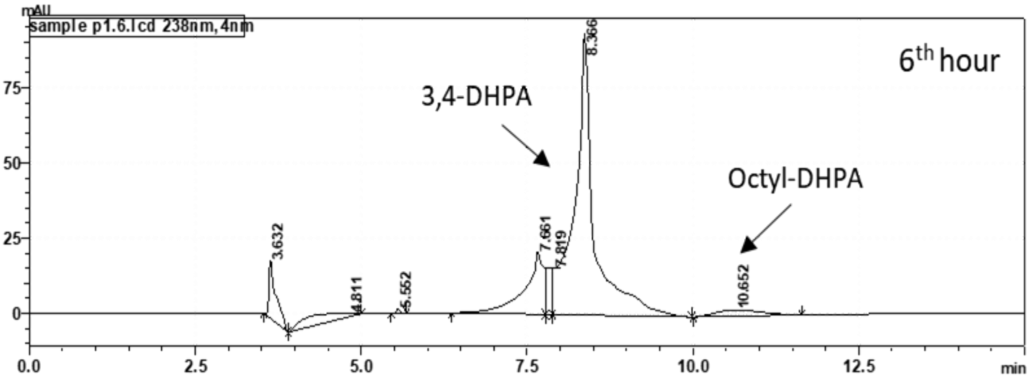
DHPA in the absence of solvent in varying molar ratios to achieve a higher yield of ester (Table 3.2). The parameters, such as the molar ratio of the antioxidant to the alcohol and catalyst loading percentage, affect the conversion of the native molecule to the ester form. The optimization of these parameters may help in obtaining higher conversion rates with minimal use of reaction components and conditions, leading to reduced wastage and easier purification steps.

**Table 3.2 Esterification trials of 3,4-DHPA using PTSA as the catalyst**

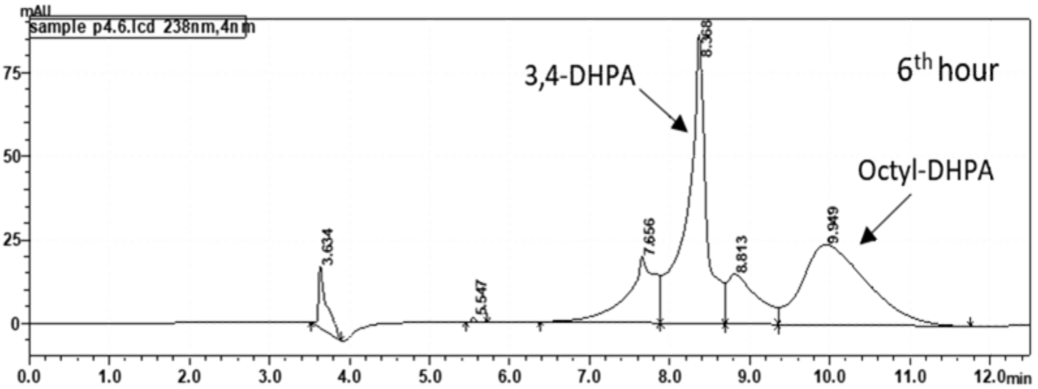
| Trial | Acyl donor | Molar ratio | Catalyst loading (mol %) | Reaction conditions | Results |
|---|---|---|---|---|---|
| 1 | Octanol | 1:30 | 1 | Solvent-free reaction with 150 rpm at 60ºC | 35-40% ester formation after 9 hours |
| 2 | Hexanol, octanol, decanol, dodecanol | 1:40 | 1 | Tert-butanol solvent-based reaction (150 rpm at 60ºC) | Severe evaporational loss observed |
| 3 | Hexanol, octanol, decanol, dodecanol | 1:30 | 1 | Solvent-free reaction for 8 hours (150 rpm at 60ºC) | Decyl and dodecyl esters observed with around 30% conversion after 6 hours |
| 4 | Decanol, dodecanol | 1:30 | 1 | Solvent-free reaction for 6 hours (150 rpm at 60ºC) | Decyl ester (30-39%) and dodecyl ester (35-46%) |
| 5 | Decanol, dodecanol | 1:30 | 10 | Solvent-free reaction with addition of molecular sieves at 2nd hour of reaction (150 rpm at 60ºC) | After 6-8 hours, a very low percentage of ester was observed (10-20%) |

| | | | | | |
|---|---|---|---|---|---|
| 6 | Octanol | 1:30 | 5 | Solvent-free reaction at 80°C for 2-3 hours | No ester formation |
| 7 | Octanol, decanol, dodecanol | 1:30, 1:50 | 1 | Solvent-free reaction with the addition of molecular sieves (150 rpm at 60°C) | 1:50 molar ratio gave a higher ester percentage than 1:30 molar ratio |
| 8 | Octanol | 1:100 | 1 | Solvent-free reaction with molecular sieves (150 rpm at 60°C) | After 12 hours, no sign of ester formation |
| 9 | Octanol | 1:100 | 2 | Solvent-free reaction with molecular sieves (150 rpm at 60°C) | Even after 16 hours, ester was not formed |
| 10 | Decanol, dodecanol | 1:100 | 2 | Solvent-free reaction with no molecular sieves (150 rpm at 60°C) | 11% of dodecyl ester obtained after 24 hours |
| 11 | Octanol, decanol, dodecanol | 1:100 | 15 | Solvent-free reaction (150 rpm at 60°C) | Consistent ester yields after 4-6 hours of reaction |

The molar ratio of the antioxidant to the alcohol was varied in order to increase the esterification efficiency since the presence of excess alcohol was found to have a positive influence on the reverse reaction of esterification rather than the forward hydrolysis reaction (Wei and Jingnan 2018). Increasing the molar ratio brought about a considerable increase in the ester formation in the study by Wei and Jingnan (2018), wherein the dihydrocaffeic acid to hexanol ratio was varied from 1:10 to 1:30 to give the desired effect. During the initial studies, the use of 3,4-DHPA to octanol in the ratio 1:30 did not yield satisfactory conversion (Fig. 3.3a), and hence, it was resolved to study the effect of molar ratio. As expected, the higher molar ratio (1:50) resulted in increased production of the ester with 1 mol% catalyst loading (Fig. 3.3b). However, the yield was still not sufficient, and hence, it was further increased to 1:100. But without enough catalyst loading, the yield was not improved, which could be attributed to the presence of higher amounts of alcohol, which dilutes the acidic catalyst present.



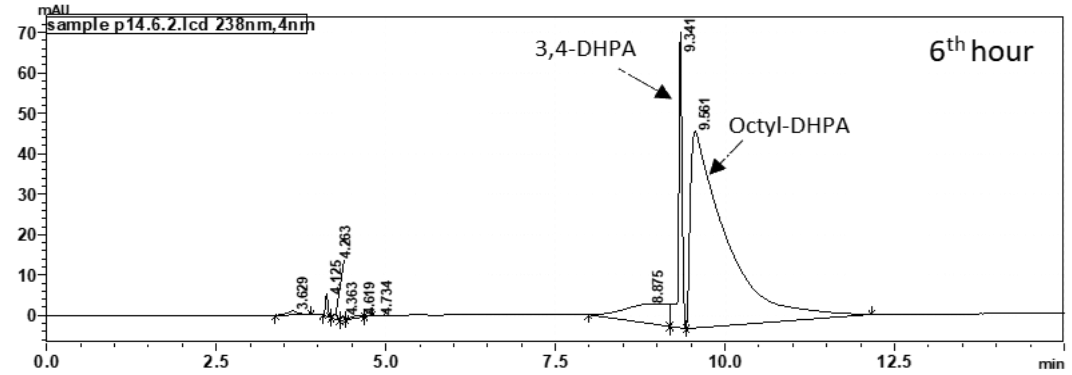
**(a) 1:30 molar ratio**



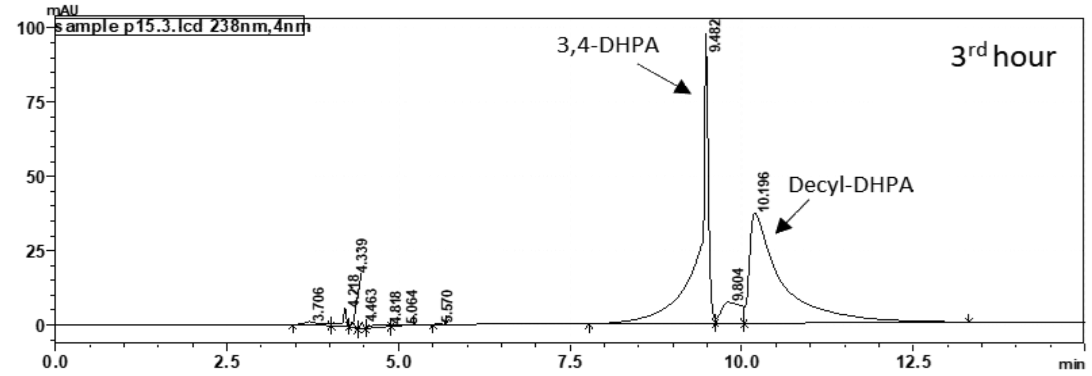
**(b) 1:50 molar ratio**

**Fig. 3.3 HPLC chromatogram of esterification of 3,4-DHPA with octanol after 6 hours**

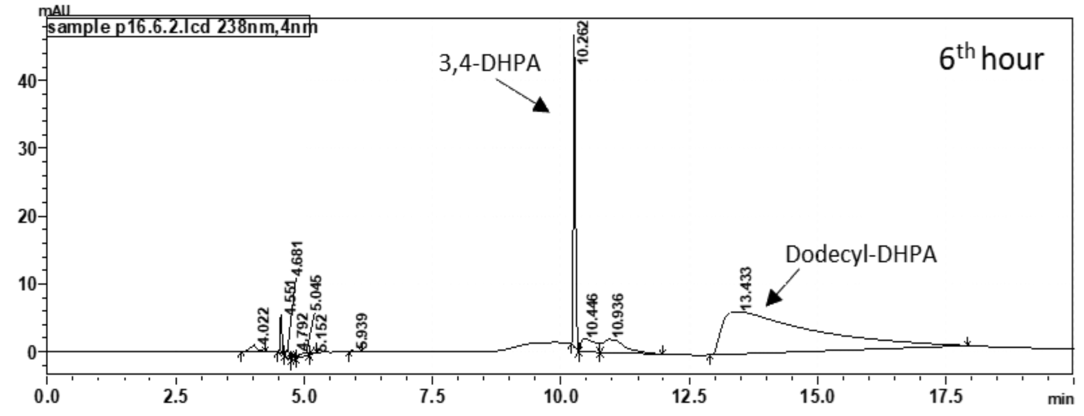
For efficient esterification, the amount of catalyst utilized plays an important role. Since esterification is a reversible reaction, the conversion of acid to ester can be improved by varying the catalyst loading in the reaction (Khudsange and Wasewar 2018). In the preliminary studies done according to Wei and Jingnan (2018), with 1% catalyst loading, the octyl ester was observed with a lower conversion percentage (around 25%) after 9 hours of reaction time. Therefore, it was decided to increase the percentage to 15 mol% for the better yield. When it was increased up to 15%, there was a considerable increase in the conversion percentage (Fig. 3.4) in shorter reaction times. Similar results were also reported by Khudsange and Wasewar (2018), wherein the conversion of valeric acid to its ester of butanol was found to improve after increasing the catalyst load, and the reaction time required also decreased.



**(a) octyl ester**



**(b) decyl ester**



**(c) dodecyl ester**

**Fig. 3.4 HPLC chromatograms of 3,4-DHPA esters using PTSA**

### 3.5.2.2 Purification of ester from the homogeneous reaction mixture

To utilise the ester in oil systems, a fairly pure compound is necessary. Since the reaction was done in solvent-free conditions with octanol as the donor and the solvent, water was used to wash the mixture three times. Following each stage of purification, the presence of ester was verified by HPLC analysis of the organic and aqueous phases. Then, the consecutive water phases were pooled after centrifugation and were subjected to liquid-liquid extraction using ethyl acetate. In order to neutralize the trace water and acids, washing with saturated sodium carbonate solution was done. Significant effervescence was observed as soon as the saturated sodium carbonate solution was introduced, signaling the start of the neutralization process and the release of carbon dioxide. Then, washing with saturated brine solution was carried out by shaking the ethyl acetate layer with brine solution. This step removes large amounts of water which might be dissolved in the organic layer. The ethyl acetate layer was separated and dried using $MgSO_4$ until it did not clump together in solution. The salt was decanted, and the ethyl acetate layer was evaporated under vacuum. The final product was analysed through HPLC which revealed that the ester was present in very low amounts. Since a homogeneous catalyst was used, its removal necessitates these tedious steps. However, the ester was present in low quantities along with a higher amount of the catalyst, which makes the extraction of the ester highly impossible. The ester was found in the initial water layer (69%), and hence, we attempted to use different solvents instead of ethyl acetate to separate the ester and the catalyst through liquid-liquid extraction.

Solvents such as diethyl ether, pentane, dichloromethane, hexane, and benzene were added to water at varying molar ratios (organic: aqueous 10:1, 50:1, 100:1) to draw the ester into the organic layer. Diethyl ether and dichloromethane layers had about 15-25% ester but were not consistent and unrepeatable further. The high concentration (15 mol%) of PTSA used makes the purification of ester from the reaction mixture difficult and complex. Additional steps are required to neutralize the acidic homogeneous catalyst, leading to the consumption of further resources and the generation of excess toxic waste products (Gupta and Paul 2014). Furthermore, the release of these waste products is not environmentally safe, making these catalysts undesirable.

### 3.5.2.3 Synthesis of esters using heterogeneous catalyst

Although several enzyme-based esterification reactions are successful in producing a lipophilic ester of different phenolic acids and flavonoids (Razak and Annuar 2015; Vaisali et al. 2017; Wang et al. 2016), the enzymatic synthesis of esters of 3,4-DHPA was quite challenging, and the yields were low in our initial studies. This could be because of the structure of 3,4-DHPA, which causes substrate binding limitations, including steric hindrance associated with the immobilized lipase enzyme (Cruz Figueroa-Espinoza and Villeneuve 2005) used for esterification during our initial studies. Homogeneous solid acid catalysis using *p*-toluenesulfonic acid resulted in a good yield of ester, but the esters were impure due to the presence of high amounts of the acidic catalyst. Using a heterogeneous catalyst like Amberlyst-15 is a much more efficient way of converting phenolic acid into ester, as it does not require complex purification steps and allows for easier catalyst recovery. This makes it a preferable option compared to enzymatic and solid acidic catalysts.

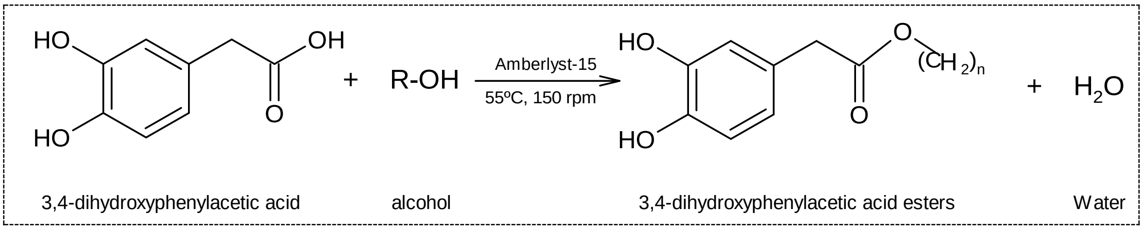
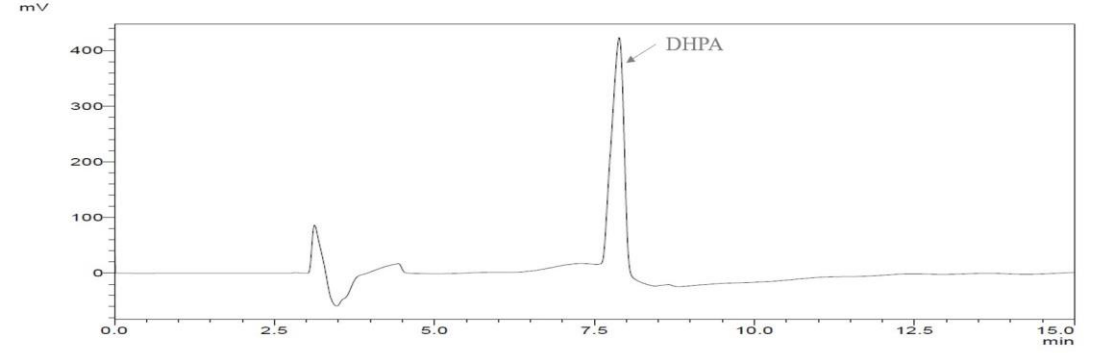


**Fig. 3.5 Esterification reaction of 3,4-DHPA**

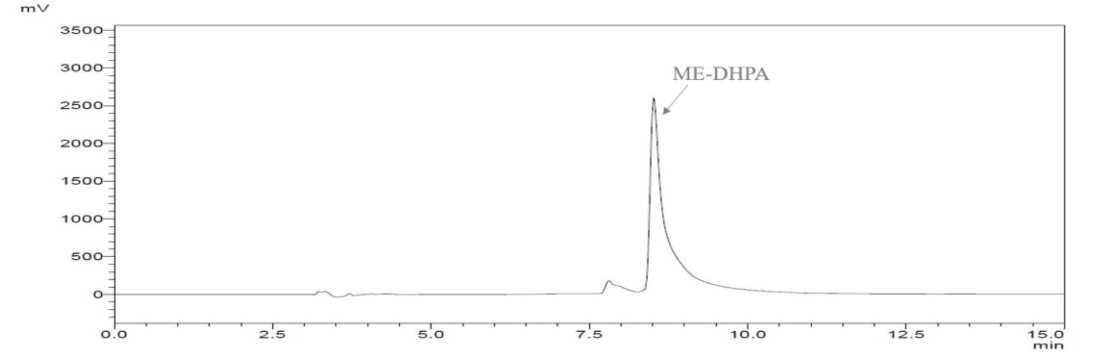
For the esterification of 3,4-DHPA with alcohols of increasing chain length, various solvent conditions were considered. During the initial studies in solvent-free synthesis, alcohols such as butanol and hexanol were used in excess to act as both the acyl donor and as a solvent for the synthesis. However, despite our best efforts, 3,4-DHPA esters (medium chain) could not be synthesized even after longer reaction times using Amberlyst-15 as a catalyst in solvent-free conditions. A recent study by Pappalardo et al. (2023) has revealed that solvent-free synthesis of chlorogenic acid esters was only possible at temperature ranges of 80–100°C where the reacting alcohol was used in minimal quantities. Since the exposure of the phenolic acid to a high temperature for a prolonged time could deteriorate its structure, the usage of a solvent system was preferred. Consequently, solvents, including tert-butanol, tert-amyl alcohol, n-hexane, and cyclohexane, were utilized for the reaction. These solvents were chosen based on previous works in our lab (Vaisali et al. 2017) and extensive literature analysis. The solvents used were inefficient for synthesizing the esters of 3,4-DHPA and were also undergoing severe evaporation loss. Single solvents were not able to support the solubility of the polar antioxidant and non-polar alcoholic groups used for the esterification (Sabally et al. 2005). For example, based on the log P values, polar solvents, including *tert*-butanol (log P 0.5) and *tert*-amyl alcohol (log P 0.9), could only solubilize the phenolic acid 3,4-DHPA while the non-polar solvents such as *n*-hexane (log P 3.9) and cyclohexane (log P 3.4) could efficiently solubilize the fatty alcohols in this study. Thus, it was proposed to use a binary solvent system, which enables better solubility of both reactants, leading to improved ester yield.

Therefore, a binary solvent system comprising *n*-hexane and 2-butanone was used in the ratios 85:15 and 75:25 for the reaction. These solvents and ratios were chosen based on previous published work on their high effectiveness (Sabally et al. 2005). *n*-hexane is a hydrophobic solvent, whereas 2-butanone is comparatively hydrophilic in nature. Adding a second solvent not only increases the solubility of both the hydrophobic and hydrophilic reactants but also stabilizes the low-boiling solvent and prevents severe evaporative loss. Here, n-hexane/2-butanone in the ratio of 85:15 experienced substantial evaporation, as evidenced by a reduction in the reaction volume when compared to the solvents in the ratio of 75:25. This could be due to the presence of a higher amount of *n*-hexane which evaporates at a faster rate during prolonged exposure to high temperatures. The ratio of 75:25 was also observed to have better conversion and subsequent yield for the medium-chain fatty alcohols employed and was thus used for all further esterifications.

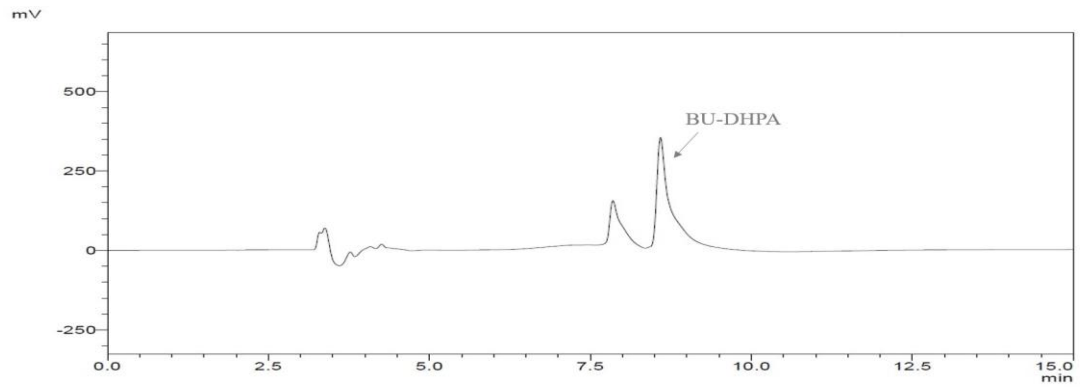
With Amberlyst-15 as the catalyst, the loading of 15% (w/w) gave higher yields in a shorter time as compared to the lower catalyst loading. Methyl ester was synthesized with a conversion of 53.9% after 24 h and 81.1% after 48 h of reaction in the presence of Amberlyst-15 in the n-hexane/2-butanone solvent system with a phenolic acid: alcohol molar ratio of 1:60 (Fig. 3.6b). Meanwhile, the direct esterification of 3,4-DHPA with butanol and hexanol was not achieved even with the use of binary solvent systems. Hence, after the removal of the catalyst and evaporation of the excess solvents, the methyl ester was used in the *trans*-esterification reaction to obtain butyl and hexyl esters. The reaction was carried out using equimolar ratio of methyl ester and corresponding alcohols, and the esters were obtained after 2 and 6 days for the butyl and hexyl ester, respectively. The percent conversion was found to be 69.3% and 78.8%, respectively, for butyl and hexyl esters from the HPLC chromatogram (Fig. 3.6 c, d). The remaining solvents were evaporated from the reaction mixture using a rotary evaporator at 60°C, and the semi-solid product obtained was stored at 4°C for further use.



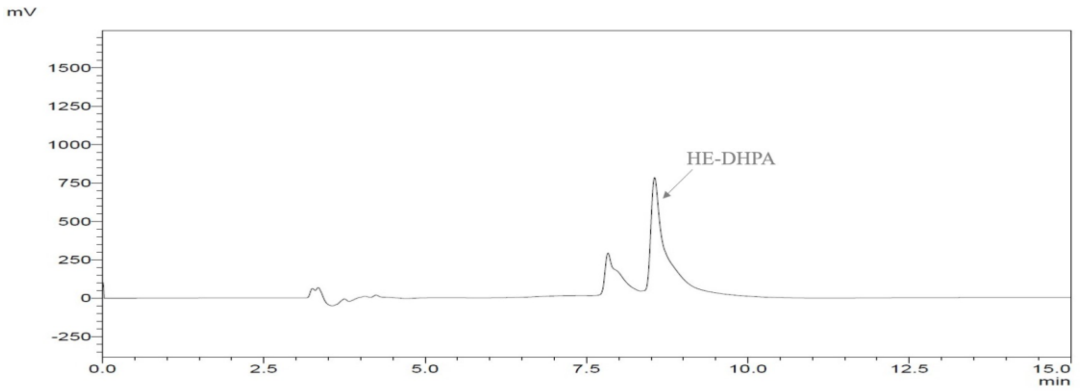
**(a) 3,4-DHPA**



**(b) methyl DHPA**



(c) butyl DHPA



(d) hexyl DHPA

**Fig. 3.6 HPLC chromatograms of 3,4-DHPA esters using Amberlyst-15**

As conjectured, the esterification of 3,4-DHPA was highly influenced by the reaction conditions, and the optimal synthesis parameters varied with respect to the acyl donor used (Table 3.3). Generally, the carboxylic acid group in the 3,4-DHPA reacts with the fatty alcohol in the presence of Amberlyst-15 (Fig. 3.5) to form lipophilic esters of different chain lengths. The acylation at the carboxylic group preserves the functional hydroxyl groups in the ortho and para positions of the phenolic ring, which is essential for the antioxidative activity but enhances the molecule’s lipophilicity (Cruz Figueroa-Espinoza and Villeneuve 2005). To ensure that the antioxidative potential of the phenolic acid remains unaffected, it was decided to maintain the optimal length of the acyl chain. Therefore, any further esterification using longer-chain alcohols was not pursued. In the current study, acylation occurs at the only carboxylic group present in the 3,4-DHPA, which was further confirmed by the MS and NMR analysis in the upcoming sections.

**Table 3.3 Comparative summary of Enzymatic and Chemical esterification trials of 3,4-DHPA using different catalysts**

| Acyl Donor | Reaction conditions | | Catalyst | Yield |
|---|---|---|---|---|
| | **Solvent** | **Molar ratio** | | |
| Methanol | n-hexane:2-butanone in 75:25 ratio | 1:4 | 2.5% w/w CALB | Around 60% conversion after 24 hours |
| | n-hexane:2-butanone in 75:25 ratio | 1:60 | 15% w/w of Amberlyst-15 | 81% conversion |
| Butanol | No solvent | 1:4 | 2.5% w/w of CALB and TLL | No detectable yield |
| | n-hexane:2-butanone in 75:25 ratio | 1:1 | 15% w/w of Amberlyst-15 | 69% conversion |
| Hexanol | n-hexane:2-butanone in 75:25, 85:15 ratio | 1:4 | 2.5% w/w of CALB and TLL | After 48 hours, 35% conversion (CALB) observed with 75:25 while 85:15 had evaporative loss |
| | n-hexane:2-butanone in 75:25 ratio | 1:1 | 15% w/w of Amberlyst-15 | 78% conversion |
| Octanol | No solvent | 1:4 | 2.5% w/w CALB, TLL | 39-43% conversion after 16 days with CALB and 9-20% with TLL |
| | n-hexane:2-butanone in 75:25 ratio | 1:100 | PTSA (15% loading) | Consistent ester yields after 4-6 hours of reaction |

| | | | | |
|---|---|---|---|---|
| Decanol, Dodecanol | No solvent | 1:4 | 2.5% w/w CALB, TLL | Maximum of 4.2% (decyl ester) after 120 hours with CALB |
| | No solvent | 1:30 | PTSA (1% and 10% loading) | Decyl ester (30-39%) and dodecyl ester (35-46%) with 1% loading |
| | No solvent | 1:100 | PTSA (15% loading) | Consistent ester yields after 4-6 hours of reaction |

Throughout the chemical esterification trials, choosing a suitable solvent system (no solvent/single/binary solvent) and the acyl chain length, the type of catalyst used had a significant impact on the yield of 3,4-DHPA esters. The identification of a solvent system compatible with all the reaction components (hydrophilic antioxidant, hydrophobic acyl donor, and catalyst) was critical. This further impacted the tediousness of the purification steps and the yield of the pure ester. When a homogeneous catalyst (PTSA) was utilized, the separation of the chemical catalyst from the reaction mixture was complex owing to the high amounts of PTSA. Lowering the amount of catalyst used resulted in lower yields with longer reaction periods.

To optimize yield and purity, heterogeneous catalysts such as Amberlyst-15 can be used. This enhances the recovery of the ester from the reaction mixture with good purity. Various antioxidant systems respond differently to acyl donors based on the length of their carbon chains. Choosing an optimal chain length was crucial for maintaining the antioxidative properties of 3,4-DHPA as well as improving its solubility in oils.

### 3.5.3 Characterization of 3,4-DHPA esters

#### 3.5.3.1 Analysis of ester using MS and NMR

The analysis of the octyl ester conducted using mass spectrometry indicated a significant peak at 171.0 m/z in the negative mode of ionization. This peak is attributed to PTSA, which has a molecular weight of 172.2 g/mol and has undergone ionization in negative mode, resulting in the observed m/z peak. Since the sample had higher amounts of PTSA and was directly analyzed in MS, the peaks corresponding to octyl ester (280 m/z) were very low. This indicates the presence of high amounts of catalyst impurities as a result of incomplete purification of the ester, which was not successful despite our best efforts. Octyl ester synthesized was analyzed using DMSO as the solvent in $^1$H NMR. PTSA consists of a benzene ring with a methyl group (-$CH_3$) attached at the *para* position and a sulfonic acid group (-$SO_3H$) also attached to the benzene ring. The methyl group attached to the benzene ring showed a peak at 2.3 ppm as a singlet. Two multiplets were observed between 7.2 – 8.0 ppm, indicating the aromatic proton peaks and a broad signal that corresponds to the sulfonic acid proton was also observed. Thus, the proton spectrum had characteristic peaks of the PTSA catalyst, further confirming the presence of major catalyst impurities after esterification. Considering these results, the homogeneous catalyst-based esterification was not pursued for the synthesis of lipophilic esters.

Methyl ester of 3,4-DHPA synthesized using the heterogeneous catalyst in binary solvent was found to have a molecular weight of 182 g/mol according to the mass spectra obtained (Fig. 3.7a). Butyl and hexyl esters were also analyzed in MS for their mass profile, and their molecular weights were confirmed to be 224 g/mol and 252 g/mol, respectively (Fig. 3.7 b, c). Butyl ester was observed with a peak at 223 m/z in negative mode while hexyl ester was observed as a peak at 220 m/z. The hexyl ester could have undergone demethylation during ionization, which could explain the removal of a 31 m/z fragment from the parent molecule. Such type of fragmentation is common with the mass analysis of esters. The acylation at the carboxylic group preserves the functional hydroxyl groups in the *ortho* and *para* positions of the phenolic ring which is essential for the antioxidative activity but enhances the molecule's lipophilicity (Figueroa-Espinoza and Villeneuve 2005). The structures of the esters were further analyzed using $^1$H NMR and $^{13}$C NMR to confirm the purity.

*Methyl 2-(3,4-dihydroxyphenyl)acetate*

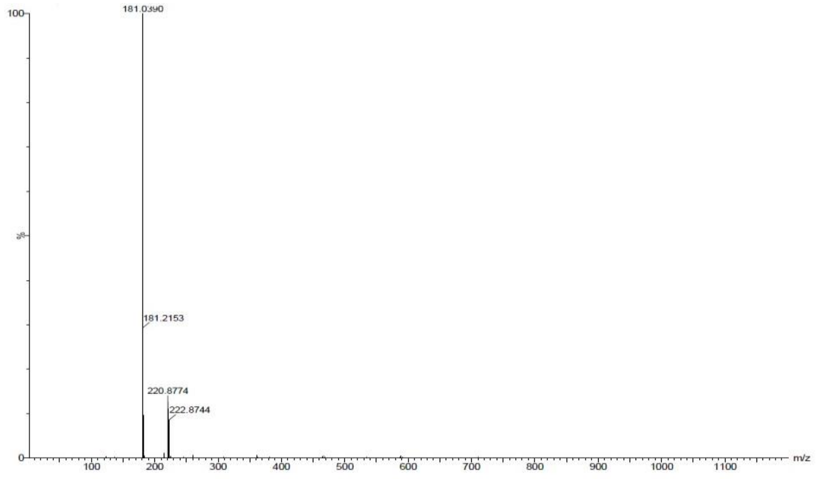
$^1$H NMR (DMSO-$d_6$, 400 MHz): δH 9.51 (2H, s), 6.66–6.62 (2H, m), 6.48 (1H, d, $J$ = 7.9 Hz), 3.59 (3H, s), 3.46 (2H, s). $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 173.8, 172.6, 145.6, 144.8, 126.3, 125.5, 120.6, 117.2, 115.9, 52.1, 40.9.

*Butyl 2-(3,4-dihydroxyphenyl)acetate*

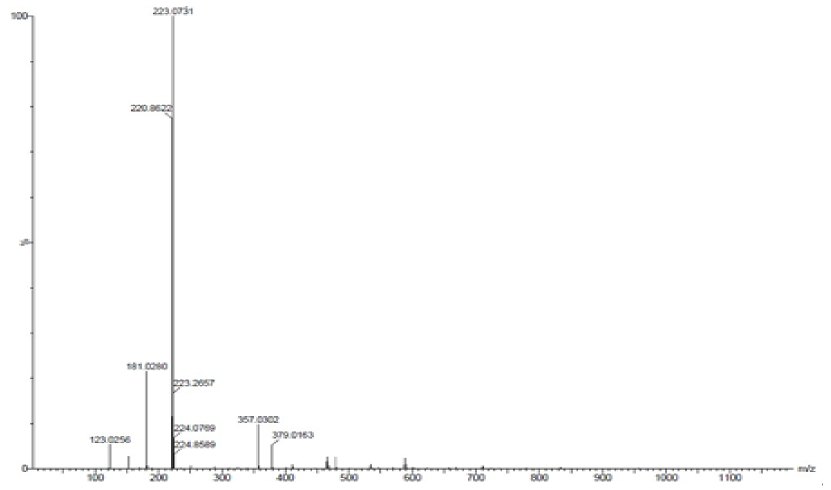
$^1$H NMR (DMSO-d$_6$, 400 MHz): δH 6.65 (2H, d, *J* = 4.1 Hz), 6.49 (1H, d, *J* = 8.0 Hz), 4.01 (1H, t, *J* = 6.4 Hz), 3.59 (1H, d, *J* = 7.4 Hz), 1.60–1.48 (2H, m), 1.36–1.21 (2H, m), 0.87 (3H, t, *J* = 7.3 Hz). $^{13}$C NMR (100 MHz, DMSO-d$_6$) *δ* 173.6, 172.1, 145.5, 144.5, 126.2, 125.6, 120.5, 117.1, 115.8, 64.3, 60.9, 40.7, 35.2, 30.7, 19.1, 14.1.

*Hexyl 2-(3,4-dihydroxyphenyl)acetate*

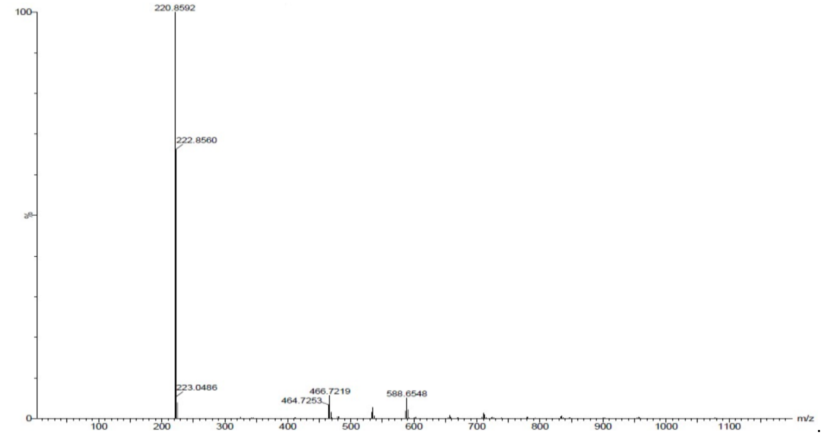
$^1$H NMR (DMSO-d$_6$, 400 MHz): δH 6.68–6.63 (2H, m), 6.49 (1H, d, *J* = 8.0 Hz), 4.00 (2H, t, *J* = 6.6 Hz), 3.59 (2H, s), 1.59–1.48 (2H, m), 1.26–1.21 (6H, m), 0.86 (3H, t, *J* = 6.4 Hz). $^{13}$C NMR (100 MHz, DMSO-d$_6$) *δ* 172.1, 145.6, 144.7, 125.6, 120.4, 117.0, 115.9, 64.5, 40.7, 31.3, 28.6, 25.5, 22.5, 14.4.



**(a) methyl DHPA**



**(b) butyl DHPA**



**(c) hexyl DHPA**

**Fig. 3.7 m/z profiles of synthesized 3,4-DHPA esters**

### 3.5.3.2 Calculation of miLog P values

Log P value refers to the log of the partition coefficient of a solute between octanol and water. In the context of antioxidants, describing the log P value might help in characterizing an ester based on its lipophilicity. They can be calculated by breaking down the molecular structure into defined fragments containing functional groups, and their contribution to the log P value is obtained from empirical studies. This also considers the effect of intramolecular hydrogen bonding on the log P value. These log P contributions are summated, and the overall estimate of log P value is calculated as miLog P. The calculated miLog P values of all antioxidants used in the current study are given in Table 3.4.

**Table 3.4 Calculated miLog P values of antioxidants**

| Antioxidant | miLog P | Molecular weight (g/mol) |
|---|---|---|
| 3,4-DHPA | 0.39 | 168.15 |
| Methyl 3,4-DHPA | 1.01 | 182.19 |
| Butyl 3,4-DHPA | 2.45 | 224.27 |
| Hexyl 3,4-DHPA | 3.46 | 252.32 |
| TBHQ | 3.08 | 166.22 |
| BHT | 5.43 | 220.35 |

Molecules that exhibit negative or very low log P values are typically characterized as hydrophilic, whereas compounds with higher log P values are considered hydrophobic. As expected, the miLog P values were found to increase with the increase in the acyl chain length. Synthetic antioxidants TBHQ and BHT are highly hydrophobic based on the miLog P values and, hence, are easily soluble in bulk oils. Hexyl ester of 3,4-DHPA has lipophilicity comparable to that of TBHQ, which indicates improvised solubility in bulk oils. Due to their higher solubility in non-polar media, these synthetic antioxidants are preferred industrially for retarding oxidation. The method used for the calculation of miLog P value is rapid and highly accurate, making it a versatile tool for precise determination.

### 3.5.3.3 DPPH radical scavenging activity

Antioxidants that are able to inhibit or delay lipid oxidation by scavenging free radicals are called primary antioxidants, and their scavenging ability is exploited in this method. It is a simple, rapid, sensitive, and highly reproducible spectrophotometric technique for the determination of antioxidant capacity (Gülçin et al. 2005). This assay is based on the ability of 3,4-DHPA to scavenge the synthetic free radical DPPH and the measurement of the consequent decolorization, which reverses the formation of free radicals. DPPH in the radical form has a deep purple color ($\lambda_{max}$ of 517 nm), and when it encounters a molecule that can donate hydrogen atoms in an alcoholic solution, it gets reduced to the colorless form (DPPH-H), leading to a decrease in the absorbance value. Hence, it indirectly measures the antioxidant potential in a fast and inexpensive way using a comparatively stable DPPH radical (Gulcin 2020).

The phenolic acid and its esters were evaluated for their radical scavenging activity using DPPH free radicals in methanolic solutions. They were reported as percent DPPH radical scavenging activity calculated using a blank solution in the absence of antioxidant (Fig. 3.8). The natural phenolic acid was shown to have the highest radical scavenging activity among the investigated antioxidants, reaching 90.89% at a concentration of 15 μM. The $IC_{50}$ value of 3,4-DHPA was 2.02-fold less than that of synthetic antioxidant TBHQ, which indicates excellent antioxidative properties of the phenolic acid (Table 3.5). The radical scavenging activity of butyl ester was better than TBHQ ($p < 0.05$) but was not higher than the native 3,4-DHPA, methyl, and hexyl esters. The modification of the phenolic acid has altered the antioxidative potential and the extent of variation differs based on the nature and length of the acyl donor used. The radical scavenging activity was shown to decrease significantly in the concentration range of 5–20 μM; however, at 25 μM, the activity of all the esters was comparable to that of the native DHPA.

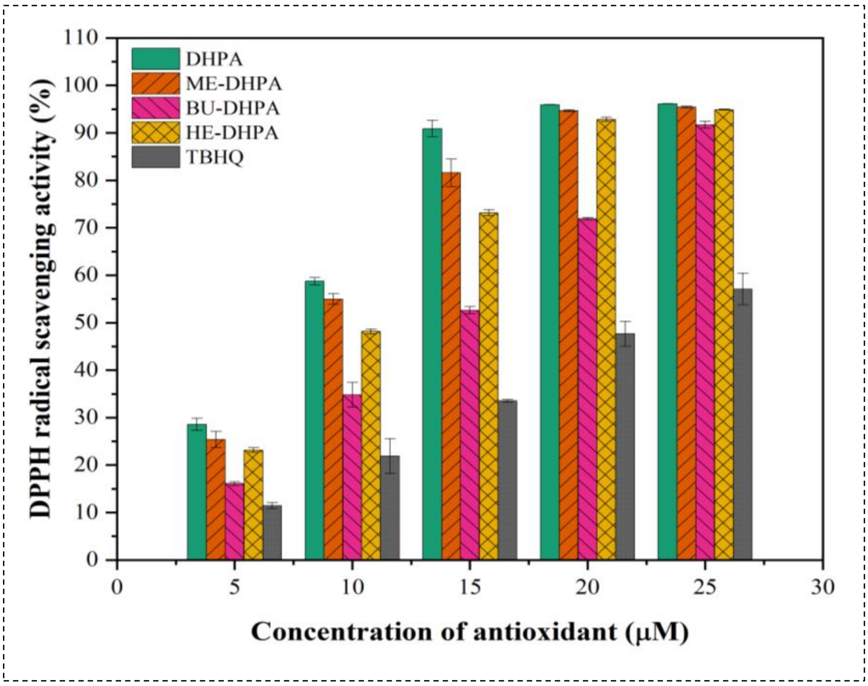


**Fig 3.8 DPPH radical scavenging activity of antioxidants**

The two phenolic hydroxyl groups in 3,4-dihydroxyphenylacetic acid have the capacity to donate hydrogen atoms to a free radical. During esterification, though these phenolic hydroxyl groups are not modified, there was a change in their antioxidative capacity (Fig. 3.8). This could be attributed to the presence of an electron-withdrawing group, say an ester group close to the electron-donating phenolic hydroxyl groups attached through a short-saturated side chain (Silva et al. 2000). The rotatability of the hydroxyl group is influenced by these bulkier ester groups, further reducing the radical scavenging activity of the synthesized compounds. However, the reduced radical scavenging activity of the esters was still superior to the potential of the commonly used TBHQ. This suggests that the 3,4-DHPA and its esters could be a safer alternative to the synthetic antioxidants in retarding oxidation.

**Table 3.5 $IC_{50}$ of the synthesized antioxidants evaluated by DPPH and ABTS radical scavenging activity assays**

| Antioxidants | $IC_{50}$ (µM) | |
|---|---|---|
| | DPPH scavenging activity | ABTS scavenging activity |
| DHPA | $10.72 \pm 0.2^c$ | $148.98 \pm 3.0^c$ |
| ME-DHPA | $11.12 \pm 0.3^c$ | $238.66 \pm 6.8^a$ |
| BU-DHPA | $13.86 \pm 0.3^b$ | $183.76 \pm 3.7^b$ |
| HE-DHPA | $11.61 \pm 0.1^c$ | $234.19 \pm 2.9^a$ |
| TBHQ | $21.76 \pm 0.9^a$ | $233.54 \pm 3.5^a$ |

$^{a\text{-}c}$ mean values with different letters in each column are significantly different, $p<0.05$, One-way ANOVA, Tukey's test

3,4-DHPA shares a similar structure with dihydrocaffeic acid in that they both have a side chain joined to the phenolic ring by a single bond. When the side chain was unsaturated as in the case of caffeic acid, the acylation of the carboxylic group increased the radical scavenging activity. Meanwhile, the antioxidant activity was eventually reduced by the acylation of the saturated side chain that contains the carboxyl group (dihydrocaffeic acid). The extent of the loss of radical scavenging activity was dependent on the size, length, and position of the acyl group attached (Silva et al. 2000). Similar results were obtained by Lecomte et al. (2010) in their study of the esterification of rosmarinic acid using enzymatic and chemical catalysts. With the exception of the dodecyl ester, all rosmarinic acid esters were found to have reduced global radical scavenging activity than the parent molecule. Additionally, Gholivand et al. (2017) observed a decrease in dihydrocaffeic acid's scavenging activity, which may have resulted from modifications to the carbonyl group's structure.

#### 3.5.3.4 ABTS radical scavenging activity

In methanolic solutions, the concentration range of 100–500 µM was used to test the scavenging activity of 3,4-DHPA and its lipophilic esters against the ABTS free radical. The results were given as the percentage of the radical scavenging activity (Fig. 3.9). The assay is simple to perform, and ABTS radicals are generated by oxidation of ABTS with potassium persulfate overnight. The phenolic hydroxyl groups interrupt the oxidation chain reaction by donating hydrogen, leading to the formation of a stable product that would not propagate the oxidation reaction any further (Gulcin 2020). At 200 µM concentration, the unmodified DHPA exhibited 100% scavenging activity, but the methyl and hexyl esters, as well as TBHQ, attained their maximum activity at only 500 µM. Surprisingly, butyl ester demonstrated greater scavenging capacity than the other esters examined, while it performed the lowest in the DPPH radical scavenging assay. Contrary to the DPPH radicals, ABTS radicals are more reactive and involve both HAT (hydrogen atom transfer) and SET (single electron transfer) based processes (Gulcin 2020). Therefore, there is a probability that the antioxidant esters' performance will differ between the two assays.

The potential of the esters was comparable to that of the synthetic antioxidant TBHQ, despite their lower activity. The modification of the 3,4-DHPA invariably brings in some changes in the antioxidative property due to steric hindrance but does not cause a complete loss of activity. Peng and researchers observed a reduction in the antioxidative activity of monoacylated epigallocatechin in ethanol and water-based assays (Peng et al. 2023). The antioxidant potential is also highly dependent on the length of the acyl donor that is attached to the phenolic acid. For example, in the aforementioned study, the epigallocatechin esters having different carbon chain lengths affected the radical scavenging assays variably. Esters with 4 to 12 carbons performed better in the water-phase ABTS assay, while modified esters with 12 to 18 carbons demonstrated more activity in the DPPH test. Their total activity, though, was not higher than that of the original epigallocatechin. Similar variation was also observed with the behavior of butyl DHPA ester, which had a higher $IC_{50}$ in DPPH but a reduced $IC_{50}$ in ABTS (Table 3.5) radical scavenging assays in our study.

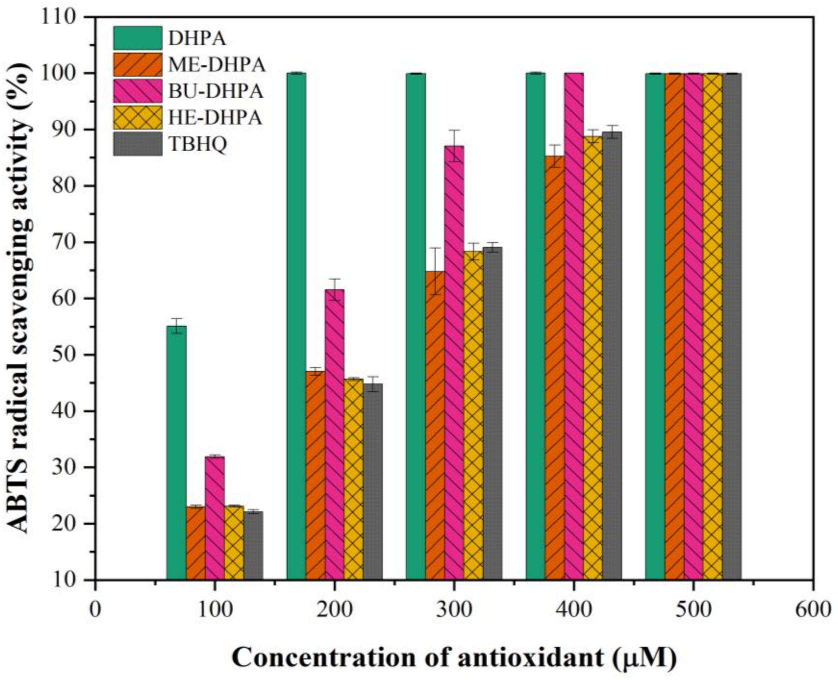


**Fig 3.9 ABTS radical scavenging activity of antioxidants**

### 3.5.3.5 Ferric reducing antioxidant power (FRAP) assay

The assay measures the ability of the antioxidant to reduce ferric ($Fe^{3+}$) to ferrous ($Fe^{2+}$) ions, and it is done in acidic media to maintain the solubility of iron. The absorbance at 593 nm increases when the ferric 2,4,6-tripyridyl-s-triazine $[Fe^{3+}\text{-}(TPTZ)^2]^{3+}$ complex is reduced to the ferrous complex (strong blue-colored $[Fe^{2+}\text{-}(TPTZ)^2]^{2+}$). The concentration range of 5–25 μM was used to examine the phenolic acid and its lipophilic esters, and the results were given as absorbance values (Fig. 3.10). An increasing trend was observed for all the antioxidants studied as the concentration increased with DHPA showing the highest absorbance at 25 μM concentration.

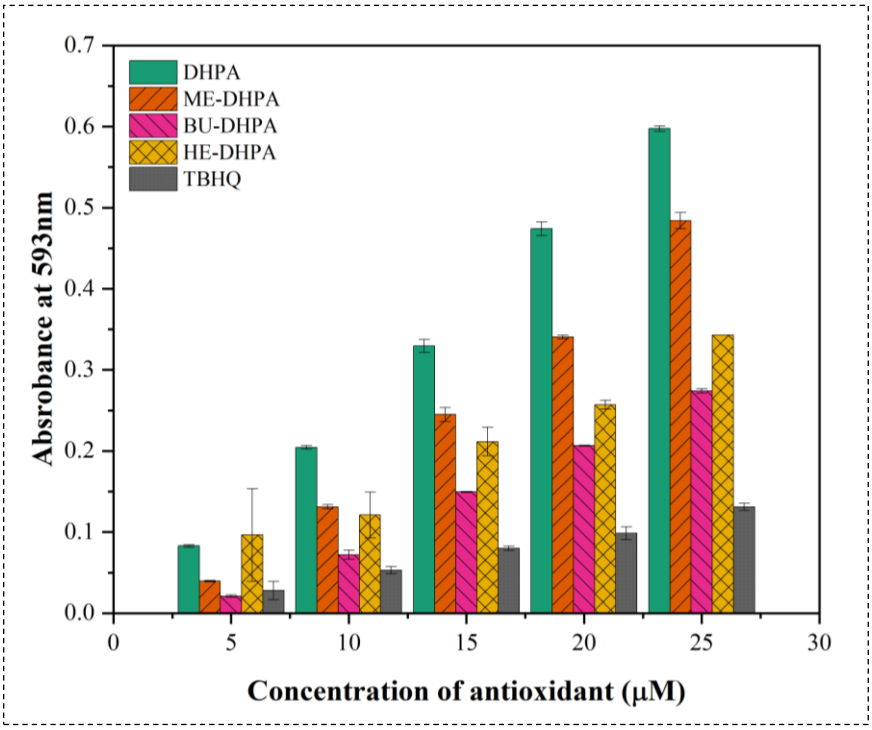


**Fig 3.10 Ferric reducing antioxidant power of antioxidants**

The ferric-reducing power decreases in the order as DHPA > me- DHPA > he-DHPA > bu-DHPA > TBHQ. The well-known synthetic antioxidant TBHQ showed very low ferric reducing power as compared to the phenolic esters. The butyl ester showed the least efficiency when correlated to the other esters in reducing ferric ions, while the same ester had the best radical scavenging activity in the ABTS assay. The results of the study indicate that the DPPH and FRAP assays had similar activity trends, while the ABTS assay showed an opposite trendline in relation to the synthesized esters. The ferric-reducing power could reflect an indirect antioxidant capacity, which could be compared to HAT-based radical scavenging assays (Gulcin 2020). As DPPH scavenging assay measures its activity using the HAT mechanism, there may be a significant correlation between these findings. This might explain the similar behavior of the different esters in these in vitro antioxidant assays, which utilize other antioxidative mechanisms. Nevertheless, the study brings out the excellent ferric-reducing ability of 3,4-DHPA and its lipophilic esters for the first time.

## 3.6 SUMMARY AND CONCLUSIONS

3,4-DHPA is a lesser-known natural phenolic acid with excellent antioxidative capacity. This compound has the potential to serve as a substitute for the extensively used synthetic antioxidants, namely TBHQ, BHT, and BHA. The enzymatic method of esterification was carried out in an attempt to synthesize amphiphilic esters of 3,4-DHPA, which could effectively retard oxidation.

- Lipase-catalyzed esterification in solvent-free conditions for the synthesis of 3,4-DHPA esters gave very low yields after prolonged reaction times. This could be attributed to the higher concentration of alcohol used which leads to reduced enzyme activity owing to water bilayer displacement. This inhibitory effect was evident with the use of primary alcohol in the study.
- In solvent-based conditions, the effect of the molar ratio of phenolic acid to the alcohol and catalyst loading percentage was found to influence the efficiency of esterification.
- Under enzymatic conditions, it was found that tert-butanol was relatively effective in lipophilizing 3,4-DHPA. Other solvents (tert-amyl alcohol, n-hexane, cyclohexane) yielded negligible results, potentially due to either evaporational loss or the lipase enzyme's inactivity in the presence of these solvents.
- A binary solvent consisting of n-hexane:2-butanone solvent was effective in the 75:25 (v/v) ratio rather than the 85:15 volumetric ratio. This ideal solvent system resulted in 73% and 35% conversion for methyl and hexyl esters, respectively.
- Due to the reversible nature of esterification, the reactions had very limited repeatability. Despite using molecular sieves to absorb the released water, the ester yield was inconsistent, which made it difficult for esterifying 3,4-DHPA under enzymatic conditions.
- Immobilization support material stability was weak in the presence of solvents in the case of lipase acrylic resin compared to CALB lipase immobilized on Immobead-150.

The chemical method of esterification using homogeneous (PTSA) and heterogeneous catalysts (Amberlyst-15) was carried out with 3,4-DHPA. Acyl donor's chain length (hexanol, octanol, decanol, and dodecanol), molar ratio, and catalyst loading were altered to optimize the synthesis of the esters.

- Ester yield was increased when the molar ratio was increased from 1:30 to 1:100 with a high catalyst loading of 15 mol % after 4-6 hours of reaction.
- Purification of the ester from the homogeneous reaction mixture was incomplete owing to the presence of high amounts of acidic catalyst. Despite our best efforts, a pure ester was not obtained, and hence, the method was not pursued further.
- Heterogeneous catalysis using Amberlyst-15 (15% w/w) was done successfully for the synthesis of methyl, butyl, and hexyl esters of 3,4-DHPA with 81.1%, 69.3%, and 78.8% conversion, respectively.
- Methyl ester was synthesized by direct esterification, while butyl and hexyl esters were obtained through a trans-esterification reaction of methyl DHPA with respective alcohols under similar conditions.
- The optimal reaction molar ratio was 1:60 for methyl ester synthesis, while for transesterification reactions, a 1:1 molar ratio was efficient.

Structural characterization of the synthesized esters was performed using mass and NMR spectrometry. Additionally, the *in vitro* antioxidative capacity of the esters was also evaluated. These characteristics are essential for estimating the ability of the esters to act as antioxidants and effectively retard oxidation.

- Octyl ester synthesized using PTSA was found to have a high quantity of the catalyst in $^1$H NMR analysis despite efforts to remove the acidic catalyst from the reaction mixture.
- Conversely, the purity of methyl, butyl, and hexyl esters synthesized using Amberlyst-15 was validated in MS, $^1$H, and $^{13}$C NMR analysis with the respective characteristic peaks observed.
- Methyl, butyl, and hexyl esters of 3,4-DHPA were found to have increasing miLog P values, indicating an increase in the hydrophobicity of the esters.

- 3,4-DHPA showed the best radical scavenging activity with the lowest $IC_{50}$ values in both DPPH and ABTS assays compared to all other antioxidants studied.
- The ferric-reducing antioxidant power decreases in the order of DHPA > me-DHPA > he-DHPA > bu-DHPA > TBHQ.
- 3,4-DHPA and all its esters were better performing than TBHQ in all *in vitro* antioxidant activity assays.
- The lipophilisation has reduced the antioxidative potential of all esters, and the extent of influence is varied for esters of different acyl chain lengths attached.
- The influence of the length of the acyl chain contrasted among the three assays, which utilize different radicals, indicating the behavior of esters in different reaction media. This suggests that the activity of the antioxidant esters in these assays might not completely describe their efficiency in all systems but only a representative prediction.

# CHAPTER 4

## APPLICATION OF LIPOPHILIC ESTERS OF 3,4-DHPA IN OXIDATIVE STABILITY STUDIES OF FISH OIL

# CHAPTER 4

Recent interest in natural antioxidants as a potential replacement for synthetic antioxidants is mainly due to the excellent antioxidative capacity of these natural phenolic acids. Since they are hydrophilic in nature, they are often lipophilised to improve application in hydrophobic food systems. The 3,4-DHPA esters synthesized were found to have good antioxidant activity in *in vitro* assays. However, these assays are carried out in aqueous solutions of methanol, which might not be comprehensive in terms of their behavior in oil-based applications. Hence, to better understand their potential as antioxidants, fish oil fortified with these modified esters was evaluated based on storage stability.

Fish oil represents a rich source of n-3 PUFAs, including EPA and DHA, which are often targets of oxidation during storage. Crude fish oil contains several impurities that could significantly accelerate oxidation reactions. Hence, refining fish oil has been done through degumming, deacidification, and bleaching to improve storage stability. The refined fish oil was characterized before oxidative stability studies to evaluate its quality. The permitted concentration of synthetic antioxidants in food products is commonly 200 mg/L. Accordingly, 3,4-DHPA and its esters were added to refined fish oil at a maximum concentration of 0.9 mM, and the stability was analyzed for 30 days at 35°C. Fish oil without any antioxidants was used as a control, and BHT and TBHQ-fortified fish oils were studied for comparison.

Oxidation in fish oil proceeds through a chain reaction involving the formation of primary and secondary oxidation products, including peroxides, 2,4-alkenals, and 2,4-alkadienals. The progress of the oxidation reaction is generally measured by determining peroxide and *p*-anisidine values and further calculation of TOTOX values. This TOTOX value gives a comprehensive estimation of oxidation considering both the primary and secondary oxidation reaction products. The presence of antioxidants delays oxidation by donating hydrogen atoms to lipid alkyl radicals while scavenging the lipid peroxy radicals, thereby breaking the chain reaction of oxidation (Mishra et al. 2021).

The ability of the antioxidant in retarding oxidation depends on the structural features, its concentration, and the presence of other active components. More than often times, antioxidants are used in combinations that combine the relevant structural features, resulting in a synergistic mixture having better potential than individual antioxidants. In this study, we have attempted to use a combination of hydrophilic and lipophilic phenolic acid in order to understand the influence of polarity on the location of these compounds at the site of oxidation.

## 4.1 MATERIALS AND METHODS

### 4.1.1 Materials

Crude fish oil without any added antioxidants was obtained from Raj Fishmeal and Oil Company, Udupi, India. After refining, fish oil was stored at -20°C until further use. Ortho-phosphoric acid (85%), methanol, hydrochloric acid, sulfuric acid, phenolphthalein indicator, chloroform, isopropyl alcohol, toluene, ethanol, iso-octane, glacial acetic acid, diethyl ether, sodium hydroxide, and hexane were of analytical grade and purchased from Loba Chemie, India. Mercury was obtained from Central Drug House, India. Ammonium molybdate, granulated activated charcoal, soy lecithin, potassium hydroxide, starch, potassium iodide, sodium thiosulfate pentahydrate, *p*-anisidine, boron trifluoride, and nitric acid were from Merck, India. EPA and DHA methyl ester analytical standards were procured from Merck, India.

### 4.1.2 Refining of fish oil

For the efficient removal of phospholipids, free fatty acids (FFAs), and metal ions from crude fish oil, a three-step method optimized previously in our laboratory was utilized (Charanyaa et al. 2017). This technique enables the removal of unwanted components without compromising the n-3 PUFA content from the fish oil.

#### 4.1.2.1 Degumming

The crude fish oil was first subjected to degumming to remove the phospholipid contents by treating it with an acidic degumming agent. Ortho-phosphoric acid was added to crude oil at 5% (w/w) and was continuously stirred for 30 minutes in a magnetic stirrer. Then, the oil was centrifuged at 10,000 rpm for 25 minutes. This resulted in a clear and less viscous supernatant, which was collected and processed further.

#### 4.1.2.2 Deacidification

The degummed oil was further deacidified to remove the free fatty acids present. Liquid-liquid extraction was carried out with methanol and oil in the ratio of 1:1 (w/w). The mixture was stirred for 60 minutes and then transferred to a separating funnel. It was allowed to settle overnight into methanol and oil layer. The oil part was collected, leaving the methanol layer.

#### 4.1.2.3 Bleaching

For the removal of trace solvents and impurities, such as oxidation products and pigments from the fish oil, bleaching was carried out with a suitable bleaching agent. Granulated activated charcoal (3% w/w) was added to the deacidified fish oil and was sealed and heated to about 80°C. The reaction was maintained under vacuum between 40-70 mmHg for about 20 minutes. Then, the mixture was centrifuged at 10,000 rpm for 15 minutes to remove the activated charcoal. The supernatant was collected as refined fish oil and was stored at -20°C for future use.

### 4.1.3 Characterisation of fish oil

The physicochemical characteristics of the refined and crude fish oil were studied in order to analyze the efficiency of the refining process and the quality of the resultant oil used for oxidative stability studies.

#### 4.1.3.1 Phospholipid content estimation

The chromogenic solution for the determination of phospholipid content was made in a number of steps as described by Hundrieser et al. (1985). 120 mL of distilled water was used to dissolve 16 grams of ammonium molybdate to make Solution I. A mixture of 40 mL of concentrated HCl, 10 mL of mercury, and 80 mL of solution I was shaken for 30 minutes and filtered to give solution II. 200 mL of strong sulfuric acid was used to dilute the remaining solution I. Solution III was obtained by combining solutions I and II. Then, 25 mL of solution III, 45 mL of methanol, 5 mL of chloroform, and 20 mL of distilled water were combined to prepare the chromogenic solution. The oil sample was initially dissolved in chloroform, and the solvent was evaporated by heating it in a water bath. To this, 0.4 mL of chloroform and 0.1 mL of the chromogenic solution was added. This solution was vortexed thoroughly and heated in a boiling water bath for 75 seconds. Then the sample was cooled to room temperature, and 4 mL of chloroform was added. The resulting mixture was vortexed, and the chloroform layer was analyzed at 735 nm in a UV-visible spectrophotometer. The concentration was calculated based on the standard graph plotted using increasing concentrations of Soy lecithin.

#### 4.1.3.2 Acid Value Estimation

Acid value represents the milligrams of potassium hydroxide necessary to neutralize FFAs in 1 gram of the oil and was determined based on the standard method (Cd 3d-63) as described by AOCS (AOCS 2009). It is calculated as,

$$\text{Acid value (mg KOH/g of oil)} = \frac{(\mathrm{A} - \mathrm{B})\ \mathrm{x}\ \mathrm{M}\ \mathrm{x}\ 56.1}{\mathrm{W}}$$

where A and B are the volumes of alkali used (mL) in the titration of the sample and the blank solution, M is the molarity of the alkali used, and W is the mass (g) of the oil sample taken.

#### 4.1.3.3 Peroxide Value

The hydroperoxide content of the fish oil was measured according to the standard method described in AOCS Cd 8b-90 (AOCS 2009). The assay measures the concentration of hydroperoxides as milliequivalents of peroxide per 1000 grams of the test sample to oxidize potassium iodide under given conditions. The method involves the quantification of peroxides by reacting them with iodide to produce iodine, which is then titrated with sodium thiosulfate. The peroxide value is then calculated using the following formula,

$$\text{Peroxide value (m Eq. of peroxide/1000 g)} = \frac{(\mathrm{A} - \mathrm{B})\mathrm{x}\ \mathrm{M}\ \mathrm{x}\ 1000}{\mathrm{m}}$$

where A and B were the samples and blank titer values, M was the molarity of the thiosulfate solution, and m represented the mass of the oil sample (g).

### 4.1.3.4 *p*-Anisidine Value

The estimation of *p*-anisidine value (PAV) was done using the standard AOCS method Cd 18-90 (2009). It measures the amount of secondary oxidation products such as aldehydes, primarily 2-alkenals and 2,4-dienals, which are formed during the oxidation of oils. *p*-anisidine value is calculated according to the following formula,

$$p - \mathrm{Anisidine\ value} = \frac{25 \times (1.2\mathrm{A_s} - \mathrm{A_b})}{\mathrm{m}}$$

where $A_b$ and $A_s$ are the absorbances of the lipid solution before and after the reaction with *p*-anisidine, and m is the mass of the oil taken for analysis.

### 4.1.3.5 TOTOX Value

The peroxide value may indicate the initial or advanced stage of oxidation and might be misleading when used alone. It is hence used in combination with the *p*-anisidine value as TOTOX value. It is calculated as,

$$\mathrm{TOTOX} = (2 \times \mathrm{PV}) + \mathrm{pAV}$$

Here, PV and p-AV represent the peroxide value and *p*-anisidine value.

### 4.1.3.6 Determination of EPA and DHA Content

In order to analyze the fatty acid profile of the fish oil, fatty acid methyl esters (FAME) were prepared using the method described by Ichihara and Fukubayashi (2010) with few modifications. The method involves the transesterification of triglycerides or the esterification of free fatty acids with methanol, resulting in methyl esters of the fatty acids, which are then analyzed by Gas Chromatography (GC). The oil samples were first dissolved in diethyl ether in screw-capped glass tubes. The lipid solution was then mixed with 2 mL of 0.5 M methanolic NaOH solution and was allowed to hydrolyze for 1 hour at 70ºC under reflux. After hydrolysis, 0.6 mL of 2 M HCl was mixed into the solution, and hexane was added. The hexane phase was then evaporated, and the residue was methylated using 2.75 mL of boron trifluoride in methanol. The mixture was heated for 20 minutes. For the extraction of methylated fatty acids, solvent extraction with hexane and water in a 1:1 volume ratio was performed. This procedure was repeated three times, and the hexane fractions were pooled and used for GC analysis.

GC of fish oil and standard FAME was analyzed using Trace 3330 GC Ultra system, Thermoelectron Corporation, USA, in a DB-5 stationary column using nitrogen as carrier gas. Detection was done using the flame ionization detector (FID) at 300°C, and the injector temperature was 280°C. The gradient conditions for the analysis include the initial temperature of 160°C for 1 minute, then the temperature was increased at 5°C/min to 185°C in ramp 1 and maintained for 10 minutes. Then, the temperature was increased at 8°C/min to 240°C during ramp 2 and maintained at 240°C for 10 minutes. For quantification, calibration curves (concentration versus area under the curve) obtained from standard EPA and DHA methyl esters were utilized.

#### 4.1.3.6 Determination of density, viscosity, and moisture content

The measurement of density before and after refining fish oil was done on a digital density meter (DDM2911, Rudolph Research Analytical). The viscosity was measured using a Rolling Ball viscometer (Lovis 2000 M/ME, Anton Paar), which is based on Hoeppler's falling ball principle. The capillary of size 1.8 mm was used for the measurement of the viscosity of the fish oil. The moisture content was determined using Karl Fischer Titrator (899 Metrohm Coulometer, Metrohm India Pvt. Ltd) for fish oil samples before and after refining.

#### 4.1.3.7 Determination of trace metals

The fish oil was first digested using acids under controlled heating; a combination of nitric acid and hydrochloric acid is often used since it helps in the complete dissolution of metals. After digestion, the clear solution obtained was taken for analysis in ICP-OES (Inductively Coupled Plasma Optical Emission Spectroscopy) to determine the concentration of these metals. Multiple elements can be analyzed in ICP-OES with high sensitivity and accuracy.

### 4.1.4 Oxidative stability studies of fish oil

The ability of 3,4-DHPA and the synthesized esters to enhance the storage oxidative stability was studied in refined fish oil. 3,4-DHPA and its esters were prepared in ethanol and were added in calculated quantities to obtain a 0.9 mM final concentration. Binary combinations of hydrophilic 3,4-DHPA and lipophilic esters were made using equimolar concentrations to obtain the maximal concentration of 0.9 mM. The solvent was evaporated by purging nitrogen and 5 g refined fish oil was added. The oil was then homogenized for 5 min to ensure better dispersion of the antioxidants throughout the oil. Refined fish oil without any added antioxidants was used as control and was treated similarly to the fish oil samples containing antioxidants. Synthetic antioxidants TBHQ and BHT were also utilized for comparative studies. The glass vials were kept at 35°C in the dark in contact with atmospheric air for 30 days. Every 3 days, the oil samples were taken, and peroxide and *p*-anisidine values were determined using methods described in sections 4.1.3.3 and 4.1.3.4, respectively. In addition, the acid values of all the samples at the end of the 30-day storage were measured by the method described in 4.1.3.2 and compared to that of the fish oil sample on the 0th day of analysis.

### 4.1.5 Statistical analysis

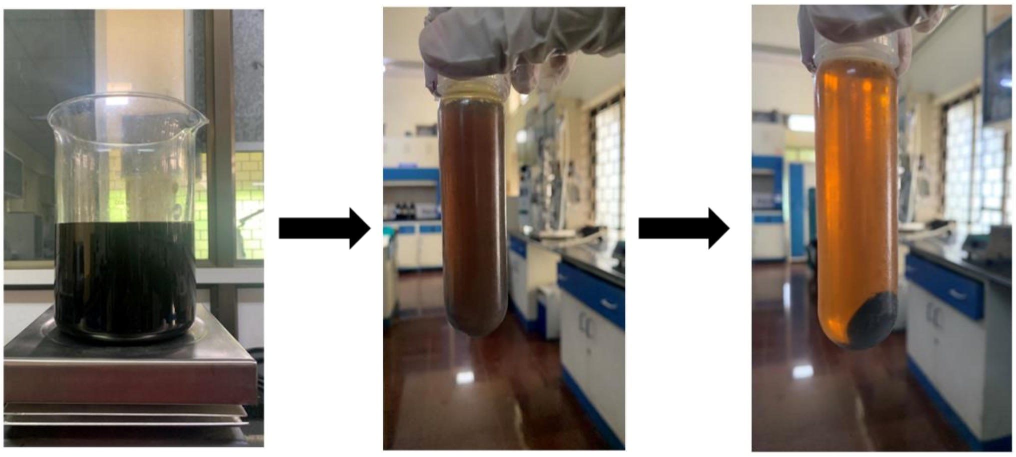
All experiments were done in triplicates and were reported as mean values with standard deviation. The obtained data mean was compared through analysis of variance (ANOVA) using OriginPro, OriginLab Corporation, Northampton, MA, USA, and significance was determined for $p < 0.05$.

## 4.2 RESULTS AND DISCUSSION

### 4.2.1 Refining and Characterisation of fish oil

Fish oil is a great source of n-3 PUFAs, particularly EPA and DHA, which offer numerous health benefits to humans. However, the presence of these unsaturated fatty acids makes them highly vulnerable to oxidative damage, which is further aggravated by impurities present in crude fish oil. To improve the shelf life and stability of fish oil, it is refined to remove these impurities, including phospholipids, FFAs, trace metals, and water. Conventional steps such as degumming, deacidification, and bleaching are utilized to refine fish oils without removing essential fatty acids. Therefore, it is often necessary to characterize the refined fish oil before conducting oxidative stability studies. Several analyses are carried out to study the physical and chemical parameters and compare them with crude fish oil.

Phospholipid content estimation after refining of fish oil is essential for several reasons. It helps to control and understand their presence in refined fish oil for purity, quality control, and product stability. The method involves the direct reaction between the chromogenic solution and the phosphorous moiety of phospholipids, resulting in the formation of a Prussian blue complex. During degumming, the majority of phospholipids, along with some trace metals and mucilaginous substances, are removed. The treatment with acid leads to the conversion of hydratable and non-hydratable phospholipids into hydrophilic due to the low pH and forms sludge (Fig. 4.1). It can be then easily removed through centrifugation (Charanyaa et al. 2017). In our study, there was a 99.69% reduction in the phospholipid content after refining fish oil (Table 4.1). A similar efficiency (96.34%) was observed by using citric acid in soybean oil by Sampaio et al. (2015). The degumming process can also remove trace metals at low pH and precipitate along with phospholipids. Charanyaa et al. (2017) have observed a considerable reduction in trace metal content after degumming using 5% orthophosphoric acid in sardine oil.

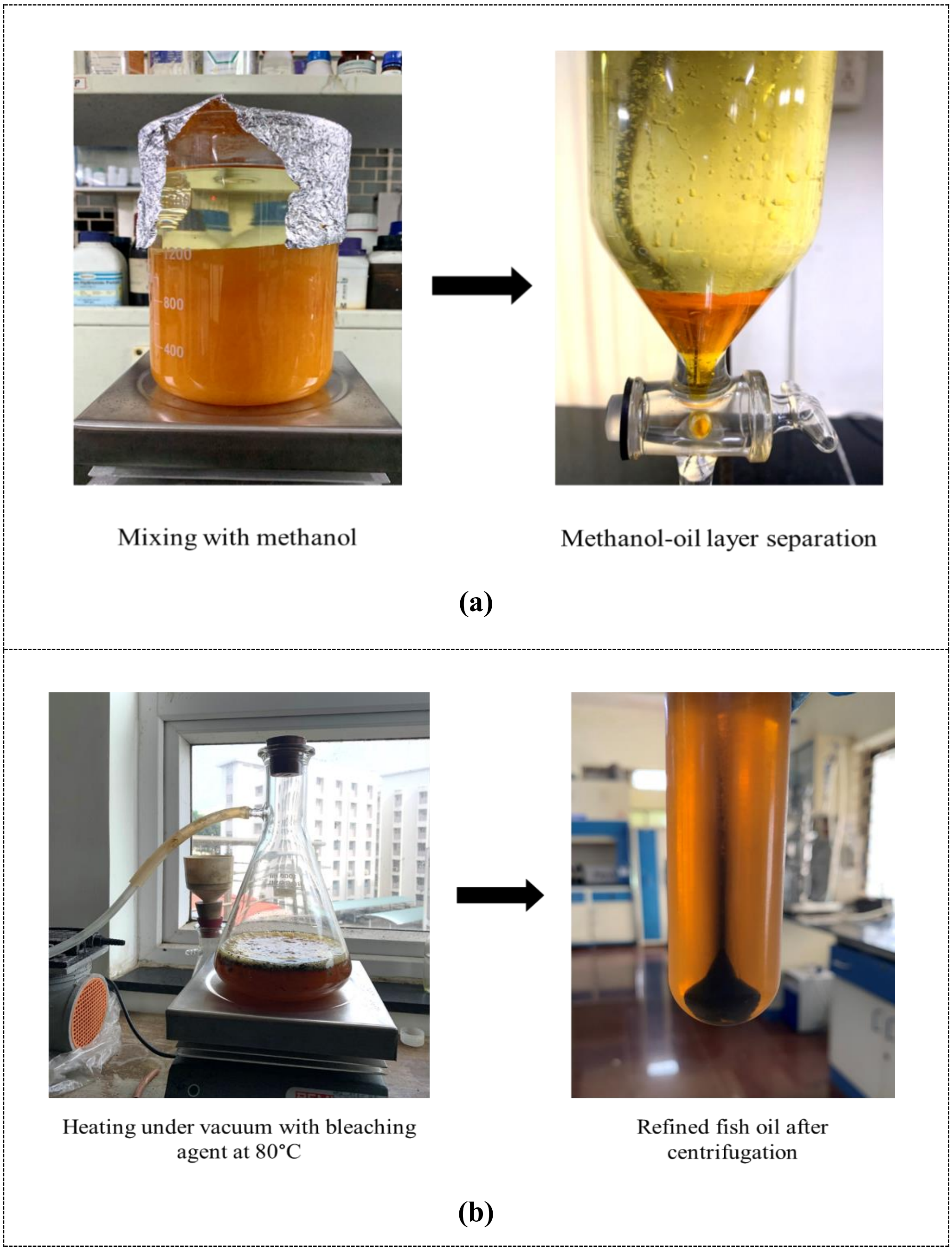


Mixing with degumming agent

Before centrifugation

After centrifugation

**Fig. 4.1 Degumming of crude fish oil**



Mixing with methanol

Methanol-oil layer separation

(a)

Heating under vacuum with bleaching agent at 80°C

Refined fish oil after centrifugation

(b)

**Fig. 4.2 (a) Deacidification and (b) bleaching of fish oil**

Free fatty acids present in fish oil act as accelerants of oxidation, leading to spoilage of fish oil during storage and transport. They are more reactive than the fatty acids which are present as esters. Hence, acid value is measured as an indicator of the quality, stability, and shelf life of fish oil. Solvent extraction using methanol was carried out instead of the conventional neutralization step to remove these FFAs from degummed fish oil. Short-chain alcohols were better at enriching EPA and DHA and were able to be completely separated from the oil phase (Charanyaa et al. 2017). The acid value reduced from 7.57 mg KOH/g to 3.64 mg KOH/g after treatment with methanol. Two distinct layers of oil and methanol were observed in the separating funnel, indicating complete phase separation (Fig. 4.2a). The method is also characterized to have higher extraction selectivity for FFAs (Charanyaa et al. 2017).

**Table 4.1 Characterisation of crude and refined fish oil**

| Parameters evaluated | Values in crude fish oil | Values in refined fish oil |
|---|---|---|
| Density (g/cm$^3$) at 35°C | 0.917 | 0.912 |
| Viscosity (mPa.s) at 35°C | 26.76 | 25.73 |
| Peroxide value (mEq.$O_2$/kg) | 7.85 | 3.83 |
| *p*-anisidine value | 1.34 | 0.84 |
| TOTOX value | 17.04 | 8.50 |
| EPA content (mg/mL) | 4.02 | 3.73 |
| DHA content (mg/mL) | 1.08 | 0.97 |
| Moisture (ppm) | 1678.3 | 939.5 |
| Phospholipid content (µg/kg) | 21.37 | 0.065 |
| Acid value (mg KOH/g) | 7.57 | 3.64 |
| Cu (mg/L) | 1.08 | 0.67 |
| Zn (mg/L) | 2.58 | 1.14 |
| Fe (mg/L) | 9.21 | 6.56 |
| Ni (mg/L) | 0.61 | 0.31 |

The overall profile of the fish oil has improved after refining, resulting in a reduced amount of impurities. The density and viscosity of the fish oil have reduced (Table 4.1), which could be due to the removal of mucilaginous substances, including phospholipids, and further bleaching has also improved the color and clarity of the oil (Fig. 4.2b). The method was also efficient in removing trace amounts of water as measured through Karl Fischer titration. The amount of trace metals such as iron, copper, zinc, and nickel has also reduced considerably after refining, indicating better quality of the oil (Table 4.1). These minor components might act as intrinsic factors and can influence the mobility of the surface-active agents, such as antioxidants, around the site of the oxidation reaction.

The fatty acid profile of the crude and refined fish oil was analyzed using GC (Fig. 4.3). The fatty acid methyl esters were extracted from oil in hexane and were detected using FID with nitrogen as the carrier gas. Peaks corresponding to EPA and DHA were found in both samples at 21.2 mins and 24.3 mins, respectively. EPA and DHA together comprised approximately 10.6% (w/w) of the total fatty acids in refined fish oil. The standard methyl esters of EPA and DHA were used to prepare the calibration curve, from which the concentration of the fatty acids was calculated. The fish oil was also found to have other saturated and unsaturated fatty acids, which are denoted by the other peaks observed. As per the temperature gradient elution, the saturated fatty acids are eluted first, followed by mono and polyunsaturated fatty acids. The refining method yielded a reasonable quantity (Table 4.1) of these n-3 PUFAs, which indicates that the loss was minimal. In addition, the TOTOX value of crude fish oil was reduced by about 50% after refining. This could be attributed to the removal of free fatty acids, oxidation products, and trace metals during the various steps of refining. Overall, the quality of refined oil has improved significantly in terms of physical and chemical properties suggesting that the method was optimal in refining locally sourced fish oil.

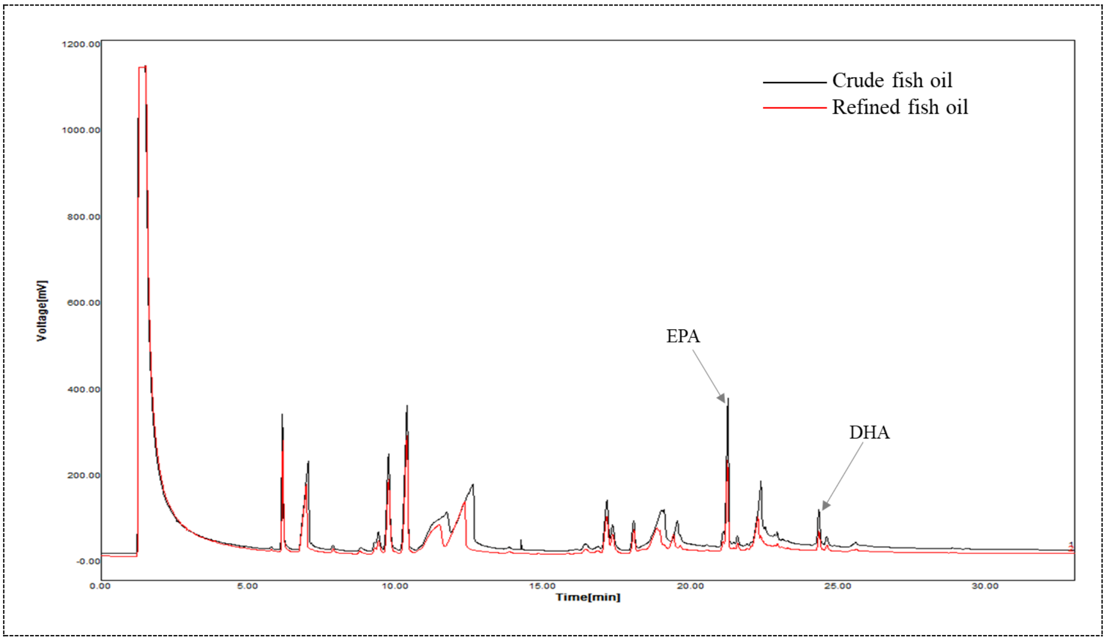


**Fig. 4.3 GC chromatogram of crude and refined fish oil**

### 4.2.2 Oxidative stability studies

The effectiveness of the 3,4-DHPA and its lipophilic esters in retarding oxidation in refined fish oil was examined for a period of 30 days at 35°C in the dark. The vials were stored uncovered, thereby allowing them to be exposed directly to the air within the surrounding environment in the orbital shaker. All antioxidants were used at the maximum permissible concentration of 0.9 mM, and TBHQ and BHT were used as a positive control for the study. The present study involved the measurement of peroxide and *p*-anisidine values at three-day intervals to assess the formation of primary and secondary oxidation products, such as hydroperoxides and aldehydes, respectively. The objective was to evaluate the extent of deterioration of the fish oil in the presence of antioxidants during accelerated oxidation. This enabled the identification and quantification of oxidation products, which are known to reduce the quality and safety of the fish oil. The analysis will provide insights into oxidative changes in the fish oil, helping to develop mitigation strategies. TOTOX value is calculated by combining the peroxide and *p*-anisidine values. It is based on the assumption that an increase of one unit in peroxide value is equal to an increase of two units in *p*-anisidine value (Shahidi and Wanasundara 2002). Hence, it provides the overall oxidative status of fish oil at a given time.

During the initiation phase of oxidation, peroxides are formed when double bonds of n-3 PUFAs lose hydrogen due to heat, light, metals, or other initiators. These free radicals react with oxygen to create peroxyl radicals, which continue the oxidation chain reaction. Aldehyde carbonyl bonds present in non-volatile α-unsaturated aldehyde compounds (2-alkenals and 2,4-dialkenals) react with amine groups of p-anisidine. This results in the formation of a Schiff base having a maximum absorbance at 350 nm, which is measured to calculate the *p*-anisidine value (Barriuso et al. 2013). The method has been widely adapted for the quantification of secondary oxidation products since it can be associated well with peroxide content and flavor acceptability scores (Shahidi and Wanasundara 2002). It is not advisable to depend on just one technique to evaluate the oxidative status of fish oil since the peroxide value by itself may be misleading and might not be a comprehensive indicator of all the oxidation products that are present in the oil. The TOTOX value is a composite index of two parameters with distinct unit dimensions for assessing the oxidation of oils and fats. The peroxide value, when used alone, can lead to erroneous results, particularly at advanced stages of oxidation, where it may be very low due to degradation of the formed hydroperoxides into secondary oxidation products. Conversely, the p-anisidine value is likely to be very high (Lembke and Schubert 2014).

#### 4.2.2.1 TOTOX values

TOTOX values of fish oil containing different antioxidants were illustrated in Fig. 4.4, and the control samples without any antioxidants reached the maximum value of 270.71 ± 8.62. The TOTOX value of methyl ester had significantly reduced, and its efficiency was similar to that of the native 3,4-DHPA and BHT on almost all days of analysis. Hradkova et al. (2013) described similar results in their study of alkyl esters of several phenolic acids in tocopherol-rich sunflower oil. In the Rancimat test, the lipophilized esters of hydroxytyrosol, a similar phenolic antioxidant with a catechol group, showed better efficiency than α-tocopherol and BHT (Trujillo et al. 2006). The OH groups located in the *ortho* position of the phenolic ring have the tendency to decrease bond dissociation energies, making it easier to abstract the H atom and stabilize the resulting radical (Wright et al. 2001). This elucidates the competent antioxidative potential of a natural phenolic acid such as 3,4-DHPA. According to the DPPH and ABTS assays, native 3,4-DHPA exhibited the highest radical scavenging potential, as evidenced by low $IC_{50}$ values. Considering such a mode of action behind the antioxidants, acylation at the carboxylic group, as confirmed by the NMR data, will not have any effect on the antioxidant potential. According to Fig. 4.4, the native and methyl DHPA performed similarly. This suggests that 3,4-DHPA and its methyl ester have comparable potential in fish oil, as they did not show any significant difference in the $IC_{50}$ values.

The relatively reduced antioxidant potential of butyl and hexyl esters suggests the involvement of factors beyond the position of the hydroxyl group in the phenolic compound. Lipid oxidation can be better understood by analyzing molecular locations in space, particularly at nanoemulsion surfaces, rather than solely by chemical reactions. Therefore, the methyl esters' potential was similar to 3,4-DHPA which could be due to the close polarity of the antioxidants. Upon further examination of the influence of the polarity of the esters, it was observed that the hexyl and butyl esters of

3,4-DHPA showcased a reduction of approximately 6.92% and 12.9%, respectively. Meanwhile, the native and methyl esters of 3,4-DHPA demonstrated a significant reduction in TOTOX values by 36.2% and 36.3%, respectively (Fig. 4.6). A lower scavenging activity was observed in *in vitro* assays for butyl and hexyl esters, which suggests that a higher concentration of the antioxidants is required to scavenge the radicals (higher $IC_{50}$ values). During the 30-day study, TBHQ proved to be the best synthetic antioxidant, with a maximum reduction of 85.6% in the TOTOX value, reaching only 38.91 ± 3.34 at the end. Based on the results of *in vitro* assays (as shown in Fig. 3.8, 3.9, 3.10), it is evident that the performance of TBHQ is relatively poor. This suggests that the oxidation occurring in a dynamic bulk oil system is influenced by multiple factors. It is possible that TBHQ's reported ability to quench singlet oxygen contributes to its ability to further prolong the induction period in bulk oil systems (Lee and Jung 2010).

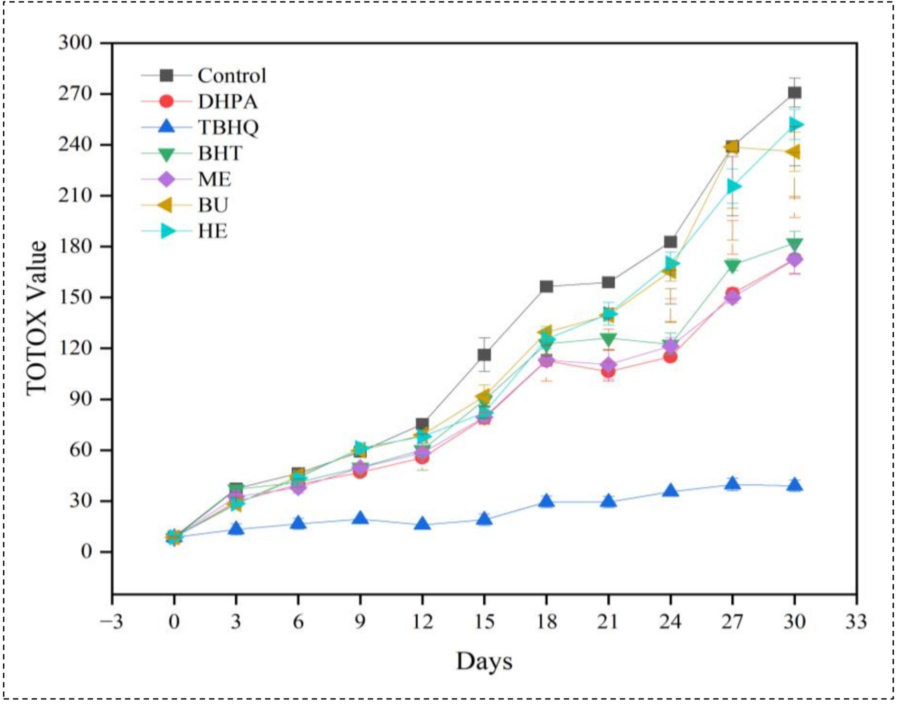


**Fig. 4.4 TOTOX Values of fish oil fortified with various antioxidants during oxidative stability study**

In order to effectively retard oxidation, it is imperative that the antioxidants present in bulk oil are capable of accessing the oxidation site that is located at the air-oil interface. The polar paradox theory posits that a hydrophilic antioxidant, when utilized in bulk oil, may concentrate at this air-oil interface. In contrast, a hydrophobic antioxidant may dissolve into the oil, resulting in reduced efficacy. This phenomenon is particularly noteworthy due to the potential ramifications it may have on the long-term stability of oils (Laguerre et al. 2015). Thus, the most polar antioxidants native 3,4-DHPA (miLog P 0.39) and its corresponding methyl ester (miLog P 1.01) exhibited similar antioxidant potential with an efficient reduction in oxidation at the end of 30 days of storage (Fig. 4.4), in accordance to the polar paradox theory.

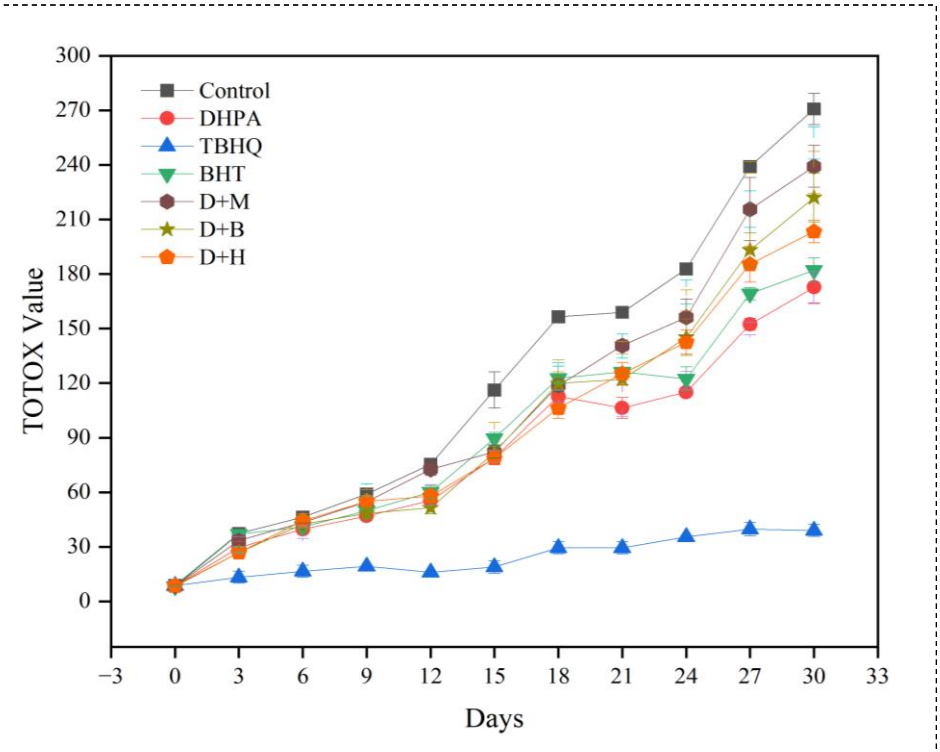


**Fig. 4.5 TOTOX values of binary combinations of 3,4-DHPA and its esters under oxidative stability study**

Increasing the length of the alkyl chain increases the hydrophobicity of the esters, which in turn increases their solubility in bulk oil. While the solubility of butyl and hexyl esters in sardine oil can be expected to increase, there was a significant reduction in their efficiency in reducing the oxidation of bulk oil (Fig. 4.6). With the increase in solubility of antioxidants, there is also a concurrent increase in the heterogeneity of the lipid system, resulting in more amphiphilic pockets of oxidation that require unique antioxidant requirements (Vaisali et al. 2022). The percentage reduction of TOTOX values (Fig. 4.6) of the methyl, butyl, and hexyl esters was found to decrease, which could indicate the effect of acyl chain length on the antioxidant potential. According to the study by Peng and Shahidi, short-chain esters of resveratrol exhibit better antioxidative potential than medium and long-chain esters (Peng and Shahidi 2022). A similar decreasing trend was observed after the esterification of ferulic acid and hydroxytyrosol in their effectiveness in bulk oil systems (Fang et al. 2006; Medina et al. 2009). The hexyl and butyl esters exhibit lower antioxidative activity compared to the methyl ester at lower concentrations in the *in vitro* assays, which is reflected in the oxidative stability studies. Meanwhile, at the highest concentrations studied in DPPH and ABTS radical scavenging assays, the performance of the esters was comparable to the native 3,4-DHPA, except for the FRAP assay. The measurement of the ability of the antioxidant to donate hydrogen atoms to free radicals such as DPPH and ABTS is crucial. It shows how well the antioxidants can combat peroxide radicals in bulk oil during oxidation (Shahidi and Wanasundara 2002). This aids in realizing the effect of concentration on the antioxidative potential, i.e., the ability to act as primary antioxidants after esterification.

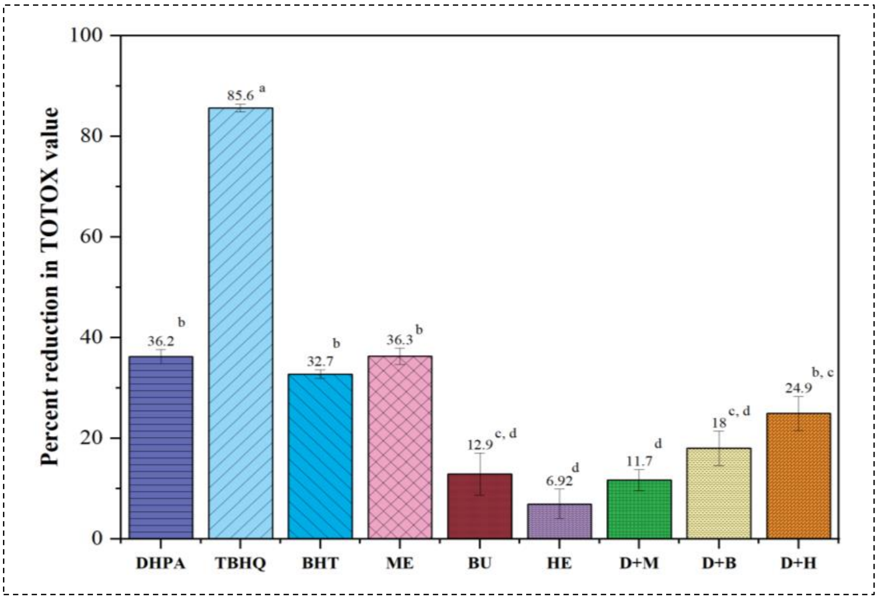


**Fig. 4.6 Percent reduction in TOTOX values at the end of 30 days of oxidative stability study in fish oil with antioxidants**

The synthetic antioxidant TBHQ, despite having lower radical scavenging activity as measured by the *in vitro* assays, performed well in the bulk oil system (Mishra et al. 2022). The results of TBHQ and 3,4-DHPA esters differ greatly between *in vitro* and bulk oil studies, suggesting that the efficacy of these antioxidants in preventing oxidation also depends on their ability to accumulate efficiently on the sites of oxidation in a given medium. The study showed that TBHQ outperformed the phenolic acid and its esters in a bulk oil system. This suggests that while *in vitro* assays can precisely measure an antioxidant's ability to scavenge free radicals, this may not accurately reflect the effectiveness of the same antioxidant when used in a bulk oil system. This is likely because the mechanisms through which antioxidants reduce oxidative damage may differ between the two systems. Moreover, the solubility of esters of increasing chain lengths in hydrophilic solvents used *in vitro* assays may differ from that of lipophilic systems like bulk oils. The study results seem to agree with the polar paradox theory, where relatively hydrophilic antioxidants (native and methyl 3,4-DHPA) showed better antioxidant activity than hydrophobic counterparts (butyl and hexyl ester). However, the hydrophobic TBHQ showed the best performance in bulk oil, contradicting the polar paradox theory. Hence, more studies need to be conducted to confirm the concurrence with the polar paradox theory.

The ability of the antioxidants to reach the potential oxidation sites in bulk oil greatly affects the formation of oxidation products. This study uses all the antioxidants in the concentration of 0.9 mM irrespective of their polarity. Increasing the concentration of lipophilic ester could effectively reduce oxidation and prolong the induction period. In this study, the control sample reached a TOTOX value of 156.42 on the 18th day. Meanwhile, other samples (except for butyl and hexyl esters) reached this maximum value only on the 24th day of analysis. This demonstrates that 3,4-DHPA, its methyl ester, and their binary combinations efficiently prolonged the induction times. After day 24, there was a considerable increase in the TOTOX values of butyl and hexyl esters, while the methyl 3,4-DHPA had a reduced value similar to 3,4-DHPA. The present study suggests that during the induction period, the butyl and hexyl esters, which exhibited lower antioxidative potential, were entirely exhausted. As a result, there might not have been any esters remaining to combat oxidation. This underscores the importance of using these esters in higher concentrations to extend their activity throughout the storage period. Due to their higher lipophilicity compared to 3,4-DHPA, the butyl and hexyl esters can be added to fish oil in higher concentrations without causing solubility issues, as suggested by Mishra et al. (2021). On the other hand, it is possible that as the concentration of oxidized products in the lipid system increases, the system becomes more heterogeneous. This may prevent the interaction of phenolic and its esters with the system. In such a dynamic system, small pockets of oxidation may form, which can drive the oxidation process further (Budilarto and Kamal-Eldin 2015). Antioxidant strategies for such a system need to be customized as they do not adhere to the typical 'polar paradox theory.'

The polar paradox theory does not take into account important factors such as the arrangement of OH groups, double bonds, and side chains of the phenolic antioxidants. It is solely based on the hydrophilic-lipophilic nature of antioxidants. For example, according to the polar paradox theory, 3,4-DHPA (milog P 0.39), which is considered hydrophilic, should be a better antioxidant than the relatively hydrophobic TBHQ (milog P 3.08) (Table 5.1). However, TBHQ is a better antioxidant in bulk oil than 3,4-DHPA. Similarly, TBHQ and hexyl ester are expected to perform similarly, but the study revealed an alternative trend (Fig. 4.6). Due to safety concerns, synthetic antioxidants, including TBHQ and BHT, have been banned from usage in certain countries (Mishra et al. 2022). Hence, 3,4-DHPA and its esters can potentially replace BHT as antioxidants in oil systems while providing equivalent oxidative stability. The "polar paradox theory" suggests that the polarity of antioxidants is a significant factor in their behavior. However, when it comes to the bulk fish oil system, this theory only partially governs the behavior of antioxidants. Hence, the increased lipophilicity of the synthesized esters enables their unrestricted usage in bulk oil systems as compared to the native 3,4-DHPA.

Interestingly, when combined with 3,4-DHPA in equimolar proportions, the trend observed with lipophilic esters was reversed (Fig. 4.5, 4.6). The hexyl ester combination DH 1:1 showed the best reduction in TOTOX, followed by butyl and methyl ester combinations, respectively (Fig. 4.6). The reduction in the potential of the methyl ester in equimolar combination with 3,4-DHPA could be associated with the close polarity of the two compounds (Vaisali et al. 2017). Rather than improving the antioxidative potential through regeneration of the primary antioxidant or sacrificial oxidation of one of the antioxidants, antioxidants with similar polarity and the same mechanism of action could reduce the efficacy of the blends due to competitive interaction at the oxidation sites when used in equal concentrations (Mishra et al. 2021). However, improved oxidative stability was observed when the polarity was varied in the presence of butyl and hexyl esters with higher log P values. Though the acyl chain length had an effect on the antioxidative ability of the esters, no synergistic effect was observed with any of the combinations. This could be attributed to the application of phenolic acid and its esters in equimolar ratio. The ability of the antioxidant combination to reach the site of oxidation is dependent on the concentration used and the interaction between the amphiphilic compounds with other surface-active agents present in oil. Hence, optimization of the molar ratio of the combinations might improve the overall interactive efficiency since the concentration of individual antioxidants plays a significant role (Mishra et al. 2023).

#### 4.2.2.2 Acid Values

The FFAs present in fish oil could act as pro-oxidants, leading to the acceleration of oxidation reaction. They can bind to trace metals and facilitate their mobility, bringing them closer to oxidation sites. Additionally, FFAs are surface-active which could modify the microenvironment of oxidation. The accumulation of these FFAs can also reduce the pH, altering the behavior of other antioxidative compounds present in fish oil. Short-chain FFAs released during oxidation through hydrolysis are the major reason for the development of rancid off-flavors (Bhosle and Subramanian 2005). By monitoring the acid value alongside other oxidative stability parameters such as peroxide value, anisidine value, and TOTOX value, a comprehensive understanding of the fish oil's stability and shelf life can be obtained. Thus, measurement of acid value is essential for ensuring the quality of fish oil and understanding how different storage conditions or antioxidants can influence the oil's stability.

The fish oil samples fortified with different antioxidants after the completion of the oxidative stability study for 30 days were measured and compared with the fish oil sample before the start of the study (Fig. 4.7). The influence of the presence of 3,4-

DHPA and its esters and their binary combinations on acid value was not significantly different ($p > 0.05$). However, control sample at the end of 30th day (4.12 mg KOH/g of oil) and the fish oil sample containing TBHQ (3.56 mg KOH/g of oil) were significantly different ($p > 0.05$).

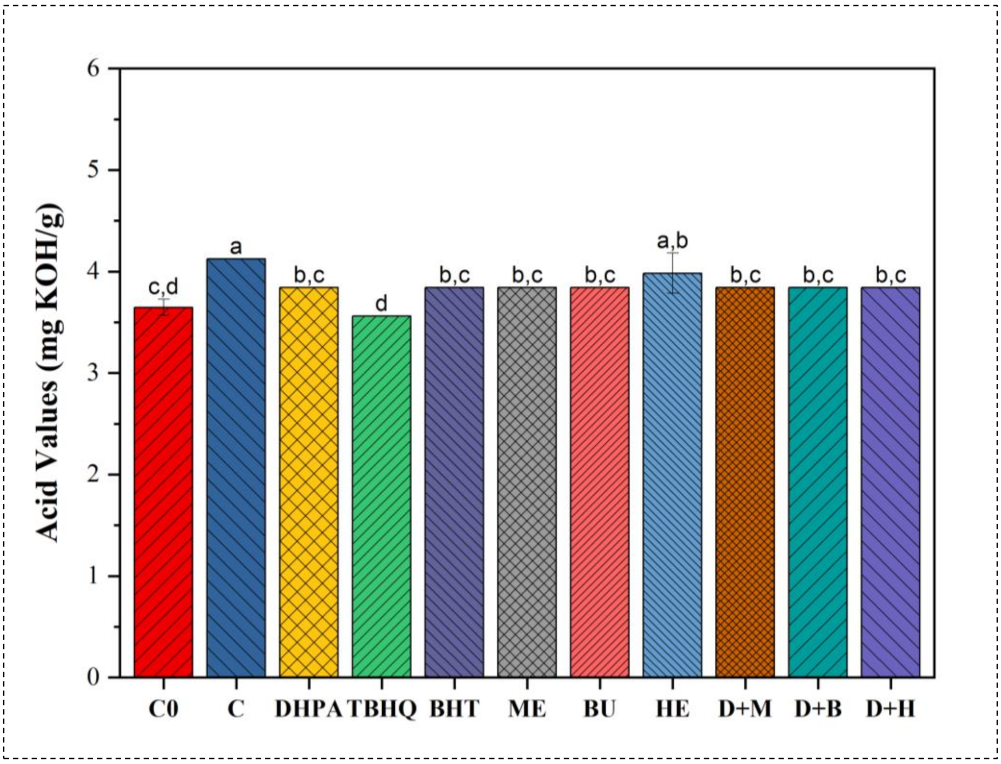


**Fig. 4.7 Acid values of fish oil samples fortified with different antioxidants. Bars represent standard deviation (n=2).**

Among the esters, the hexyl DHPA showed the highest acid value of 3.98 ± 0.19 mg KOH/g of oil. This trend was similar to the observed TOTOX values wherein the hexyl ester showed the least protective effect on fish oil oxidation. Meanwhile, all other samples had an acid value of 3.84 mg KOH/g of oil, which indicates that all antioxidants, including BHT, were equally able to reduce the formation of FFAs from the hydrolysis of triglycerides of fish oil. Though during refining, there were significant variations in acid values, during oxidation, the increase in acid value was minimal. This further suggests that FFAs are released as a result of the hydrolytic degradation of triglycerides caused by the presence of trace amounts of water (Charanyaa et al. 2019).

## 4.3 SUMMARY AND CONCLUSIONS

- The refining of crude fish oil obtained without any added antioxidants was successfully carried out using the method developed in our laboratory previously with good yields.
- The physico-chemical properties of refined oil were better than the crude fish oil in various aspects, indicating the good quality of fish oil.
- Primary and secondary oxidation indicator parameters, including acid value, peroxide value, p-anisidine value, and TOTOX values, were found to decrease considerably after refining.
- Oxidative stability studies in bulk oil revealed that among the phenolic esters, methyl ester showed higher efficacy relative to butyl and hexyl esters at the end of 30 days at 35°C.
- The efficiency of the phenolic acid and its esters does not entirely depend on the polarity but also on the physical location of the esters at the site of oxidation.
- This study further showed compliance with the polar paradox theory, with hydrophilic compounds (3,4-DHPA) showing better antioxidant potential than their lipophilic counterparts.
- However, the hydrophobic TBHQ showed the best oxidative stability throughout the storage study, contradicting the polar paradox theory.
- The binary combination of 3,4-DHPA with the hexyl ester was better performing than the combinations with methyl and butyl ester in equimolar ratio.
- Minimal changes in acid value were found at the end of the 30-day storage study indicating that the reduced release of FFA is due to the absence of water in the fish oil samples.

# CHAPTER 5

# APPLICATION OF LIPOPHILIC ESTERS OF 3,4-DHPA IN OXIDATIVE STABILITY STUDIES OF FISH OIL CONTAINING TRACE WATER

# CHAPTER 5

The presence of several intrinsic and extrinsic factors accelerates oxidation in a bulk oil system. For example, storage of crude fish oils at reduced temperatures (-18°C) led to an increase in shelf life two-fold compared to the fish oil stored at 4°C (Boran et al. 2006). Additionally, among the extrinsic parameters influencing hydrolytic and oxidative stability studied in refined fish oil, the amount of moisture present had a significant impact (Charanyaa et al. 2019). In the presence of moisture, the triglycerides present in fish oil could be hydrolyzed into mono- and di-acylglycerols, glycerol, and fatty acids. The release of these products results in the formation of association colloids, which act as sites of oxidation in a bulk oil system. These reverse micelle-like structures become the major site of oxidation rather than the oil-air interface, where oil comes in contact with oxygen from the atmospheric air. In such sites, the behavior of antioxidants varies from that of their effectiveness in a homogeneous bulk system.

Oftentimes, the refined fish oils stored for a longer time exceed the limit of maximum moisture content of about 0.3%, which can result in the formation of harmful hydrolytic oxidation products. In addition, these oils contain minor components such as free fatty acids, monoacylglycerols, diacylglycerols, sterols, phospholipids, phenolics, and lipid oxidation products (hydroperoxides, aldehydes, and ketones). These surface-active compounds react with trace water to form reverse micelles resulting in a change in the microenvironment of oxidation. These surface-active components are molecules that are amphiphilic in nature, comprising a hydrophilic part and a hydrophobic tail (Wang et al. 2024). They can arrange themselves into reverse micelles at concentrations above their critical micelle concentration (CMC) in non-polar media. Since the presence of minor components varies widely, these association colloids are structurally and compositionally complex. Hence, the study on the presence of trace water to examine the efficiency of 3,4-DHPA and its lipophilic esters was carried out in fish oil.

Mixtures of antioxidants have been shown to perform better than single antioxidants, indicating a synergistic effect. However, designing a mixture of antioxidants specific to fish oil would require optimizing the concentration of each antioxidant component to bring a synergistic effect on their overall potential (Mishra et al. 2023). Thus, the current study focused on the optimization of the molar concentrations of the antioxidants to improve their efficiency as a mixture in fish oil containing trace water. The results were compared with synthetic antioxidants TBHQ and BHT and also with the oxidative stability study without trace water adjustment.

## 5.1 MATERIALS AND METHODS

### 5.1.1 Materials

The water content of the fish oil samples was measured using a Karl Fischer titrator (899 Metrohm Coulometer, Metrohm India Pvt. Ltd).

### 5.1.2 Oxidative stability studies in fish oil containing trace water

The ability of 3,4-DHPA and its esters in varying molar combinations in retarding lipid oxidation in fish oil containing trace water was studied along with synthetic antioxidants TBHQ and BHT. Ethanolic solution of antioxidants in calculated quantities (according to Table 5.1) was added to amber glass vials, and the solvent was evaporated using nitrogen. All fish oil samples had a final antioxidant concentration of 0.9 mM in 5 mL of refined fish oil. Trace water content in all oil samples was adjusted to 0.3% (w/w) using Karl Fischer titrator. The oil samples were homogenized and incubated at 35°C in darkness in contact with atmospheric air for 30 days. Samples were withdrawn every 3 days to measure peroxide and *p*-anisidine values using methods described in sections 4.1.3.3 and 4.1.3.4, respectively. The acid value was measured using the method described in 4.1.3.2 at the end of 30 days and was compared with control samples at the 0th and 30th days of analysis.

**Table 5.1. Antioxidants analyzed in different molar concentrations for oxidative stability studies in fish oil with 0.3% trace water**

| Sample ID | Phenolic acid | Ester | Molar Ratio (Phenolic acid: Ester) |
|---|---|---|---|
| C | - | - | - |
| DHPA | 3,4-DHPA | - | - |
| ME | - | Methyl 3,4-DHPA | - |
| BU | - | Butyl 3,4-DHPA | - |
| HE | - | Hexyl 3,4-DHPA | - |
| TBHQ | TBHQ | - | - |
| BHT | BHT | - | - |
| DM 1:1 | 3,4-DHPA | Methyl 3,4-DHPA | 1:1 |
| DB 1:1 | 3,4-DHPA | Butyl 3,4-DHPA | 1:1 |
| DH 1:1 | 3,4-DHPA | Hexyl 3,4-DHPA | 1:1 |
| DM 2:1 | 3,4-DHPA | Methyl 3,4-DHPA | 2:1 |
| DB 2:1 | 3,4-DHPA | Butyl 3,4-DHPA | 2:1 |
| DH 2:1 | 3,4-DHPA | Hexyl 3,4-DHPA | 2:1 |
| DM 1:2 | 3,4-DHPA | Methyl 3,4-DHPA | 1:2 |
| DB 1:2 | 3,4-DHPA | Butyl 3,4-DHPA | 1:2 |
| DH 1:2 | 3,4-DHPA | Hexyl 3,4-DHPA | 1:2 |
| AE | - | Methyl + Butyl + Hexyl-3,4-DHPA | 1:1:1 |
| AED | 3,4-DHPA | Methyl + Butyl + Hexyl-3,4-DHPA | 1:1:1:1 |

### 5.1.3 Statistical analysis

All experiments were done in triplicates and were reported as mean values with standard deviation. The obtained data mean was compared through analysis of variance (ANOVA) using OriginPro, OriginLab Corporation, Northampton, MA, USA, and significance was determined for $p < 0.05$.

## 5.2 RESULTS AND DISCUSSION

Lipid oxidation in bulk oil was initially thought to be a homogeneous system governed by the presence of antioxidants. However, recent research has revealed that lipid oxidation involves a balance between the antioxidants and prooxidants present. In addition to these chemical reactions, physical properties, including the partitioning effect of antioxidants in a food matrix, diffusion of oxygen, and interaction of the pro-oxidative factors with lipids, also play a crucial role in oxidation reactions (Decker 2010). Considering these interactive effects, bulk oil oxidation can be defined as a heterogeneous system comprising several minor components that impact its physical properties. Examples include amphiphilic surface-active components such as MAGs, DAGs, phospholipids, sterols, FFAs, and polar oxidation products, including hydroperoxides, aldehydes, and ketones (Chen et al. 2011).

Refined oils contain these minor amphiphilic molecules, which can form association colloids in the presence of small amounts of water. The structures formed in a non-polar media are generally reverse micelles wherein they have a hydrophilic core containing water (Fig. 5.1). Among other factors, the presence of water is an important deciding factor for the formation of these reverse micelles in bulk oils (Wang et al. 2024). Water can be absorbed from the atmosphere during exposure, and it is also produced during the molecular decomposition of hydroperoxides during peroxidation (Budilarto and Kamal-Eldin 2015).

The reverse micelles formed in non-polar media contain a hydrophilic core wherein the polar head groups extend while the aliphatic chains extend into the lipid medium. Their stability and composition vary depending on a large variety of surface-active components. The presence of these reverse micelles can affect the oxidation mechanism as well as the antioxidant activity. A pro-oxidative effect can be observed when the association colloids present can reduce the surface tension which leads to increased oxygen transfer to the oil (Wang et al. 2024). Also, lipid hydroperoxides and water-soluble metal ions can concentrate on the interface of the association colloids which could accelerate the conversion of lipid hydroperoxides to free radicals.

In some cases, synergistic effects were observed in bulk oils containing association colloids (Wang et al. 2024). The ability of these structures to accumulate different antioxidants at the interface could enhance their interactive effects significantly. Additionally, the surface-active nature of the amphiphilic antioxidant enables it to replace some of the DAGs and other minor components from the interface. Hence, the study of the potential of antioxidants in the presence of these reverse micellar structures is essential to efficiently utilize them for retarding lipid oxidation in such a complex system. The concentration of these amphiphilic antioxidants present in the mixture can be a deciding factor for their accumulation at the oxidation sites and their cumulative effect on oxidation. Therefore, the molar concentration of 3,4-DHPA and its lipophilic esters are varied in binary combinations in refined fish oil containing 0.3% trace water.

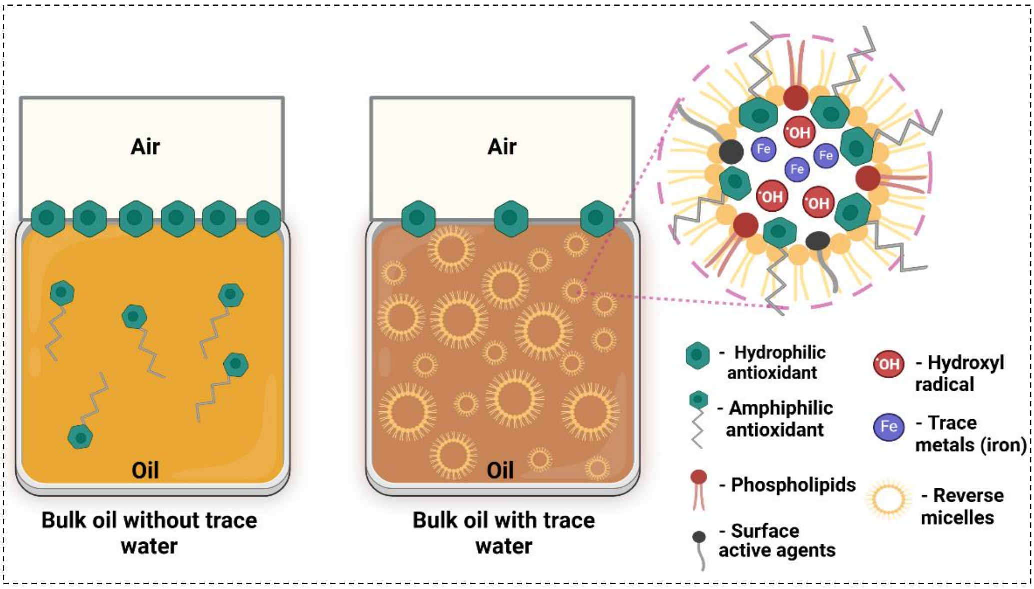


**Fig. 5.1 Reverse micelles in fish oil with trace water**

### 5.2.1 TOTOX values

Peroxide and *p*-anisidine values of fish oil samples with 0.3% trace water were measured every 3 days, and TOTOX values were calculated. TOTOX values of fish oil in the presence of different antioxidants for 30 days are illustrated in Fig. 5.2, 5.3. The control sample without any antioxidants was found to have a TOTOX value of 1386.64 ± 17.09 on the 30th day of storage. The value is 5.13 folds higher than the control sample without trace water adjustment (Fig 4.4). This indicates that the oxidation rate in control samples was higher in the presence of 0.3% trace water. The role of trace water is often underestimated in bulk oil, but the results of the fish oil sample without any antioxidants suggest that the oxidation is heavily influenced by the presence of trace water.

In fish oil without trace water adjustment, the interface between air and oil in bulk oil is the major site of oxidation (Medina et al. 2009). When trace water is introduced in bulk oil, oxidation is further accelerated by the water-oil interface formed due to the development of association colloids/reverse micelles (Kim et al. 2014; Park et al. 2014). Additionally, trace water can also hydrolyze triacylglycerols, resulting in the release of MAGs, DAGs, glycerol, and FFAs, causing further increase in the rate of oxidation (Budilarto and Kamal-Eldin 2015). It is evident from the TOTOX values that the formation of reverse micelles increases the surface area available for oxidation and improves the contact of the unsaturated lipids with pro-oxidative factors. This physical phenomenon influences hydrolytic oxidation, explaining the rapid and exponential increase in TOTOX values at the end of 30 days of the study.

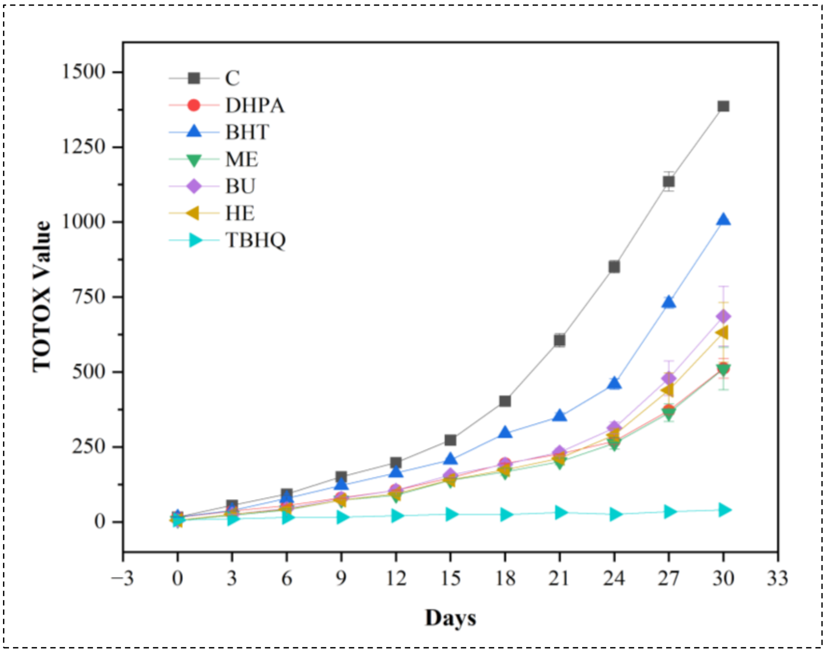


**Fig 5.2 TOTOX values of fish oil (0.3% trace water) containing individual antioxidants**

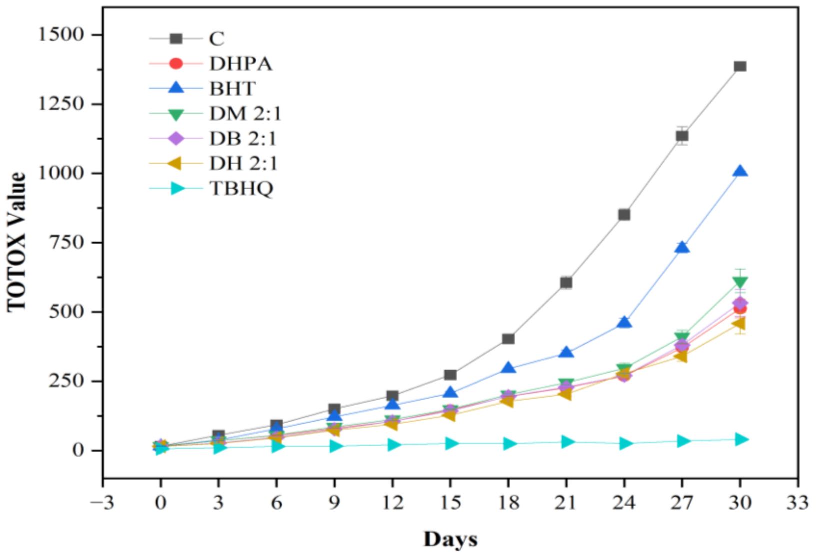
In the study of individual esters, methyl ester was found to have a reduced TOTOX value, followed by hexyl and then butyl ester. This seems to be in agreement with the polar paradox theory that hydrophilic antioxidants are efficient in bulk oils. However, the more non-polar hexyl-3,4-DHPA had the highest improvement in reducing TOTOX value in fish oil with trace water. The pattern observed was similar to their behavior in the FRAP and DPPH radical scavenging assay, which measures the potential of the antioxidants in aqueous solutions. It is also noteworthy that the lipophilic esters were able to interact better with the free radicals in the fish oil containing trace water rather than the fish oil with no trace water adjustment. This suggests that the *in vitro* assays are good indicators of the antioxidative potential of phenolic compounds.

Interestingly, the potential of BHT (Fig. 5.4) did not vary when compared to the oxidative stability studies in fish oil without trace water, as described in the previous chapter (Fig. 4.6). Meanwhile, the TOTOX value on the 30th day for 3,4-DHPA was much less than this synthetic antioxidant BHT. The efficiency of 3,4-DHPA was comparable to that of BHT in the previous study while it has significantly improved (49% higher than BHT) in the current study in fish oil containing 0.3% trace water. This indicates that the presence of trace water does not negatively affect the antioxidative potential of 3,4-DHPA. Hence, it could be a potential replacement for synthetic antioxidants, including BHT, for application in fish oil products containing trace amounts of water.

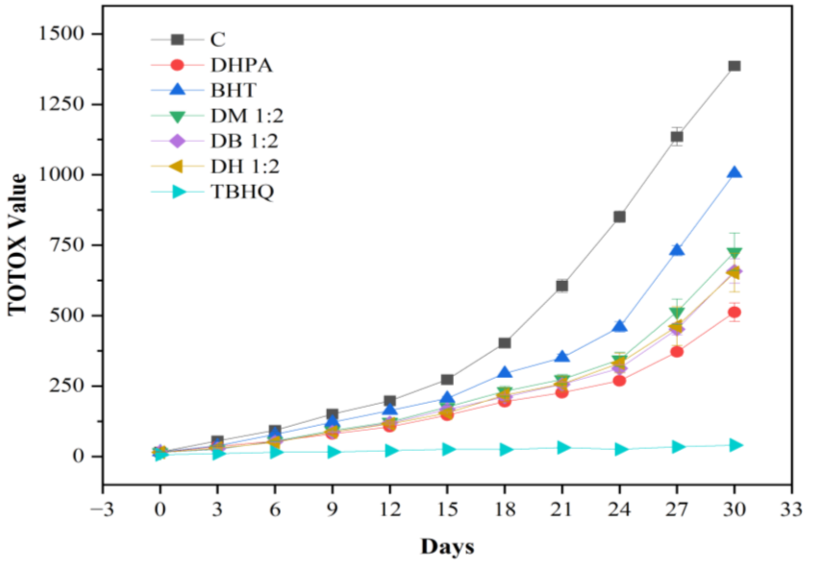
#### 5.2.1.1 Effect of binary combinations of 3,4-DHPA with its esters

Among the combinations, the hexyl ester showed the best synergistic effect with 3,4-DHPA when used in the molar ratio of 2:1 phenolic acid to the ester. A TOTOX value of 458.31 ± 38.44 was observed for DH 2:1, while 3,4-DHPA alone showed 512.22 ± 33.50, and hexyl ester alone showed 632.06 ± 57.53 at the end of 30 days. This is in agreement with the hypothesis that with increasing complexity in the oxidation process contributed by amphiphilic reactants, an equally complex and amphiphilic mixture of antioxidants is needed to counter it (Vaisali et al. 2022).

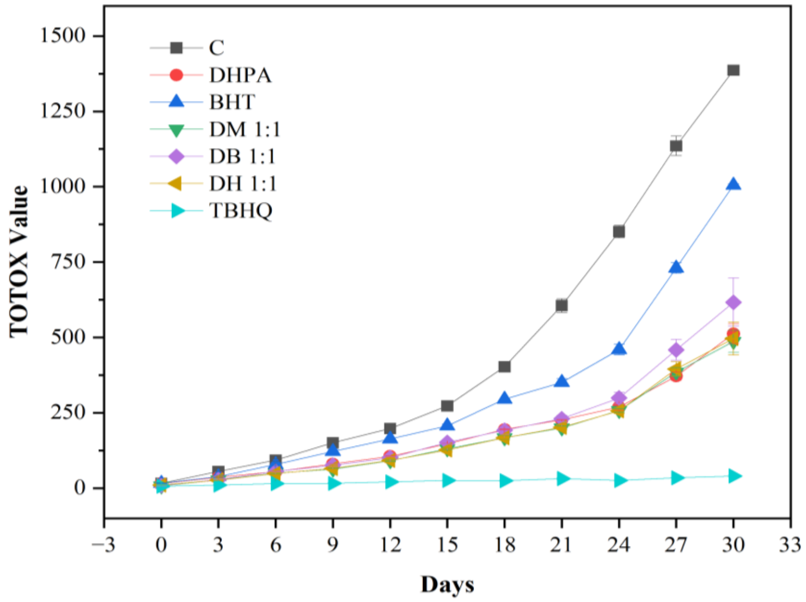
An extension in the induction period was observed in the presence of 3,4-DHPA and its lipophilic esters, separately and in their combinations. From day 15 to day 18, the control reached a TOTOX value of 402.51 ± 3.26 from 272.87 ± 2.97. Meanwhile, among the fish oil fortified with 3,4-DHPA, methyl, butyl, and hexyl esters and their binary combinations, a maximum TOTOX value of only 231.47 ± 17.39 was observed in the DM 1:2 with all other samples having lesser TOTOX values. Moreover, these samples exceed the TOTOX value of 400 only on the 27th day of storage (Fig. 5.3), indicating a significant prolongation of shelf life.



**(a)**



**(b)**



**(c)**

Fig. 5.3 TOTOX values of fish oil samples (0.3% trace water) containing binary combinations of antioxidants in different molar ratios

Although BHT is a synthetic antioxidant with good antioxidative capacity (Mishra et al. 2022), its potential was inferior to the natural phenolic acid 3,4-DHPA and its esters. The TOTOX values of BHT showed a similar trend to that of the control sample without antioxidants towards the end of the study. This indicates that the antioxidant might have been depleted over the storage period and resulted in an increased rate of oxidation. There was also a visible settling of oil as layers in the bottom of the glass vials in both control and BHT samples after 24 days, but it was not observed in other samples. This could suggest that polymerization of unsaturated fatty acids might occur in the absence of antioxidants, eventually resulting in the formation of large polymer structures affecting the physical properties of the bulk oil.

A comparison was made for hydrophilic and hydrophobic antioxidants in fish oil with and without 0.3% trace water adjustment (Fig. 5.4). All samples, except those containing BHT, showed a significant increase in reduction of TOTOX values with the addition of 0.3% trace water ($p < 0.05$). The presence of trace water did not affect the antioxidative capacity of the BHT, while there was a significant increase in lipophilic esters of 3,4-DHPA and their combinations. Meanwhile, 3,4-DHPA showed the next best reduction after TBHQ, which was similar to the pattern observed in the study in fish oil without trace water adjustment. Though the trend was similar, their potential has improved significantly in combinations, even in the presence of trace water. For example, quercetin and α-tocopherol showed antagonistic effects in stripped sunflower oil systems, while the same combination showed synergistic effects in methyl linoleate emulsions (Bayram and Decker 2023). The authors have attributed this effect to the presence of a lipid-water interface in the latter, which could enhance regeneration-induced synergism compared to the homogeneous sunflower oil system. Here, the fish oil with trace water can be considered a heterogeneous system containing a lipid-water interface, and thus, antioxidants behave differently in such a system than in bulk oil. This implies the vital role of minor components, including phospholipids and trace water, in regulating oxidation in oils. These surface-active agents can form association colloids in the presence of water, which further act as sites of oxidation (Chen et al. 2011).

Other than BHT, the percent reduction has considerably increased, which indicates the role of amphiphilic antioxidants in oxidation. TBHQ showed relatively little increase compared to other samples (esters and their binary combinations). The esters, being amphiphilic in nature, have an influence on their accumulation at the oxidation sites in the reverse micelles. The extent of the influence varied based on the length of the acyl chain attached to 3,4-DHPA. Interestingly, the pattern observed was similar for the methyl ester as well as for its binary equimolar concentrations with 3,4-DHPA. This could be attributed to the equal concentration of the ester with 3,4-DHPA and the close polarity of the ester with 3,4-DHPA as determined by miLog P values. Hence, their combination does not have any considerable additive or synergistic effect on lipid oxidation.

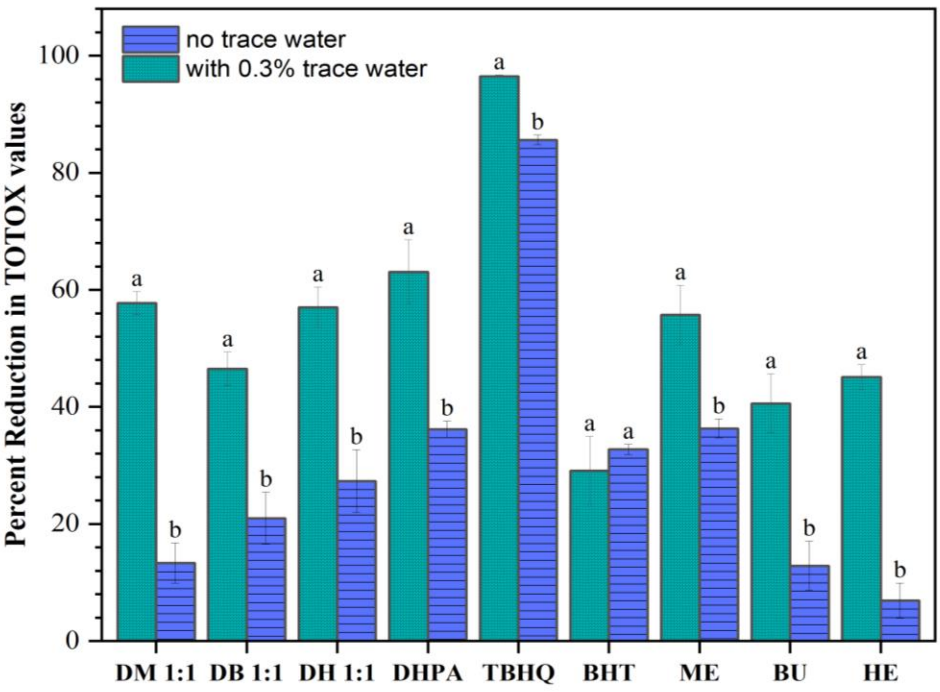


**Fig. 5.4 Comparison of the percent reduction in TOTOX values in fish oil with and without trace water adjustment**

Percent reduction in TOTOX value on the 30th day is depicted for all antioxidants in fish oil with 0.3% trace water (Fig. 5.5). The antioxidative activity decreased in the following order, TBHQ > DH 2:1 > DHPA > DB 2:1 > DM 1:1 > DH 1:1 > DM 2:1, AE, ME > DH 1:2 > DB 1:2 > DM 1:2 > DB 1:1, AED, HE > BU > BHT. The plausible reason for the improved potential of esters and their combinations can be explained by the partitioning of the amphiphilic compounds between the water and oil phases. All the esters have a hydrophilic phenolic ring and a hydrophobic alcohol chain, making them amphiphilic. These amphiphilic esters could effectively interact in the water-oil interfaces rather than the hydrophilic 3,4-DHPA, which reacts at the air-oil interface. Hence, the effectiveness of phenolic compounds in bulk oils cannot be based solely on their lipophilicity but influenced by surface active components (Budilarto and Kamal-Eldin 2015; Vaisali et al. 2017; Villeneuve et al. 2015).

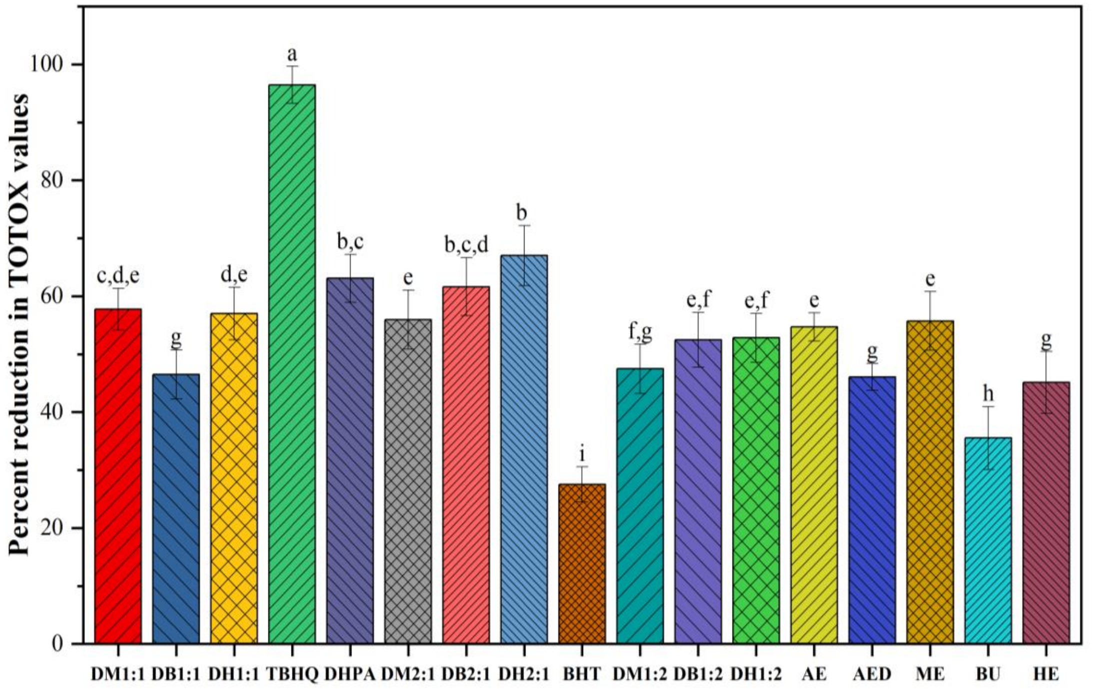


**Fig. 5.5 Percent reduction in TOTOX values for fish oil samples (0.3% trace water) at the end of 30 days of oxidative stability study**

Synergism might be obtained by different routes, including 1) regeneration of one antioxidant by the other, 2) formation of stable intermolecular antioxidant complexes which has higher activity than the parent compounds, 3) differences in solubility or phase distribution of antioxidants around the site of oxidation (Olszowy-Tomczyk 2020). In addition, the synergistic effect is dependent on the type and concentration of the antioxidant in the mixture (Shi et al. 2007). In our study, the synergistic effect between hexyl ester and 3,4-DHPA was not observed in molar ratios of 1:1 and 1:2 but in 2:1 of phenolic acid: ester. This indicates the role of the concentration of the antioxidants with different polarities and its influence on the distribution between the sites of oxidation. Moreover, in stoichiometric ratios other than 1:1, antioxidants might regenerate each other, resulting in synergistic effects in physical structures such as association colloids (Bayram and Decker 2023). Thus, the location and efficacy of antioxidants should be considered when designing a mixture of antioxidants. The location of the antioxidant in a heterogeneous system can be determined based on the log P values. In this study, the esters had relatively higher log P values indicating higher lipophilicity and thus were combined with the hydrophilic 3,4-DHPA with lower log P values. However, the esters obtained could be amphiphilic since they have an alkyl chain and this could result in their accumulation in higher concentrations in oil-water interfaces.

Similarly, butyl ester in molar ratios 1:1 and 1:2 had lower potential in controlling TOTOX value, while the molar ratio of DB 2:1 exhibited improved effectiveness. This indicates that an antagonistic effect was observed with the former molar ratios, while the molar ratio 2:1 showed additive effects. When the antioxidants in a mixture do not interfere with each other's potential, an additive effect of their individual potential is observed. This additive effect could also be due to other interactions that have not yet been fully understood since research in combinations of antioxidants is very limited (Olszowy-Tomczyk 2020). Meanwhile, the methyl ester showed the best activity in equimolar combination, which could be attributed to the closer polarities of 3,4-DHPA and methyl-3,4-DHPA.

#### 5.2.1.2 Effect of ternary and quaternary combinations of 3,4-DHPA and its esters

It can be hypothesized that for an oxidation process influenced by several factors, an equally complex antioxidant system can be effective (Mishra et al. 2021). Hence, the ternary and quaternary combinations of the esters were evaluated in equimolar ratio with a final concentration of 0.9 mM. The ternary combination had a similar potential to that of the methyl ester, but no synergistic effect was observed (Fig. 5.6). The quaternary combination of all esters with 3,4-DHPA showed the least effectiveness among the combinations, though it was significantly better than the antioxidant BHT ($p < 0.05$). Compared to the binary combinations, there was no significant improvement in the oxidative stability of these combinations. The effectiveness of a synergistic mixture depends on the optimal concentration of individual antioxidants depending on their mechanism of action (Mishra et al. 2021).

The equimolar ratio of the esters did not provide a synergistic effect, with no evident antagonistic effect as well. The ternary combination without 3,4-DHPA was better than the quaternary combination with 3,4-DHPA, even though the native phenolic acid showed the best antioxidative potential in *in vitro* studies previously (Arunachalam et al. 2024). This could be attributed to the higher lipophilicity of the esters, which could cause competitive distribution of these antioxidants around the oxidation sites. This suggests that oxidation cannot be fully explained based on the polarity of antioxidants alone. Rather, the physicochemical properties involved in the formation and stabilization of physical structures also play a role.

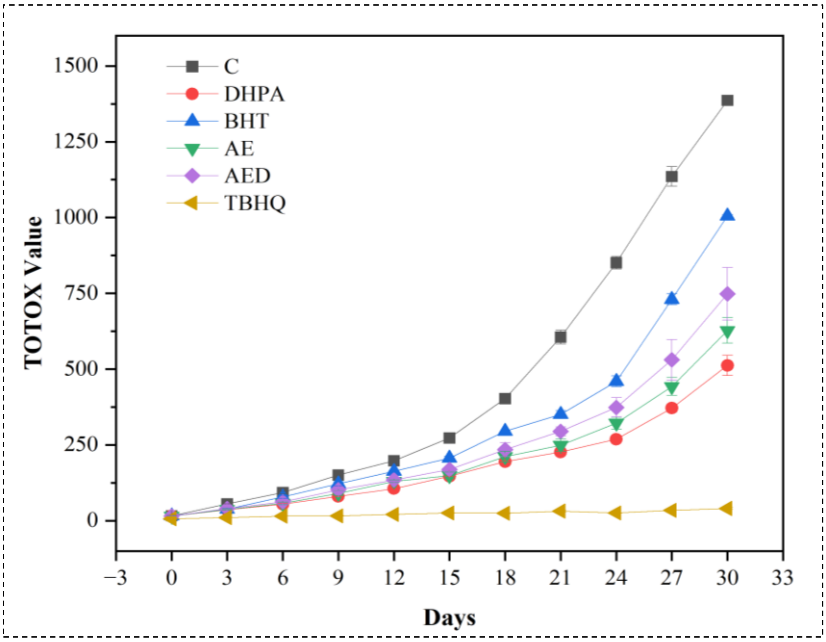


**Fig. 5.6 TOTOX values of fish oil samples containing ternary and quaternary combinations**

Since concentration plays a crucial role in determining the antioxidative or pro-oxidative effect, further statistical optimization of the concentrations of the individual esters in combinations might improve the overall effect on lipid oxidation (Mishra et al. 2023). Industrially, fish oil is bound to have a considerable amount of trace water during storage and transportation. Through the current study, it has been established that trace water content up to 0.3% does not negatively affect the efficacy of 3,4-DHPA and its lipophilic esters. The final concentration of the antioxidants in the current study did not exceed 0.9 mM, which is the maximal acceptable concentration for synthetic antioxidants. Since 3,4-DHPA is a naturally occurring antioxidant, it can be employed for long-term storage. The study opens new research opportunities regarding the design of a synergistic mixture of esters of 3,4-DHPA.

### 5.2.2 Acid values

The stability of fish oil might be affected through hydrolytic and oxidative degradation pathways or a combination of both. During oxidative degradation, the unsaturated fatty acids are targeted, and their degradation leads to the formation of primary and secondary oxidation products. However, during hydrolytic degradation, water present reacts with triglycerides, leading to the release of di- and mono-glycerides, glycerol, and free fatty acids. Thus, the release of FFAs could cause an increase in the acid values in fish oil containing trace amounts of water. In this study, the acid values of the fish oil samples fortified with different antioxidants after 30 days of storage study were studied and compared with the control sample on the 0$^{th}$ day of storage (Fig. 5.7).

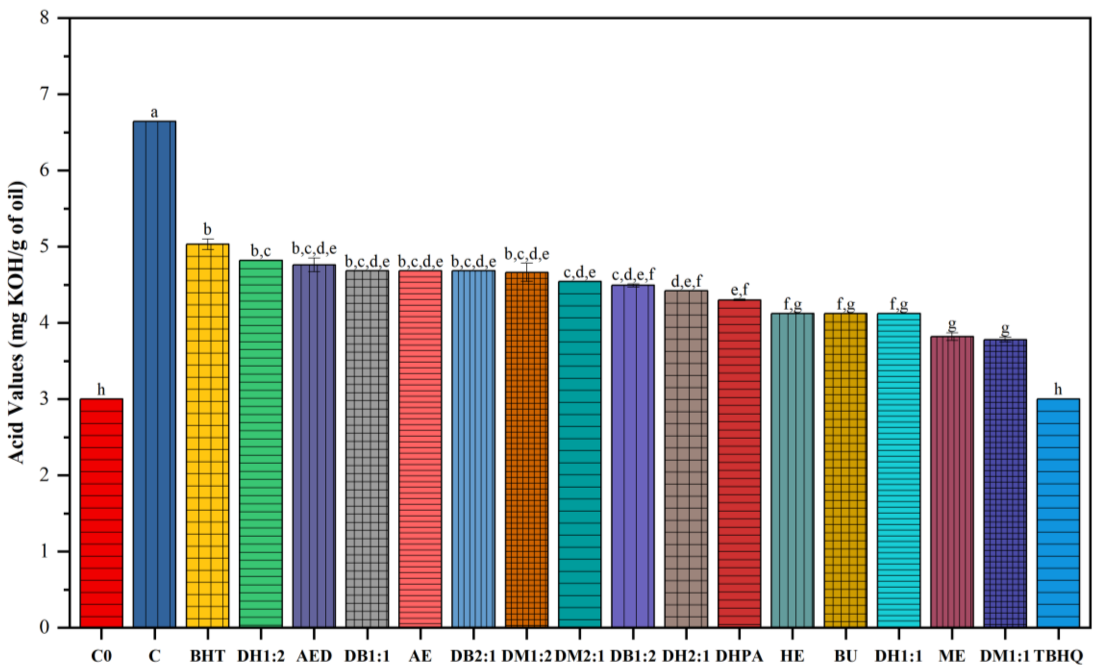


**Fig. 5.7 Acid values of fish oil samples (0.3% trace water) fortified with different antioxidants. Bars represent standard deviation (n=2).**

The control sample without any antioxidants showed a maximum acid value of 6.64 mg KOH/g of oil at the end of the 30-day study. Meanwhile, the control sample on the zeroth day had an acid value of 3.0 mg KOH/g of oil, which was twofold less. On comparing these values, it is evident that the presence of trace water has significantly affected the release of free fatty acids, leading to an increase in acid values. However, fish oil samples containing different antioxidants exhibited a lesser increase in acid values. Though there were no significant differences among the antioxidants studied except for TBHQ and BHT, methyl ester and its equimolar combination with DHPA had the lowest acid values at the end of the study. It is noteworthy that fish oil samples containing 3,4-DHPA and its esters and their combinations were better performing than the synthetic antioxidant BHT. This indicates that the phenolic acid and its esters were able to control the hydrolytic lipid rancidity, which is one of the major problems in the storage of such foods (Wang et al. 2011). A similar trend was observed with free fatty acid contents in fish oil fortified with carnosic acid, as reported by Wang et al. (2011). They measured FFA contents after storage of fish oil at 30°C and 4°C for 66 days. The control sample had a significant increase, while the samples with carnosic acid and vitamin E had lower FFA contents at 30°C, similar to our results. Interestingly, the FFA content was higher in the samples studied at 30°C than the samples at 4°C, indicating the role of temperature in oxidation as well.

The release of FFAs in fish oil could lead to the formation of additional physical structures, which would further enhance the rate of oxidation. Thus, the presence of trace water exerted the most influence on lipid oxidation, as previously studied in our laboratory (Chranayaa et al. 2019). These association colloids improve the contact between the amphiphilic antioxidants (phenolic esters) and the radicals due to improved solubility and dispersion. This helps in retarding oxidation by controlling oxidative and hydrolytic instability.

## 5.3 SUMMARY AND CONCLUSIONS

The assessment of the role of trace water on the oxidation of refined fish oil was evaluated using the storage stability study. The influence of 3,4-DHPA and its lipophilic esters separately and in combinations were measured using TOTOX values. The study evaluates the impact of the formation of association colloids and its effect on the hydrolytic and oxidative stability of fish oil containing 0.3% trace water. This study is the first of its kind wherein the binary, ternary, and quaternary combinations of 3,4-DHPA and its methyl, butyl, and hexyl esters are studied.

- The presence of trace water significantly increases the rate of oxidation in all samples, 5.13 folds higher than the fish oil control sample without trace water adjustment.
- TBHQ and BHT were the best and worst-performing antioxidants, respectively, among all the samples analyzed.
- Hexyl ester of 3,4-DHPA showed a synergistic effect with the native phenolic acid with a molar ratio of 2:1 (phenolic acid: ester) in reducing TOTOX values at the end of 30 days.
- The molar ratio of 2:1 of 3,4-DHPA and its esters was more effective than the other binary combinations, with molar ratios of 1:2 and 1:1 for the butyl and hexyl esters.
- Butyl and hexyl esters performed well in combinations rather than when they were used separately, while for methyl ester, there was not much improvement when used in combinations.
- The ternary combination without 3,4-DHPA showed better efficiency in retarding TOTOX values than the quaternary combination with 3,4-DHPA. However, there were no significant combinatorial effects observed with ternary and quaternary combinations.
- The acid values of samples with trace water were significantly higher than those without trace water. Except for TBHQ and BHT, no considerable variation was observed with samples containing 3,4-DHPA and its esters, similar to the trend observed with fish oil samples without trace water.
- Methyl ester and its equimolar binary combination with 3,4-DHPA had the lowest acid values among the phenolic acids studied.

# CHAPTER 6

# SUMMARY AND CONCLUSION

# CHAPTER 6

The use of synthetic antioxidants in PUFA-rich products to reduce oxidation could be harmful to human consumption owing to the toxic nature of TBHQ, BHT, and BHA, among others. Natural phenolic compounds are sought for their excellent antioxidative properties, but their hydrophilic nature limits their application in oil-based products. To overcome the solubility issues, phenolic acids are often lipophilized with long-chain alcohols to improve their hydrophobicity. 3,4-DHPA, an effective antioxidant, was esterified, and its esters were evaluated in oxidative storage stability studies in refined fish oil. The combinations of the phenolic acid and its esters were also studied to understand their interactive effects on oxidation better. The chapter includes the overall summary and conclusions of the current study.

## 6.1 SUMMARY

- The enzymatic method of esterification was attempted for the lipophilization of 3,4-DHPA with acyl donors of different chain lengths. Varying reaction conditions were employed to achieve esterification enzymatically using immobilized Lipase from *Candida antarctica* and *Thermomyces lanuginosus*. Despite our best efforts, the enzymatic route of synthesis of 3,4-DHPA esters was not satisfactory.
- The synthesis through chemical methods using homogeneous and heterogeneous catalysts was optimized for successful esterification. However, the purification of the ester after esterification using the homogeneous catalyst PTSA was complex, and the complete removal of the catalyst from the ester could not be achieved.
- The heterogeneous catalyst Amberlyst-15 was efficient in esterifying 3,4-DHPA with methanol, butanol, and hexanol with good yields. The structure of the purified esters was characterized and confirmed using LC-MS, $^{1}H$, and $^{13}C$ NMR studies.
- *In vitro* antioxidant activity assays revealed the excellent antioxidative property of 3,4-DHPA and its esters compared to synthetic antioxidant TBHQ. The

unmodified phenolic acid had the best antioxidant activity compared to the lipophilized esters. Though the esterification has reduced the esters' potential, it was still superior to that of TBHQ.

- The lipophilicity of the methyl, butyl, and hexyl esters was improved after esterification, as calculated using miLog P values.

Crude fish oil was refined, and physicochemical properties were studied before oxidative stability studies. 3,4-DHPA and esters were studied at a maximal concentration of 0.9 mM in the storage study, which was done at 35°C for 30 days in darkness in contact with atmospheric air. Synthetic antioxidants TBHQ and BHT were used for comparative purposes. A binary combination using the equimolar ratio of 3,4-DHPA and its esters was also evaluated for its potential.

- The antioxidative potential of DHPA was on par with the synthetic antioxidant BHT but not better than TBHQ.
- Methyl, butyl, and hexyl esters had reduced potential than DHPA, and the length of the acyl chain was found to have an influence on their potential. The phenolic acid was found to behave according to the polar paradox theory in bulk fish oil, but the effect of hydrophobic TBHQ was still better than the hydrophilic DHPA. Hence, the efficiency of the antioxidants in bulk oil systems cannot be predicted based only on their lipophilicities.
- The binary combinations revealed an alternative trend, with hexyl ester and 3,4-DHPA showing better potential, followed by butyl and methyl ester combinations.
- No significant synergistic effect was observed with the binary combinations of phenolic acid and its esters.

The presence of minor components, including trace water, has been found to influence the rate of oxidation in oils considerably. Thus, 3,4-DHPA and its esters were evaluated for their ability to retard oxidation in fish oil containing 0.3% trace water. Binary combinations of varying molar ratios and ternary and quaternary equimolar combinations were also studied to comprehend the overall interactive effect on oxidation.

- ➢ Trace water increased the TOTOX values of the 30th-day control samples without antioxidants by 5 folds compared to fish oil without trace water adjustment.
- ➢ 3,4-DHPA and hexyl ester in the binary combination with a molar ratio of 2:1, respectively, showed a synergistic effect in reducing TOTOX values.
- ➢ No significant improvement in overall activity was observed with ternary and quaternary combinations, but the ternary combination was better than the quaternary mix containing 3,4-DHPA.
- ➢ Acid value determination revealed that the presence of trace water significantly increases the release of free fatty acids due to hydrolytic degradation of triglycerides in the absence of antioxidants, as observed in control samples (6.65 mg KOH/g of oil).
- ➢ Among the phenolic acid and esterified antioxidants, methyl ester and its equimolar combination with 3,4-DHPA had the least acid values (3.84 mg KOH/g of oil) after 30 days of storage.

The synthesis of 3,4-DHPA esters was carried out using medium-chain alcohols, considering the resulting ester's antioxidative potential. However, further increase in acyl chain length can be exploited as an alternative using different catalysts and reaction conditions. The structural characterization of this ester could be studied, and the antioxidative potential could vary depending on the lipid system used. In the current study, the maximal concentration used for all antioxidants was kept at 0.9mM, which is the accepted limit for synthetic antioxidants. The study can also be expanded to higher concentrations since 3,4-DHPA is a natural antioxidant, and the ester has a higher solubility.

## 6.2 SIGNIFICANT FINDINGS

- ✓ Enzymatic esterification of 3,4-DHPA was possible with shorter chain acyl donors such as methanol, but with the increase in chain length, the esterification efficiency was substantially reduced.
- ✓ Esterification and trans-esterification reactions using the heterogeneous catalyst Amberlyst-15 were successful, with high yields only in a binary solvent system consisting of polar and non-polar solvents under optimal conditions.

- ✓ Refined fish oil oxidative stability (with no trace water) was improved by 3,4-DHPA, and its potential was on par with BHT but not higher than that of TBHQ.
- ✓ Lipophilisation of 3,4-DHPA influences its antioxidative activity, and it depends on the length of the acyl chain in bulk fish oil without trace water.
- ✓ The presence of trace water up to 0.3% increases the oxidation by 5 folds in bulk fish oil due to the formation of reverse micelles stabilized by surface-active minor components.
- ✓ Binary combination of 3,4-DHPA with hexyl ester in a molar ratio of 2:1 showed a synergistic effect in reducing TOTOX values in refined fish oil containing 0.3% trace water.
- ✓ 3,4-DHPA was efficient in fish oil without trace water, while its combination with hexyl ester was better performing in fish oil containing 0.3% trace water.

Commercial application of the synthesized esters of 3,4-DHPA could be further explored for usage in fish oil-rich matrices, specifically during fish oil storage on an industrial scale. Similarly, the synergistic combination of the hexyl ester and 3,4-DHPA can also be utilized to extend the shelf life of fish oil exposed to trace amounts of water. The health effects of 3,4-DHPA, including anti-inflammatory, anti-microbial, and anti-cancer potential, can be an additional benefit when they are used in nutraceuticals and pharmaceutical components rich in n-3 PUFA.

## 6.3 CONCLUSION

Methyl ester of 3,4-DHPA was obtained through an esterification reaction in a binary solvent comprising n-hexane and 2-butanone in the presence of 15% w/w of Amberlyst-15 as a catalyst. The obtained methyl ester was trans-esterified to synthesize butyl and hexyl esters using the same catalyst. *In vitro* antioxidant assays indicate the best potential of 3,4-DHPA in reducing oxidation, while the activity of esters has reduced relatively after modification. Oxidative stability studies carried out in refined fish oil in the presence of TBHQ, BHT, 3,4-DHPA, and the synthesized esters show partial compliance with the polar-paradox theory. Lipophilic esters of 3,4-DHPA were studied in binary, ternary, and quaternary combinations in refined fish oil containing 0.3% trace water. Hydrolytic oxidation triggers the degradation of triglycerides into mono- and diglycerides, with the release of free fatty acids contributing to the increase of acid values. The formation of reverse micelles in the presence of trace amounts of water brings the amphiphilic esters in contact with the oil-water interfaces, wherein the oxidation occurs accelerated. This resulted in the synergistic effect between 3,4-DHPA and hexyl ester (2:1 molar ratio) in reducing oxidation compared to the other combinations in different molar ratios. This indicates the importance of the molar concentrations of individual antioxidants in designing a mixture to obtain optimal protection against oxidation in specific oil systems. Therefore, 3,4-DHPA, a natural phenolic antioxidant, could be a potential replacement for the synthetic antioxidants in bulk and emulsion-based food systems rich in n-3-PUFAs.

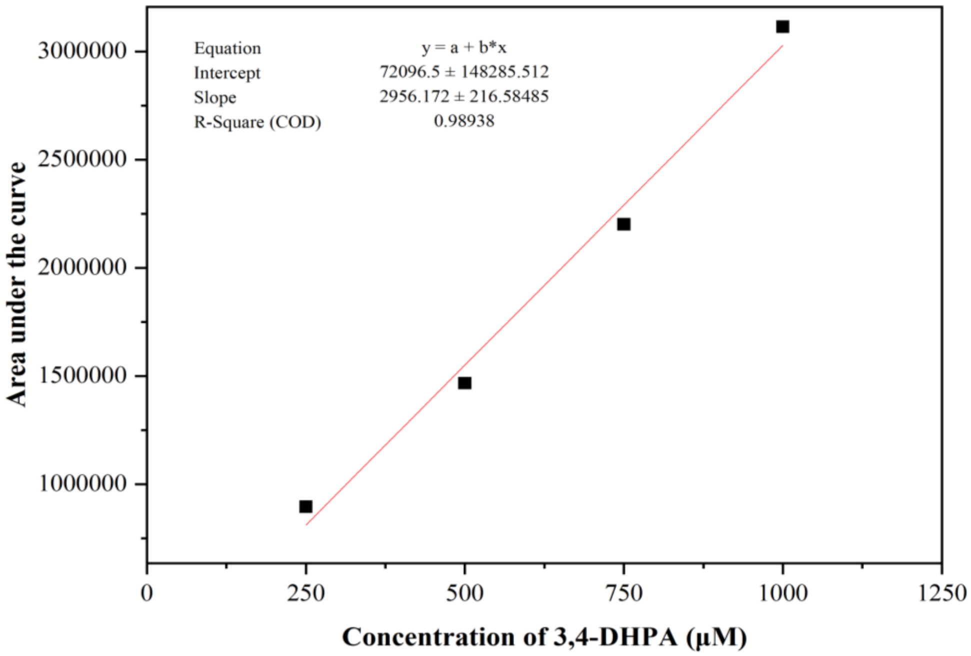
## 6.4 SCOPE FOR FUTURE WORK

- 3,4-DHPA and its esters were utilized in the maximal permitted concentration for synthetic antioxidants of 0.9 mM in fish oil in the current study. However, since the phenolic acid has been lipophilized and has no toxic effects on health, the application of these antioxidants in higher concentrations could be further studied.
- Optimization of molar concentrations of hydrophilic phenolic acid and hydrophobic esters could be carried out in higher final molar concentrations using statistical tools to further improve the potential in retarding oxidation.
- 3,4-DHPA being a radical scavenging antioxidant, its combination with antioxidants having metal chelating or singlet oxygen quenching capacity could bring further improvisation of its efficiency in different oil systems.
- Study of 3,4-DHPA and its esters in n-3 PUFA-rich emulsion systems could be carried out for various applications. This could be explored for systems that are fish oil-enriched food emulsions with potential health benefits.
- Exploration of alternate esterification methods and alternative acyl group donors can be carried out to expand the properties of the 3,4-DHPA and its application.

# APPENDICES

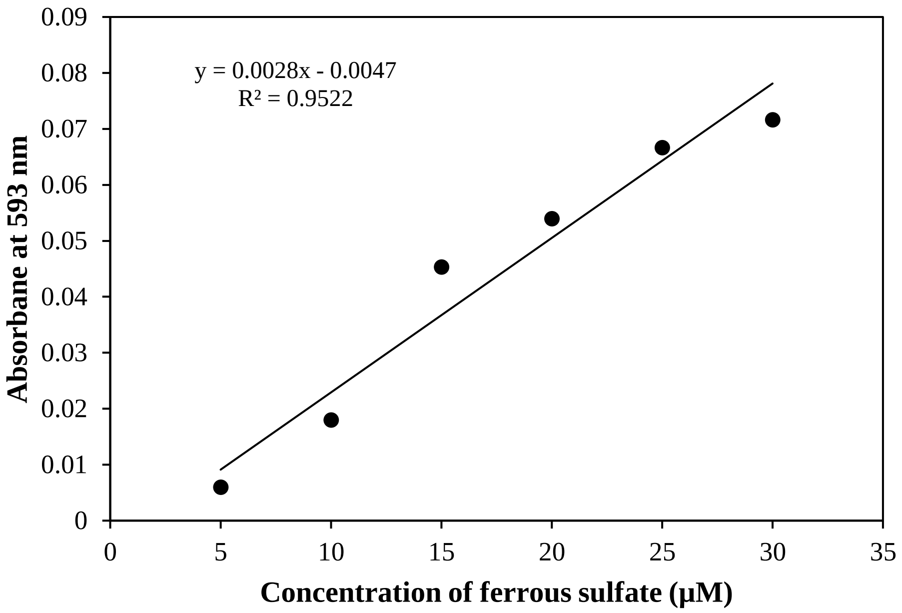
# Appendix I

## 3,4-DHPA standard plot in HPLC



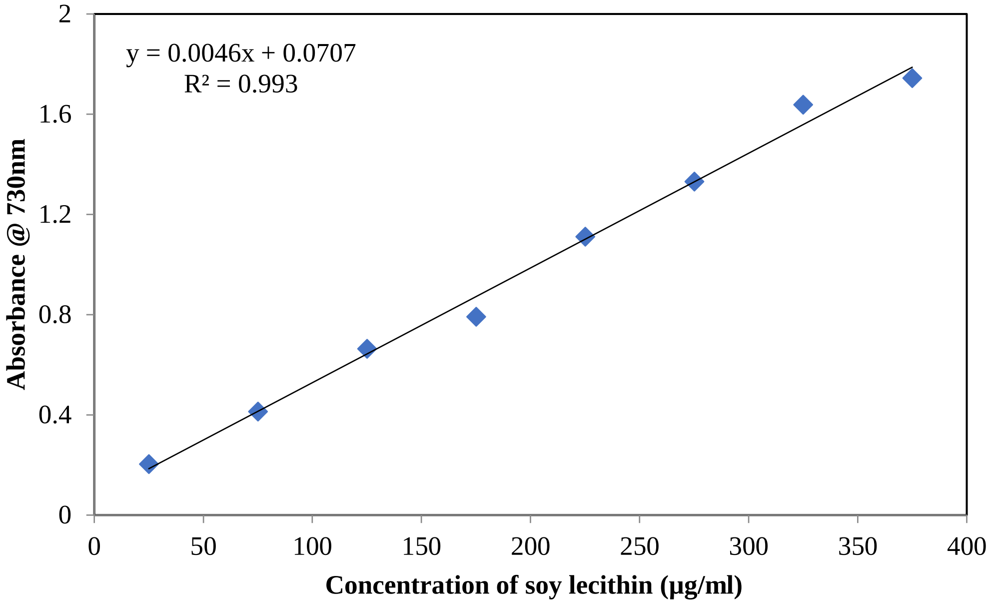
# Appendix II

## Standard plot of ferrous sulfate for FRAP assay



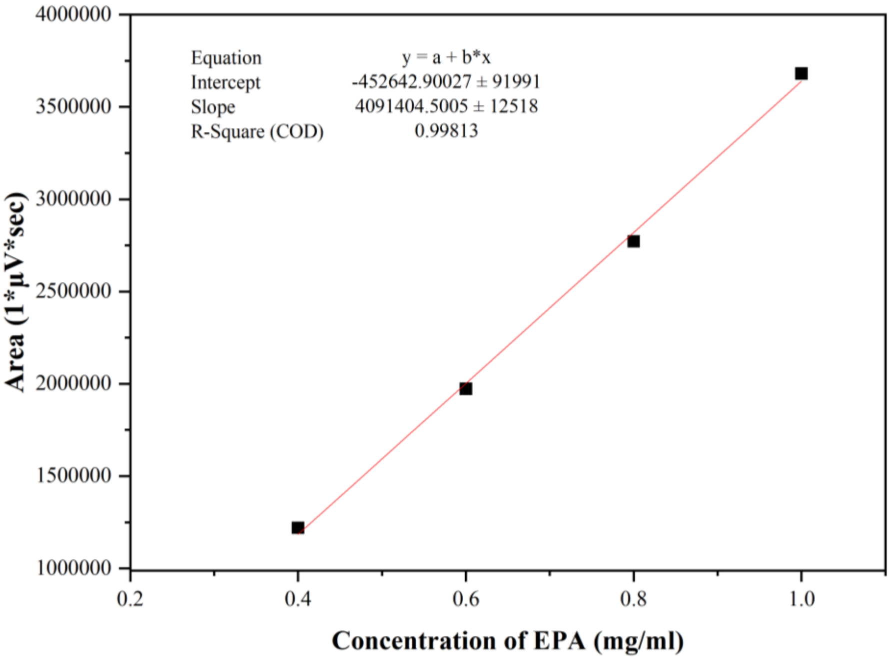
# Appendix III

## Standard plot of soy lecithin for phospholipid estimation



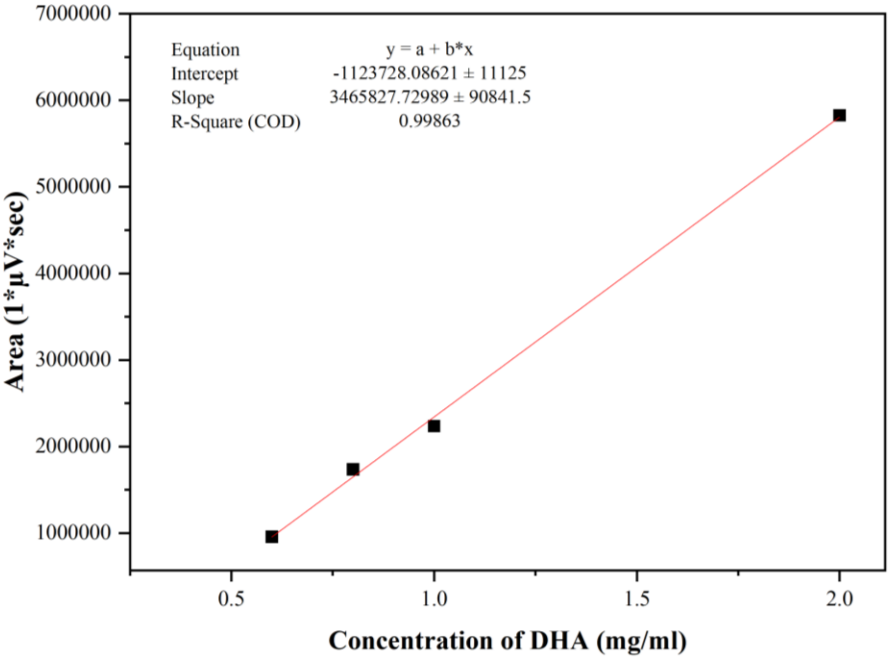
# Appendix IV

## Standard plot of EPA methyl ester



# Appendix V

## Standard plot of DHA methyl ester



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## LIST OF PUBLICATIONS BASED ON PRESENT WORK

1 Selva Sudha A, Vaisali C., Prasanna D. Belur (2024). “Effect of combinations of 3,4-dihydroxyphenylacetic acid and its esters on storage stability of fish oil containing trace water.“*Eur. J. Lipid Sci. Technol.*, e202400089. Q2 Journal with IF: 1.8.

2 Selva Sudha A, Vaisali C., Prasanna D. Belur (2024). “Synthesis and characterization of 3,4-dihydroxyphenyl acetic acid esters and study of their efficacy in bulk fish oil.” *Food Chem.*, 441(December 2023), 138380. Q1 Journal with IF: 8.8

3 Vaisali, C., Selva Sudha A**,** Hari, H., Shinkar, A., Prasanna D. Belur, Iyyaswami, R. (2022). “Probing the synergistic effects of rutin and rutin ester on the oxidative stability of sardine oil.” *J. Food Sci. Technol.*, 59(11), 4198–4209. Q1 Journal with IF: 3.1.

# CURRICULUM VITAE

**SELVA SUDHA A**

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## ACADEMIC DETAILS

I. **Ph.D. Chemical Engineering -** (2018-2024)

National Institute of Technology, Surathkal, Karnataka, India

- Advisor : Dr. Prasanna B.D. (Professor)
- Thesis : "Studies on the lipophilisation of 3,4 - dihydroxyphenylacetic acid and its application in enhancing the oxidative stability of fish oil."

II. **Master of Technology in Biotechnology -** (2016-2018)

P.S.G College of Technology, Coimbatore, Tamilnadu, India

- Project title : Biotransformation of 3-cyanopyridine to nicotinic acid by using nitrilase enzyme from *Nocardia globerula* NHB-2
- Score : 8.85 CGPA

## JOURNAL PUBLICATIONS

1 Selva Sudha A, Vaisali C., Prasanna D. Belur (2024). "Effect of combinations of 3,4-dihydroxyphenylacetic acid and its esters on storage stability of fish oil containing trace water."*Eur. J. Lipid Sci. Technol.*, e202400089. Q2 Journal with IF: 1.8.

2 Selva Sudha A, Vaisali C., Prasanna D. Belur (2024). "Synthesis and characterization of 3,4-dihydroxyphenyl acetic acid esters and study of their efficacy in bulk fish oil." *Food Chem.*, 441(December 2023), 138380. Q1 Journal with IF: 8.8

3 Vaisali, C., Selva Sudha A**,** Hari, H., Shinkar, A., Prasanna D. Belur, Iyyaswami, R. (2022). "Probing the synergistic effects of rutin and rutin ester on the oxidative stability of sardine oil." *J. Food Sci. Technol.*, 59(11), 4198–4209. Q1 Journal with IF: 3.1

## PROJECT AND TRANINGS

- A mini project on “A small scale study of physic-chemical parameters of aquaculture pond water.”
- Completed a main project on “Immobilization of Protease from *Bacillus pumilus* B20”. Major immobilization techniques based on four different support materials.
- Underwent an implant training in the department of Bio-Medical at Apollo Multispecialty Hospitals, Madurai.
- Underwent hands on training at Anna University, Trichy on “Sophisticated Spectroscopic Instruments” 2014.

## CONFERENCES

- Presented a paper titled “Immobilization of Protease from *Bacillus pumilus* B20” in the International Conference on Advances in Biotechnology, Civil and Mechanical Sciences (ICABCMS-2016) conducted by Selvam College of Technology, Namakkal.
- Attended a 2-day international workshop on “Current trends in diagnostics and therapeutics for Lifestyle diseases” conducted by Anna University, Chennai between 27.02.2018-28.02.2018.
- Presented a paper titled “Influence of different immobilization support materials on the enzymatic synthesis of 3,4-dihydroxy phenylacetic acid esters” at the International Conference on Technologies and Innovations for Sustainable Development (TISD-2023) conducted by MNNIT, Allahabad between 27-29 October 2023.

## LABORATORY SKILLS

- Chromatographic skills such as HPLC-ESI-MS, Gas Chromatography (GC), and FPLC.
- Rotatory evaporator, Pressure homogenizer, Rolling ball viscometer, Density meter, Refractometer, Tensiometer, Coulometer, Ultrasonicator.
- Oil refining and quality and stability parameter studies
- Bacterial Growth Kinetics, Enzyme purification and immobilization techniques