STUDY OF OXIDATIVE STABILITY OF SARDINE OIL IN THE PRESENCE OF ANTIOXIDANTS

Thesis

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

DOCTOR OF PHILOSOPHY

By

SUMIT KUMAR MISHRA

DEPARTMENT OF CHEMICAL ENGINEERING

NATIONAL INSTITUTE OF TECHNOLOGY KARNATAKA,

SURATHKAL, MANGALURU, INDIA – 575025

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DECLARATION

I hereby declare that research thesis entitled "**STUDY OF OXIDATIVE STABILITY OF SARDINE OIL IN THE PRESENCE OF ANTIOXIDANTS**" 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.

Place: Surathkal
Date:

Sumit Kumar Mishra
Register No. (177127CH007)
Department of Chemical Engineering

CERTIFICATE

This is to certify that the research thesis entitled "**STUDY OF OXIDATIVE STABILITY OF SARDINE OIL IN THE PRESENCE OF ANTIOXIDANTS**" submitted by **SUMIT KUMAR MISHRA (Register Number: 177127CH007)** as the record of the research work carried out by him, *is accepted as the thesis submission* in partial fulfilment of the requirements for the award of the degree of **Doctor of Philosophy**.

Research Guide
Dr. Prasanna B. D.
Professor
Dept. of Chemical Engineering
NITK, Surathkal

Research Co-Guide
Dr. Regupathi I.
Professor
Dept. of Chemical Engineering
NITK, Surathkal

Chairman - DRPC

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ABSTRACT

The Indian sardine fish (*Sardinella longiceps*) oil is highly valued for its affordability and abundance as a source of n-3 polyunsaturated fatty acids (n-3 PUFA), which includes the beneficial eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These compounds have positive effects on the prevention and treatment of various health conditions, such as coronary, neuromuscular, immunological, and allergic disorders. However, it is important to note that the n-3 PUFA contains many -C=C- double bonds, which are susceptible to oxidation due to their low bond dissociation energies (80kcal/mol). This oxidation process produces primary (hydroperoxide) and secondary oxidation (aldehydes and ketones) products that can result in an unpleasant taste. The extrinsic and intrinsic factors that contribute to the oxidation process, such as light, temperature, degree of saturation, free fatty acids, phospholipids, and pigments, leading to a decline in the nutritional value of the oil. Antioxidants are substances present in natural and synthetic forms and prevent several oxidation processes by various antioxidant mechanisms, such as free radical scavenging, metal chelating, and singlet oxygen quenching. A comprehensive study was conducted, and a tailor-made strategy was developed for one-factor at-a-time analysis that utilizes thirteen natural and synthetic antioxidants from various classes with different mechanisms with 0.9mM at 25°C under darkness in the presence of air for 50 days to maintain the oxidative stability of the oil. catechin, sinapic acid, caffeic acid, resveratrol, quercetin, and vanillic acid were found to be highly effective factors in preventing oxidation. A full factorial design (2^4) was utilized to choose a combination of natural antioxidants (catechin, resveratrol, sinapic acid, and vanillic acid) and synthetic antioxidants (TBHQ, BHA, and BHT) which exhibit interactive effects and response surface modelling (RSM) was used to identify the optimal concentration of the selected antioxidant mixture, which exhibits the synergistic effect. Natural antioxidants, catechin and resveratrol at 0.5mM and 0.625mM, respectively and synthetic antioxidants, TBHQ and BHT at 0.18mM and 0.18mM, respectively exhibited a strong synergistic effect. The kinetic and thermodynamic study of synthetic antioxidant (TBHQ and BHT) combination at several temperatures (25, 35, and 45°C) shows that the decomposition follows a zero-

order reaction irrespective of the presence/absence of antioxidants, their concentrations, and storage temperatures. The thermodynamic analysis revealed that the formation of peroxides in the sardine oil is an endothermic, endergonic, and non-spontaneous reaction at ambient temperature.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABRREVIATIONS

ALA	α-Linoleic acid
n-3 PUFA	n-3 Polyunsaturated fatty acid
DHA	Docosahexanoic acid
EPA	Eicosapentonic acid
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
FFD	Full factorial design
RSM	Response surface methodology
CCD	Central composite design
ANOVA	Analysis of variance

NOMENCLATURE

Symbol	Description
°C	Degree Celsius
K	Kelvin
J	Joule
H	Hour
g	Acceleration due to gravity
G	Grams
M	Molar
mM	Millimolar
RPM	Revolutions per minute
%(w/w)	Percentage weight by weight
%(v/v)	Percentage volume by volume
K	Rate constant
E_a	Activation energy
A	Pre-exponential factor
k_B	Boltzmann constant
H	Planck's constant
ΔH^{++}	Enthalpy
ΔS^{++}	Entropy
ΔG^{++}	Gibb's free energy
$^{++}$	Transition state

CHAPTER – 1

INTRODUCTION

CHAPTER 1

1. INTRODUCTION

1.1 Fish oil and PUFA

Indian oil sardine (*Sardinella longiceps*) is a chief pelagic fishery resource of India and one of the richest and cheapest sources of n-3 polyunsaturated fatty acids. The beneficial effects of n-3 PUFA in the prevention and treatment of coronary, neuromuscular, immunological disorders and allergic conditions are well documented. Due to its nutritional value and industrial use, this is considered as one of the most important marine resources for the nation. Sardine fish oil is one of the abundant and inexpensive sources of essential fatty acids, especially eicosapentaenoic acid (EPA 20:5n-3) and docosahexaenoic acid (DHA 22:6n-3). The sardine fish has high obtainable oil content (18 wt. %) with a high content of PUFAs (~40%). The quantity of n-3 PUFA (polyunsaturated fatty acid) in sardine oil is generally in the range of 20 to 50% depending upon the type of species, geographic location of fish, and the season (Chakraborty and Joseph, 2015; Chandrasekar et al., 2015).

The beneficial effect of n-3 PUFAs in patients with numerous health issues and diseases, such as cardiovascular disease (atrial fibrillation, atherosclerosis, thrombosis, inflammation, and sudden cardiac death, among others), diabetes, cancer, depression, and various mental illnesses, age-related cognitive decline, periodontal disease, and rheumatoid arthritis, has been investigated (Finley and Shahidi, 2001; Lopez *et al.*, 2011). Several researchers have observed that n-3 PUFAs significantly alter blood lipid profiles and membrane lipid composition and affect eicosanoid biosynthesis, cell signaling cascades, and gene expression, thereby influencing health (Shahidi and Ambigaipalan 2015, 2016).

n-3 PUFAs are generally present in the esterified form. It is associated with phospholipids and triacylglycerol (TAG) in cellular membranes and storage lipids, respectively. The position at which n-3 PUFAs are attached to TAGs plays an essential role in their absorption. Long-chain (LC), PUFAs of fish oils, are distributed primarily in the sn-2 position of TAG. In contrast, marine mammal lipids contain LC PUFAs predominantly in

the sn-1 and sn-3 positions of TAG (Shahidi and Miraliakbari 2004, 2005). Since n-3 PUFAs of fish oil is in the sn-2 position, its bioavailability is higher than marine mammal oils, where n-3 PUFAs are located in sn-1 and sn-3 positions (Laidlaw *et al.,* 2014). However, the presence of a large number of double bonds (up to six –C=C– double bonds) in n-3 PUFAs in fish oil increases its propensity to auto-oxidation and free radical formation (Shahidi and Zhong, 2010). The oxidation reduces the fish oils' shelf life, palatability, functionality, and nutritional quality.

1.2 Oxidative stability of fish oil

Oxidation stability is an essential indicator in determining the quality and shelf life of oils. Oxidation produces low molecular weight off-flavor compounds during storage (Guillen and Cabo 2002). Oxidation also destroys essential fatty acids and produces toxic compounds and oxidized polymers. Oxidation stability is essential for product palatability and nutritional quality (Hamilton, 1994).

Oxidation occurring at the double bond sites of fatty acid molecules is the primary route of deterioration of oil quality. The deterioration process proceeds via. - initiation, propagation and termination steps. The deterioration process can be broadly classified into two types; viz., autoxidation and photosensitized oxidation. In the case of autoxidation oxidation, unsaturated fatty acid molecules lose a hydrogen atom and thereby produce lipid alkyl radicals in the presence of certain initiators such as heat, light/ionizing radiation, and metal ions/metalloproteins (Shahidi and Zhong, 2010). This lipid alkyl radical reacts with available triplet oxygen and forms lipid peroxy radical. In turn, this lipid peroxy radical abstracts hydrogen from other lipid molecules to form hydroperoxide and another lipid alkyl radical and the chain reaction can continue further. When alkyl radicals react with each other, non-radical species are produced which results in the termination of chain reaction (Choe and Min, 2006). Oxygen availability and temperature determine the formation rate of lipid peroxy radicals and hydroperoxide (Velasco et al., 2004). In the presence of metal ions or at high temperature, hydroperoxides readily decompose to alkoxy

radicals and then form aldehydes, volatile organic acids, esters, ketones, short-chain hydrocarbons and alcohols.

In the presence of sensitizers such as chlorophylls, oil oxidation is accelerated by light. Sensitizers absorb light energy rapidly, get excited, and in turn, some can be converted to triplet state sensitizers. Excited triplet sensitizers can transfer excitation energy to adjacent triplet oxygen to form singlet oxygen by triplet-triplet annihilation, and return to the ground singlet state. Each triplet sensitizer can generate up to 10^3-10^5 molecules of highly reactive singlet oxygen before becoming inactive if sufficient triplet oxygen molecules and light energy are available (Kochevar and Redmond, 2000). The presence of photo-sensitizers such as pheophytins, chlorophyll, myoglobin, riboflavin and heavy metals induce the production of singlet oxygen under light (Shahidi and Zhong, 2010). Singlet oxygen, thus produced can react with unsaturated fatty acids to form allyl hydroperoxides (Fukuda et al., 1986). These hydroperoxides can trigger a chain reaction similar to the autoxidation reaction explained earlier. The fatty acid composition of the oil, refining technology utilized, temperature, light, the concentration and type of oxygen species, free fatty acids, metals, moisture, phospholipids, pigments, and antioxidants influence the oxidation of oil during processing and storage (Choe and Min, 2006).

1.3 Antioxidants and their applications

Oxidative stability of oils can be enhanced by using antioxidants. Antioxidants are substances capable of delaying, retarding or preventing oxidation processes (Chandrasekar et al., 2017). Antioxidants enhance oxidative stability by one or other functions such as scavenging free radicals and reactive oxygen species, inactivating peroxides, chelating metal ions, quenching secondary oxidation products, and inhibiting pro-oxidative enzymes. Antioxidants exhibit inhibitory effects against oxidation through various mechanisms with varied rates. Antioxidants confer several diversified biological effects, such as antibacterial, antiviral, anti-inflammatory, anti-allergic, anti-thrombotic, and vasodilatory action, as well as anti-mutagenicity, anti-carcinogenicity, and anti-aging effects, among

others. Antioxidants are categorized as natural and synthetic antioxidants depending on the source of antioxidants.

Natural antioxidants are widely distributed in plant materials, animal tissues, and microorganisms. Fruits, vegetables, cereals, legume seeds, oil seeds, tea, and certain spices are rich sources of antioxidants. These sources contain various antioxidant molecules including tocopherols, carotenoids, flavonoids, phenolic acids, polyphenols, and lignan compounds. There is a growing trend towards using extract of natural antioxidants from fruits, herbs, and spices that contain multiple antioxidants to suppress the effect of intrinsic factors (degree of unsaturation, free fatty acid) and extrinsic factors (light, temperature) of oxidation (Choe and Min, 2006). Such natural extracts of tea, grape seed, rosemary, sage, thyme, oregano, ginger, turmeric, black pepper, chili pepper, clove, marjoram, cumin, among others, contain multiple antioxidants with various mechanisms to retard the different routes of oxidation.

Synthetic antioxidants are chemically synthesized and toxicologically examined for their safe use in the human diet. They have been used in a wide variety of food industry products. These are mainly phenolic compounds, the common ones being tertiary-butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), octyl gallate (OG), and dodecyl gallate (DG).

1.4 Mechanisms of oxidation and role of antioxidants

Antioxidants exhibit an inhibitory effect against oxidation through various mechanisms with varied rates. The antioxidants act as free radical scavengers, metal ion chelators, singlet oxygen quenchers and photo-sensitizer inhibitors, and lipoxygenase inhibitors, thereby suppressing the oxidation and maintaining the color, flavor, and odor of the product during storage (Choe and Min, 2009). Based on their mode of action, antioxidants may be broadly classified into primary and secondary antioxidants. Primary antioxidants can neutralize free radicals by donating a hydrogen atom to lipid alkyl radicals and scavenge the lipid peroxy radicals, breaking the chain reaction of oxidation. Secondary antioxidants

prevent or retard oxidation by suppressing oxidation promoters, including metal ions and singlet oxygen, pro-oxidative enzymes, and other oxidants (Shahidi and Zhong, 2010).

Combining antioxidants with various mechanisms to suppress the different oxidation phases by the combined effect of antioxidants creates synergism. The synergistic effect significantly increases food preservation effectiveness and reduces the number of food additives needed, particularly synthetic antioxidants that may present health concerns. Synergism categories as homo-synergism, which has a combination of free-radical scavenging (FRS) antioxidants that react more rapidly with free radicals than the other due to differences in bond disassociation energies and or stearic hindrance of FRS/ROO˙ interactions. These differences will result in one antioxidant being consumed faster than the other. However, it may be possible for this FRS to be regenerated by transfer of its radical to a different scavenger. Hetero-synergism is a combination of two or more antioxidants with different mechanisms, where a combination of metal chelators and FRS often results in synergistic inhibition of lipid oxidation. The metal chelators will decrease oxidation rates by inhibiting metal-catalysed oxidation, and fewer free radicals will be generated in the food system. It means the FRS reaction will be slower with auto-oxidation or termination, thus making its concentration greater. The combination of metal-chelator and FRS thus decreases free radical generation and increases radical scavenging potential (Reische et al., 2002).

1.5 Design of experiments and statistical optimization

The science of statistics deals with collecting, classifying, and tabulating numerical facts as the basis for explaining, describing, and comparing phenomena. Statistical methods include all the general principles and techniques commonly used in collecting, analysing, and interpreting data relating to any sphere of inquiry. Full factorial design and Response surface modeling are statistical design models used for research analyses. The utilization of factorial design allows for optimal process optimization. This approach not only identifies the impact of each factor on the response, but also help us to understand how the effects of each factor differ with changes in other factors. By employing experimental

designs, interaction effects between various factors can be determined. The studies conducted using this methodology have proven its significance. The full factorial design investigates all factors and their main and interactive effect on the outcome. 2^k full factorial design creates experimental points with all the possible combinations of the factors in each complete trial or replication of the experiments. The experimental design points in a full factorial design are the vertices of a hypercube in the n-dimensional design space defined by each factor's minimum and maximum values (Zhang et al., 2022). Response surface modeling is a statistical technique for empirical model building and exploitation of the model that helps better understand and optimize the response. Response surface modelling is often used to optimize the models after determining important factors using screening or factorial designs, incredibly when uncertain about the curvature in the response surface (Zhang et al., 2022).

Kinetic modeling is a very useful tool in the understanding of the mechanism of reactions. If applied, kinetic models can help in deciphering the reaction mechanisms involved in the oxidation of edible oils during storage and their temperature dependence. Determining the reaction order for hydrogen peroxide formation is done through the kinetic model. The first step involves analyzing the natural logarithm of peroxide values over time with linear regression. To identify reactions that cause food quality loss, they are generally classified as zero, first, or second order. It is important to keep in mind that kinetic equations may differ depending on the specific food and temperature under study. In order to create top-notch formulations, it is crucial to have a deep understanding of the oxidation reaction in the relevant product and to anticipate lipid oxidation in different situations. It is imperative to have a comprehensive knowledge of the system's characteristics, including the oxidation process, to determine whether it's endothermic/exothermic, exergonic/endergonic, and spontaneous/non-spontaneous.

The difference in energy between the ground state and the transition state in a chemical reaction is represented by ΔH^{++}. If the activation enthalpy is higher, then the products require more energy to form in an activated state. The change in entropy (ΔS^{++}) shows the

increase in randomness after the adsorption is completed, resulting from the change in energy distribution. Exothermic reactions release energy as heat, leading to a temperature rise, while endothermic reactions absorb energy from the surroundings in the form of heat. Energy is absorbed from the surroundings in endergonic reactions, whereas energy is released to the surroundings in exergonic reactions. Spontaneous reactions proceed without energy input, and the reaction occurs slowly with negative ΔG^{++}. Non-spontaneous reactions require energy input, and the reaction occurs with positive ΔG^{++}.

1.6 Scope of work

India is one of the largest producers of sardine oil that contains a significant amount of n-3 PUFA. Due to the oxidative instability of fish oil, the value proposition of the produced oil has decreased enormously. There is a necessity to find most efficient antioxidants and their optimal concentration to enhance oxidative stability of n-3 PUFA rich oil. Considering the fact that the oil undergoes multiple simultaneous diverse mechanisms of oxidations, combining two or more antioxidants of different natures is expected to be a logical and feasible solution. However, studies on the formulation of such mixtures using pure antioxidants to produce synergistic antioxidant mixture is rather scarce.

Full factorial design (FFD) is a robust statistical technique used for screening variables for their main and interactive effects. Once the variables are selected based on their main and interactive effects, response surface methodology (RSM) can be adapted to determine the optimal concentration of the variables, which gives the highest desired response. RSM has been predominantly used in optimizing the extraction of natural antioxidants from their sources. However, use of FFD in exploring the interactive effect of different natural and synthetic antioxidants is a relatively understudied process. Further, there are no studies where the optimal values of interacting antioxidants are determined using RSM. This optimal value is expected to elicit the synergistic antioxidant effect.

The current study aims to employ statistical methods to design and develop a synergetic mixture which exhibits the highest antioxidant effect at a much lower concentration in a bulk oil system. To meet the above aim, following objectives are formulated;

1.7 Objectives

1. To study the effect of various natural and synthetic antioxidants for improving the storage stability of Indian sardine oil.

2. To determine the interactive effect among the natural and synthetic antioxidants separately in bulk oil systems using full factorial design.

3. To find the optimal concentrations for the selected combination of antioxidants using response surface modeling (RSM).

4. To study the kinetics of oxidative deterioration of Indian Sardine oil in the presence of the optimal combination of antioxidants.

1.8 Thesis organization

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

CHAPTER 1: Introduction

This chapter suggests the foundation of the research work, highlighting the importance of n-3 PUFA-rich sardine oil and its health and industrial benefits. It also describes the oxidation process and parameters. The discussion of antioxidants also mentions various mechanisms. The synergism effect plays a vital role to retard oxidation with a combination of antioxidants details describe. Statistical and kinetic-thermodynamic analysis importance also mention in this section. The scope and motivation for the current work have been stated, and the formulated objectives have been presented.

CHAPTER 2: Review of Literature

This chapter presents a comprehensive review of the literature involved in research work. The beneficial applications of n-3 PUFA have been stated briefly, followed by the importance of sardine oil. The properties of crude oil and the need to remove impurities to stabilize sardine oil through various refining stages have been discussed. Oxidation factors and mechanisms in fat and oils have been discussed. The antioxidant role, importance in fat and oil industries, and mechanisms to suppress oxidation are also detailed. The importance of antioxidant combination and its synergistic effect has been reviewed. Statistical approaches to analyses and interpretation of data and the effect of that investigation are also explained in detail. Kinetic-thermodynamic method to develop and regulate the reaction conditions under different temperature stages is also summarized in this chapter.

CHAPTER 3: Comparison of natural and synthetic antioxidants for improving oxidative stability of refined sardine oil.

This chapter has discussed the screening results of 13 different categories of antioxidants (natural/synthetic) examined in refined sardine oil for improving oxidation stability. Efficacy of catechin, resveratrol, β-carotene, caffeic acid, sinapic acid, ellagic acid, ferulic acid, vanillic acid, quercetin, rutin, TBHQ, BHA, and BHT were studied in the refined sardine oil at a concentration of 0.9 mM, stored for 50 days exposed to air at 25º C under darkness. The extent of primary and secondary oxidation of the stored oil was determined every 5 days and were compared.

CHAPTER 4: Full factorial design and Response surface modeling of the selected antioxidants.

This chapter discusses the results of full factorial design (2^4) study and the "main and interactive effects" of the selected natural and synthetic antioxidants. Further, the response surface methodology (RSM) was used to identify the optimal concentration of the selected antioxidants. The synergistic and prooxidant effects of selected natural and synthetic

antioxidants were studied in storage studies performed over 50 days exposed to air at 25ºC under darkness in bulk oil systems.

CHAPTER 5: Kinetic and thermodynamic analyses of enhanced oxidative stability at optimized values of synthetic antioxidants.

This chapter explains the rate of deterioration of the bulk oil with and without the presence of antioxidant mixtures by analysing the kinetics of the oxidative reactions. The significance of thermodynamic parameters on the oxidation of bulk oil with optimized synergistic synthetic antioxidant mixtures at three different temperatures were also reported.

CHAPTER 6: Summary and Conclusions

This section offers a brief summary and conclusions of the findings of this research work on the studies of refining and storage stability enhancement of refined sardine oil using synergistic mixture of natural and synthetic antioxidants with some recommendations for future work.

CHAPTER – 2

REVIEW OF LITERATURE

CHAPTER 2

REVIEW OF LITERATURE

This section provides a detailed literature survey concerned to the objectives laid as mentioned in chapter 1. Firstly, a brief account of Indian sardine oil production statistics is given, followed by an introduction to essential fatty acids and their importance in the human diet. A thorough review of various sources of n-3 PUFA and the molecular mechanisms involved in the rancidity of oils were provided. Further, a detailed review of various stages and processes involved in the refining of edible oil and recent advances in the field of edible oil processing were provided. The last part of the chapter commentates on various classes of antioxidants, their structure-function relationships, and their mechanism of actions in the food systems, and a thorough review of published reports on the use of antioxidants to enhance the oxidative stability of bulk oil systems.

2.1 INDIAN SARDINE OIL

Indian sardines (*Sardinella longiceps*) are a primary marine pelagic fisheries resource from multiple regions of India. The Southwest coast (Kerala, Karnataka, and Goa) exported 7,20270 tons in 2012. The average annual landing was 161,960 tons during 1985-2015, a 74.5% contribution from southwest coast. The southeast coast (Tamil Nadu, Pondicherry, and Andhra Pradesh) contributed 33% during 2015; In the Northwest coast, Maharashtra state contributed 33,388 tons during 2012, and the average contribution range was 14,693 tons from 1985-2015. Gujarat contributed only 6,891 tons in 2014; the Northeast coast, Odisha contributed 2,617 tons in 2012, and West Bengal contributed 79 tons during 2011-2015.

Sardine fish oil is one of the abundant and inexpensive sources of essential fatty acids, especially eicosapentaenoic acid (EPA 20:5n-3) and docosahexaenoic acid (DHA 22:6n-3). The beneficial effects of n-3 PUFA in the prevention and treatment of coronary, neuromuscular, immunological disorders and allergic conditions are well documented. Due

to its nutritional value and industrial use, this is considered as one of the most important marine resources for the nation. The sardine fish has high obtainable oil content (18 wt. %) with a high content of PUFAs (~40%). The quantity of n-3 PUFA (polyunsaturated fatty acid) in sardine oil is generally in the range of 20 to 50% depending upon the type of species, geographic location of fish, and the season (Chakraborty and Joseph, 2015; Chandrasekar et al., 2015).

2.2 Lipids – importance and health benefits

Lipids comprise a broad group of chemically diverse compounds distributed in plants and animal sources, playing a vital role in human nutrition. Lipids can be classified as nonpolar (e.g., triacylglycerol and cholesterol) and polar lipids (e.g., phospholipids) to indicate differences in their solubility and functional properties. They contribute texture, flavour, nutrition, and caloric value to the food. The alteration of lipid composition changes the texture, alters the fatty acid and cholesterol composition, decreases total fat, alters bioavailability, and changes oxidative stability. Some lipid compounds are indispensable as food emulsifiers, while others are important as fat- or oil-soluble pigments or food colorants.

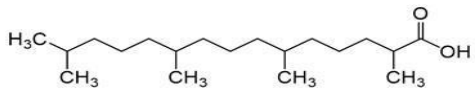
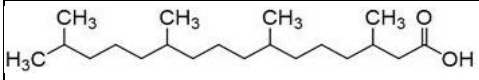
2.3 Fatty acids

Hydrolysis of acyl chains of triacylglycerols and phosphoglycerides releases fatty acids. Most of the natural fatty acids have an even number of carbons due to the biological process of fatty acid elongation, where two carbons are added at a time. The majority of fatty acids in nature contain 14–24 carbons. However, some fats contain small amounts of fatty acids having less than 14 carbons. Significant levels of such short-chain fatty acids are mainly found in tropical oils and dairy fats. In Table 2.1, major types of fatty acids are mentioned in groups according to chain length, number, position, configuration of their double bonds, and the occurrence of additional functional groups along the chains are tabulated. The fatty acids present in most of the vegetable oils and animal fats are saturated, monounsaturated, and polyunsaturated. The saturated fatty acids have a straight chain, even number of carbons, and chain length of C12 to C22 (e.g., myristic acid 14:0, palmitic acid 16:0, stearic

acid 18:0). The monounsaturated with one double bond in the *cis* configuration in the 9-carbon position (relatively to the carboxyl group) is designated as oleic acid 18:1. The polyunsaturated fatty acids are characterized by having two or more *cis* double bonds separated by a single methylene group, or a 1,4-diene structure (e.g., linoleic acid 18:2).

Table 2.1: Saturated fatty acids

Abbreviated designation	**Structure**	**Common name**
A. Even number of straight chain fatty acids		
4:0	$CH_3(CH_2)_2COOH$	Butyric acid
6:0	$CH_3(CH_2)_4COOH$	Caproic acid
8:0	$CH_3(CH_2)_6COOH$	Caprylic acid
10:0	$CH_3(CH_2)_8COOH$	Capric acid
12:0	$CH_3(CH_2)_{10}COOH$	Lauric acid
14:0	$CH_3(CH_2)_{12}COOH$	Myristic acid
16:0	$CH_3(CH_2)_{14}COOH$	Palmitic acid
18:0	$CH_3(CH_2)_{16}COOH$	Stearic acid
20:0	$CH_3(CH_2)_{18}COOH$	Arachidic acid
22:0	$CH_3(CH_2)_{20}COOH$	Behenic acid
24:0	$CH_3(CH_2)_{22}COOH$	Lignoceric acid
26:0	$CH_3(CH_2)_{24}COOH$	Cerotic acid
B. Odd number straight chain fatty acids		
5:0	$CH_3(CH_2)_3COOH$	Valeric acid
7:0	$CH_3(CH_2)_5COOH$	Enanthic acid
9:0	$CH_3(CH_2)_7COOH$	Pelargonic acid
15:0	$CH_3(CH_2)_{13}COOH$	Pentadecylic acid
17:0	$CH_3(CH_2)_{15}COOH$	Margaric acid
C. Branched-chain fatty acids		

	$C_{19}H_{38}O_2$	Pristanic acid
	$C_{20}H_{40}O_2$	Phytanic acid

(Adapted from Food Chemistry 4th edition)

2.3.1 Saturated fatty acids

Saturated fatty acids are unbranched, straight-chain molecules with even number of carbon atoms. These short-chain (<14:0) fatty acids are the constituents of lipids present in milk, coconut, and palm seed etc. Fatty acids with an odd number of carbon atoms naturally occur in bacteria, higher animals, and plants, such as pentadecylic acid (15:0) and margaric acid (17:0), which are positively associated with health. These fatty acids are inversely correlated to the risk of cardiovascular disease, long-chain fatty acid oxidation disorders, and type-2 diabetes (Zhang et al., 2019). Methanoic, ethanoic, and propanoic acids are uncommon saturated fatty acids because of their water solubility and presence in nonesterified food products.

2.3.2 Unsaturated fatty acids

These fatty acids are characterized by having one, two, or three allyl groups in their acyl residues. The unsaturated fatty acids with methylene groups inserted between the two *cis*-double bonds are usually denoted as non-conjugated fatty acids. The double bonds of fatty acids influence melting point. Unsaturated fatty acids are not linear, making it difficult to orient themselves into tight packing configurations because of van der Waals interactions between the molecules. In unsaturated fatty acids, the *trans* configuration in double bond is more linear than the *cis* configuration. In monounsaturated fatty acids, double bond is commonly present at Δ9 in the *cis* configuration, i.e., oleic acid (18:1). The elaidic acid and vaccenic acid are having the same molecular mass, but differ in the position of the double bond at Δ9 and Δ11 position, respectively, in the *trans* configuration. They are commonly found in milk fats. Polyunsaturated fatty acids (PUFA) are characterized by having double bonds (≥2 double bonds) at the *cis* configuration that are separated by a 1,4-

diene structure or methylene group. The n-6 family contains linoleic acid (18:2) at 9,12 position, linolenic acid (18:3) at 9,12,15 position, and arachidonic acid (20:4) at 5,8,11,14,17 position. Linoleic and Linolenic acids are considered as essential fatty acids for humans as it is not synthesized in the body. The n-3 family contains eicosapentaenoic acid (EPA) (20:5) having double bonds at 5,8,11,14,17 positions and docosahexaenoic acid (DHA) (22:6) having double bonds at 4,7,10,13,16,19 positions. The EPA and DHA are also essential in modulating the biosynthesis of oxygenated derivatives of arachidonic acid called eicosanoids, which have hormone-like functions and play an essential role in inflammation processes. DHA has nutritional benefits and found to play important roles in visual and brain functions. Generally, infant foods are supplemented with fish and selected microalgae oil to supplement EPA and DHA.

Table 2.2: Unsaturated fatty acids (Adapted from Food Chemistry 4th edition)

Abbreviated designation	**Structure**	**Common name**
A. Fatty acids with non-conjugated *cis* double bonds		
	n-9 Family	
14:1 (9)	$CH_3-(CH_2)_3-$	Myristoleic acid
16:1 (9)	$CH_3-(CH_2)_5-$	Palmitoleic acid
18:1 (9)	$CH_3-(CH_2)_7-CH=CH-CH_2-(CH_2)_6-COOH$	Oleic acid
22:1 (13)	$-(CH_2)_{10}-COOH$	Erucic acid
24:1 (15)	$-(CH_2)_{12}-COOH$	Nervonic acid
	n-6 Family	
18:2 (9,12)	$CH_3-(CH_2)_4-(CH=CH-CH_2)_2-(CH_2)_6-COOH$	Linoleic acid

18:3 (6,9,12)	$-(CH=CH-CH_2)_3-(CH_2)_3-COOH$	γ-Linolenic acid
20:4 (5,8,11,14)	$-(CH=CH-CH_2)_4-(CH_2)_2-COOH$	Arachidonic acid
n-3 Family		
18:3 (9,12,15)	$CH_3-CH_2-(CH=CH-CH_2)_3-(CH_2)_6-COOH$	α-Linolenic acid
20:5 (5,8,11,14,17)	$-(CH=CH-CH_2)_5-(CH_2)_2-COOH$	EPA
20:6 (4,7,10,13,16,19)	$-(CH=CH-CH_2)_6-CH_2-COOH$	DHA
B. Fatty acids with non-conjugated *trans*-double bonds		
18:1 (tr9)	$CH_3-(CH_2)_7-CH^{tr}=CH-(CH_2)_7-COOH$	Elaidic acid
18:2 (tr9,12)	$CH_3-(CH_2)_4-CH^{tr}=CH-CH_2-CH^{tr}=CH-(CH_2)_7-COOH$	Linolelaidic acid
C. Fatty acids with conjugated double bonds		
18:2 (9,tr11)	$CH_3-(CH_2)_5-CH^{tr}=CH-CH^{c}=CH-(CH_2)_7-COOH$	Conjugated linoleic acid
18:3 (9,tr11,tr13)	$CH_3-(CH_2)_3-CH^{tr}=CH-CH^{tr}=CH-CH^{c}=CH-(CH_2)_7-COOH$	α-Eleostearic acid
18:3 (tr9,tr11,tr13)	$H_3-(CH_2)_3-CH^{tr}=CH-CH^{tr}=CH-CH^{tr}=CH-(CH_2)_7-COOH$	β-Eleostearic acid
18:4 (9,11,13,15)[a]	$CH_3-CH_2-(CH=CH)_4-(CH_2)_7-COOH$	Parinaric acid

[a] Geometry of the double bond was not determined.

2.3.3 Acylglycerols

Acylglycerols are the predominant constituent of oils and fats of commercial importance. Acylglycerols are the glycerol esters of fatty acids, wherein one, two, or three fatty acids are esterified at three different carbons of glycerol.

Monoacylglycerols consist of a single substitution of one hydroxyl group of the glycerol molecule with an acyl residue via an ester linkage. Three positional isomers are therefore possible for the substitution: the initial carbon position sn-1, the central carbon position sn-2, or the terminal carbon position sn-3. The *sn* term indicates stereospecific numbering.

Diacylglycerols occur when two hydroxyl groups of the glycerol molecule are substituted with two acyl residues via an ester linkage. Diacylglycerols can contain three isomers containing a single FA (*sn*-1, *sn*-2; *sn*-1, *sn*-3; and *sn*-2, *sn*-3). Where two different FA are involved, six positional isomers are possible.

Triacylglycerols (TAG) contain full substitution of all three hydroxyl groups of the glycerol molecule with ester-linked acyl residues. They have commonly been referred to as "oils" or "fats," depending on their melting point, and represent the storage lipids of both plants (in seeds) and animals (in adipose tissue). TAG derived from the seeds of tropical trees is unusual in possessing a large ratio of saturated to unsaturated fatty acids. The more unsaturated components are concentrated in the sn-2 position, with saturated fatty acids are more common at the sn-1 and sn-3 locations. TAG from animals is more dominated by C16 and C18 saturated fatty acids, including significant proportions of fully saturated TAG giving rise to solid substances at room temperature (e.g., tallow, lard). In general, the saturated fatty acids occupy sn-2 position contrary to the plant lipids.

2.3.4 Phospholipids

The phospholipids consist of a glycerol molecule, two fatty acids, a phosphate, and an alcohol (e.g., choline, ethanolamine). These are the modified triacylglycerols where the phosphate groups are found in the sn-3 position. Phosphatidic acid is the simplest phospholipid found in nature, where the hydroxyl group at C1 and C2 of glycerol are

esterified to two fatty acids chains, and the C3 hydroxyl group is esterified to phosphoric acid. Modifications of the other substitution group on the phosphate at the sn-3 position result in phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylinositol (PI).

2.4 n-3 Polyunsaturated Fatty Acid (PUFA)

The fatty acids are classified according to their carbon-chain length and the number of double bonds. The fatty acids are categorized into three forms; saturated fatty acid (e.g., palmitic acid (C16:0), stearic acid (C18:0)), Monosaturated fatty acids (e.g., oleic acid (C18:1)), and polyunsaturated fatty acids (PUFA). PUFA have been categorised into two groups; n-3 and n-6 based on the position of the first double bond on the methyl terminal end. α-L acid (ALA, C18:3 cis-9,12,15), an n-3 PUFA has the first double bond at the third position from the methyl terminal end. Linoleic acid (LA, C18:2 cis-9,12), an n-6 PUFA has the first double bond at the sixth position from the methyl terminal end. EPA and DHA belong to n-3 PUFA, and has significant health benefits that protect against numerous metabolic disorders.

2.4.1 Influence of n-3 PUFA on human health

PUFAs are found to elicit various essential structural and regulatory functions in human body. The human body cannot synthesize all these PUFAs due to the limitation of the enzyme responsible for inserting *cis* double bonds. PUFA is found in various marine sources such as anchovy, sardine, cod liver fish, mussels, oysters, and shrimp, and some plant sources are available such as walnuts and sesame/flax seed, and soybean/olive/canola oil. PUFA is found to exhibit therapeutic functions in various health conditions including hyper blood pressure, blood vessel function, heart function, and they have antithrombotic, anti-inflammatory, and anti-oxidative actions.

Several studies have revealed that n-3 PUFA effectively reduces the risk of being positive for SARS-CoV-2 infection and the duration of symptoms, overcome renal and respiratory dysfunction and increases the survival rate in COVID-19 patients. The supplements of n-3

PUFA were thought to have a potential effect on preventing and treating COVID-19 (Fadiyah et al., 2022). Panigrahy et al. (2020) showed that n-3 PUFA, through specialized pro-resolving mediators (SPM), could regulate inflammation in COVID-19 by growing phagocytosis of viruses and debris by macrophages and inhibit the production of eicosanoids, proinflammatory cytokines, and leukocyte infiltration, and increasing anti-SARS-CoV-2 antibodies production. Louca et al. (2021) showed a 12% lower risk of SARS-CoV-2 infection by taking supplements of n-3 PUFA three times a week for at least three months. Berger et al. (2020) mentioned that oral IPE (icosapent ethyl) 2g was consumed twice daily could effectively reduce the symptoms in COVID-19 patients. IPE is an n-3 PUFA derivative, produced from eicosapentaenoic acid (EPA). The randomized meta-analysis trials unequivocally show that supplements of fish meals/oil n-3 PUFA reduces the hyper blood pressure. The blood pressure-lowering effects of n-3 fatty acids are potentiated by sodium restriction and antihypertensive effect (Mori, 2017). The n-3 fatty acids are found to help in weight reduction in overweight-treated hypertensive patients (Bao et al., 1998).

The n-3 PUFA is essential in altering plaque morphology and increasing stability. Thies et al. (2003) mention that n-3 PUFA is effortlessly incorporated in the atherosclerotic plaque of carotid atherosclerotic disease patients undergoing carotid endarterectomy, associated with decreased number of macrophages in the plaque. These results represent the critical mechanism for reducing ischaemic cardiovascular by n-3 PUFA.

The n-3 PUFA significantly affected anti-inflammatory and immunomodulatory functions by attenuating inflammatory eicosanoids, leukotrienes, cytokines, and oxidative stress by altering endothelial and cell-cell activation and immune cell function. EPA of n-3 PUFA derived from arachidonic acid is a potent chemotactic factor for leukocytes because it is the preferred substrate of the lipoxygenase pathway leading to the formation of the relatively inactive leukotriene B5 (LTB5) at the expense of leukotriene B4 (LTB4) (Calder, 2003).

Icosapent ethyl (IPE) is an n-3 PUFA derivative given to hypertriglyceridemia patients and has been shown to suppress cardiovascular risk with treatment targeted at inflammation. Mozaffarian et al. (2005) showed in studies that n-3 fatty acids reduce heart rate by −1.6 bpm, with a more significant reduction in individuals with a baseline heart rate > 69 bpm (−2.5 bpm) in meta-analysis. Bao et al. (1998) showed that the dietary n-3 PUFA and weight loss process effectively reduce the heart rate of overweight-treated hypertensive patients. The meta-analysis showed that short-term n-3 PUFA favorably affects the frequency domain of heart rate variability, as indicated by the enhancement of vagal tone (Xin et al., 2013).

The abnormal production of free radicals increases stress on cellular structures and causes changes in molecular pathways that support several human diseases, including cardiovascular, neurological, and cancer. In this regard, Mori et al. (2010) have shown that n-3 PUFA reduced the plasma and urinary F2-isoprostanes. F2-isoprostanes are lipid peroxidation products originating from the non-enzymatic free radical oxidation of arachidonic acid in membrane lipids, and it is a reliable biomarker of *in-vivo* lipid peroxidative damage.

The potent lipid mediators stimulate the resolution of inflammation with a consequent return to tissue homeostasis. Serhan (2017) has described the E-series resolvins derived from EPA via P450 metabolism or aspirin-acetylated cyclooxygenase (COX- 2), and D-series resolvins, protectins and maresins derived from DHA via lipoxygenase or aspirin acetylated COX-2 mediators act via G-coupled protein receptors, and it has potent anti-inflammatory and pro-resolving actions that increase with time during the inflammatory process. Mori et al. (2016) have mentioned plasma lipid mediators increased by n-3 PUFA supplementation for inflammation resolution in individuals with metabolic syndrome, arthritis, chronic kidney disease, and pregnant women's placental tissue.

2.5 Sources of n-3 PUFA

Commercially, customer demand is rapidly increasing for natural dietary sources of n-3 PUFA. n-3 PUFA can be derived from marine sources, plant sources, micro and macroalgal sources, and genetically modified crop sources.

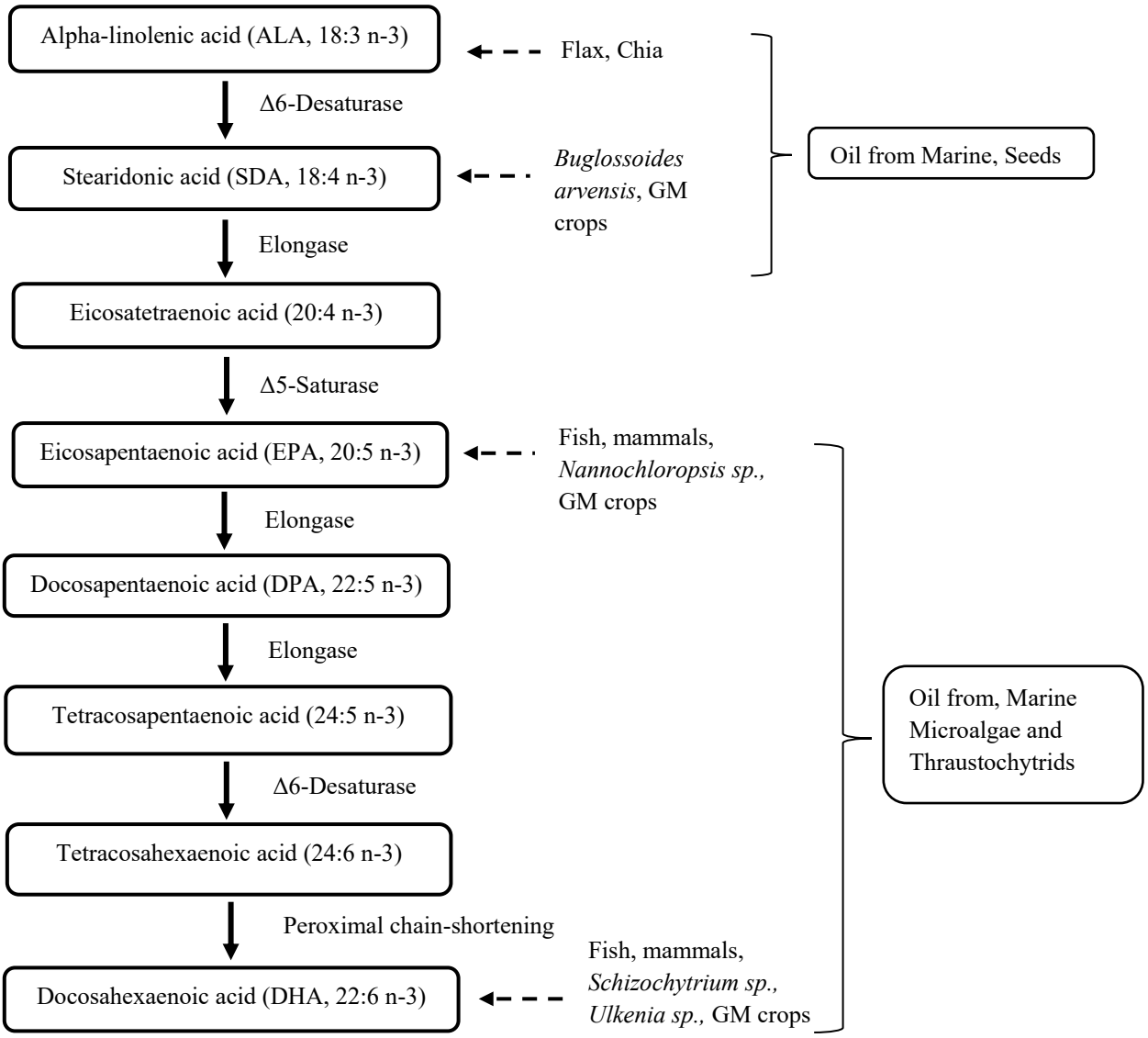


Fig 2.1 Metabolic pathway for the synthesis of n-3 polyunsaturated fatty acids from α-linolenic acid. (Adapted from Saini et al. 2021)

2.5.1 Marine sources of n-3 PUFA

Fish lipids are one of the richest sources of n-3 PUFA (EPA and DHA). The fatty acid composition is found (Table 2.3) to differ due to climatic influence, diet, age, maturity, and type of species (Osman et al., 2007). Fish have lower total lipids in tropical climates than in the Arctic. Freshwater food sources are characterized by their high lipid content, which can be identified by the presence of linoleic acid (C18:2 n-6), linolenic acid (C18:3 n-3), and EPA.

Justi et al. (2003) reported that marine plankton exhibits low levels of n-6 PUFA, wherein EPA and DHA are the dominant acids. As a result, marine fish are known to possess high levels of n-3 due to their consumption of marine plankton. On the other hand, freshwater fish are predominantly composed of n-6 fatty acids. According to Osman et al. (2007), fin fish contained higher AA and DHA levels than menhaden fish oil. Among the fish species analysed, "bagi" (*Aacnthurs nigrosis*) had the highest amount of DHA at 23.95 wt. %, while menhaden fish oil had 7.9 wt. % DHA. The EPA content in menhaden oil was 12.5 wt. %, while different fish species showed EPA levels ranging from 7.51 to 11.53 wt.%. Osman et al. (2001) analysed the fatty acid content of Malaysian marine fish. They found high levels of polyunsaturated fatty acids (56-92 wt.%) and low monounsaturated fatty acids (1-10 wt.%). Saturated fatty acids ranged from 3.63-11.4 wt.%. Striped sea catfish (0.68%), silver pomfret (0.60%), and black pomfret (0.52%) had higher AA contents than menhaden oil (0.47%). Interestingly, an earlier study (Suriah et al., 1995) found that AA contents in freshwater fishes were higher in some cases than those in marine fishes.

Table 2.3 n-3 PUFA content of marine sources

Marine sources	EPA (%)	DHA (%)	DPA (%)	Reference
Fish				
Menhaden oil	18.3	9.6	1.8	Ackman, 2005
Herring oil	7.5	6.8	0.75	
Cod liver oil	12.2	12.7	1.7	Copeman and Ma Parrish 2004
Cod flesh oil	19.1	32.6	2	
Capelin oil	9.3	4.1	0.9	
Skipjack tuna oil	11.1	29.1	0	Tanabe et al. 1999
Butterfish oil	5.1	10.8	2.4	Budge et al. 2002
Yellowtail flounder oil	15	18.7	3.3	
Winter flounder oil	14.4	20.1	3.8	
Haddock oil	14.8	24.8	1.9	
Halibut oil	9.6	30.6	2.6	
Mackerel oil	8	19.3	1.6	
Salmon oil	6.2	9.1	1.8	Aursand et al. 1994
Sardine oil	19	11	1.9	Kolakowska et al. 2006
Marine mammals				
Bearded seal oil	9.27	13.38	4.76	Shahidi 1998
Grey seal oil	5.23	7.12	4.94	
Harbor seal oil	9.31	7.76	4.22	
Harp seal oil	6.41	7.58	4.66	
Hooded seal oil	4.29	7.47	3.48	
Ringed seal oil	10.57	26.19	14.55	
Crustacean				
Shrimp	15.26	11.37	0.74	Budge et al. 2002

Red crab	12.13	11.93	2.25	
Rock crab	20.74	10.35	2.06	
Lobster	17.04	7.69	1.29	
Bivalves				
Surf clam	22.9	14.3	Trace	Copeman and Parrish 2004
Greenland cockle	22.6	16.5	0.1	
Blue mussel	19.6	13.2	0	
Icelandic scallop	26.9	25.9	0	
Cephalopod				
Common octopus	16.1	20.6	1.8	Arts et al. 2001
European squid	14.3	31.6	0.4	
Squid	13.9	16.9	1.3	

(Adapted from Shahidi and Ambigaipalam, 2018)

2.5.2 Plant sources of n-3 PUFA

It is widely known that vegetable oil is a significant source of lipid in the human diet. This type of oil is typically extracted from seeds, and in some cases, from fruits such as palm and olives. On an average, individuals consume 18.15 kg of vegetable oil annually worldwide.

Sunflower, soybean, and palm oil are the most commonly used vegetable oils, with rapeseed oil closely behind. Palm oil, also known as palmolein, is composed of oleic acid (43%), palmitic acid (40%), and LA (11%). Soybean oil is where LA (54.17 wt.%) is primarily found, with a small amount of ALA (n−6/n−3 PUFAs ratio of 10.5) (Dorni et al. 2018). Canola oil, on the other hand, is composed mainly of oleic acid (54.0–61.0 wt.%), followed by LA (20.6–25.0 wt.%) and ALA (8.7–9.5 wt.%), with an n−6/n−3 PUFAs ratio of 1.9–2.5 (Beyzi et al. 2019). Canola oil is produced from low erucic-acid (<2%) rapeseed cultivars.

The seeds of Flax (also known as linseed; *Linum usitatissimum L.*, from the family Linaceae) contain 35–50 wt.% oil (Zuk et al.2015) which is an excellent source of ALA, making up 39.0% to 60.4 wt.% of total FAs. Moreover, they have low SFAs (around 9–11%). Flax stem is another important source of high-strength fiber for industrial purposes (Zuk et al. 2015), as well as providing tocopherols, proteins, and antioxidants, which are beneficial for our health (Goyal et al. 2015). Flax or flaxseed oil is frequently used as a functional food ingredient in baked goods, juices, dairy products, and dry pasta products (Goyal et al. 2015; Zamani et al. 2020).

Oil extracted from seeds of chia (*Salvia hispanica*), camelina (*Camelina sativa*), and garden cress (*Lepidium sativum*) is becoming increasingly popular due to their high content of ALA. Chia, a yearly herbaceous plant belonging to the Lamiaceae family, is known for its Chilean variety that contains 30-33 wt.% oil rich in ALA (62-64 wt.% of total FAs) (Marineli et al. 2014; Knez et al. 2019). The Indian-grown chia variety CHIAmpion-B contains 28-30 wt.% oil with ~65% ALA of total FAs (Gopalam et al. 2021; RV et al. 2015). Besides, chia seeds are a great source of protein (15-25%), total dietary fiber (34-37%), minerals, and natural antioxidants such as carotenoids, tocopherols, polyphenols, and phytosterols (Ullah et al. 2016; Saini et al. 2021).

Camelina sativa is a valuable Brassicaceae oilseed crop that is often overlooked. Its seeds contain high levels of ALA, making up 19-43% of total FAs, and have low SFA content of around 5-10 wt. %. Garden cress (*Lepedium sativum*) is a fast-growing edible belonging to the Cruciferae family. Garden cress seeds contain about 21–24 wt.% oil with 32 wt.% of ALA, a balanced ratio of MUFA/PUFA (~1:1) (Campbell and Camelina, 2018; Vollmann and Eynck,2015).

Various sources of n-3 PUFA, such as tree peony (Paeonia section Moutan DC.), sacha inchi (*Plukenetia volubilis* Linneo), perilla (*Perilla frutescens*), and *Eucommia ulmoides* seeds, have been examined for their abundance in ALA. Tree peony (Paeonia section Moutan DC.), which is native to China and is commonly cultivated for ornamental and medicinal purposes, has seeds that are noteworthy for their high oil content (27 wt.%), with

more than 90 wt.% unsaturated fatty acids, notably ALA (26.1–54.7% of total FAs) (Li et al. 2015).

The Inca peanut, also known as sacha inchi (*Plukenetia volubilis* Linneo), is a longstanding crop of the Peruvian Amazon region (Wang and Zhu, 2018). It is popular due to its balanced and abundant n−3/n−6 PUFAs (Wang and Zhu, 2018). The seeds of sacha inchi cultivars reportedly contain a high oil content of 33.4–37.6%, with ALA composing 37.3–44.2% of total FAs and LA making up 35.2–41.0% (Chirinos et al. 2013).

Perilla (*Perilla frutescens*, family Lamiaceae) is a highly valued annual herb native to Southeast Asia and the Indian highlands. It is also used as a condiment and garnish due to its abundant pharmacological properties. Perilla seeds are rich in ALA (50-64%) and contain 30-45% oil making it a nutritious and bioactive ingredient (Saini et al. 2020; Prabu et al. 2019).

Basil (*Ocimum basilicum* L.) is primarily grown as a fragrant and therapeutic herb in tropical areas of Asia, Africa, and Central and South America. The basil seeds' oil content is 33.0 wt.%, which is abundant in ALA (57-71 wt.%) (Nazir et al. 2021; Dhama et al. 2021).

The *Eucommia ulmoides* Oliver belongs to the Eucommiaceae family. It is predominantly grown in Japan, Korea, and China for its valuable bark and foliage, which are widely used for medicinal purposes. It is noteworthy that these seeds contain a large amount of oil (30-40 wt.%), which is abundant in ALA (56-63 wt.%) (Zhang et al.2018).

The English walnut (9-10 wt% of ALA) and hemp seeds (8.8 wt.% of ALA) can also serve as alternative sources of ALA (Burns-Whitmore et al. 2019).

In plant sources, herbs possess photosynthetic leaves, which are highly rich in ALA, accounting for more than 50% of total FAs. However, purslane, a common weed in field crops and lawns, has a rich source of ALA, accounting for 41-66 % of total FAs in its leaves. Additionally, it has an appropriate balance with n-6 fatty-acid gamma-linolenic acid (GLA) (Uddin et al. 2014).

SDA, an intermediate of ALA, is a rate-limiting step for producing EPA and DHA in mammals. Interestingly, certain families of plants, including Onagraceae, Saxifragaceae, Scrophulariaceae, Boraginaceae, Primulaceae, and Cannabaceae, possess high levels of Δ6-desaturase activity. By providing a direct source of SDA, the rate-limiting step of Δ6-desaturase can be bypassed, resulting in the production of EPA and DHA. As a result, oils that are rich in SDA are becoming increasingly popular as a sustainable source of n-3 LC-PUFA, particularly for EPA (Prasad et al. 2020).

The oil extracted from the seeds of *Echium plantagineum*, *Buglossoides arvensis*, and *Ribes spp*. have been extensively studied as a source of SDA-rich oil. The seeds of purple viper's bugloss (*Echium plantagineum*, family Boraginaceae) are composed of 24% of the oil that is abundant in ALA (34.5% of total FAs), SDA (11.0%), and GLA (9.6%), and phytosterols. This oil is commercially sold as n−3, n−6, and n−9 PUFA-containing oil, which could be a promising substitute for fish oil. Among various Boraginaceae species, *Echium's* seed oil (14.7%) and *Lappula patula* (13.6%) have the highest contents of SDA (Rincón-Cervera et al.2020; Guil-Guerrero et al. 2014).

The seeds of *Buglossoides arvensis* (corn gromwell; Ahiflower®, Boraginaceae family) are highly valued due to their oil content, which ranges from 16% to 21%. This oil is rich in SDA (17% to 21%) and ALA (42% to 50%) (Prasad et al. 2020; Sreedhar et al. 2017).

The GLA content in *Borage officinalis* L. is between 15.7% to 34.5% of the total fatty acids. SDA and ALA are also present in small amounts, ranging from 0.1% to 0.3% and 0.1% to 0.6%, respectively. *Borage officinalis* L. is a highly valuable source of GLA (Guil-Guerrero et al. 2018).

Jostaberry (*R. nidrigolaria* Bauer), blackcurrant (*R. nigrum* L.), redcurrant (*R. rubrum* L.), and gooseberry (*R. uva-crispa* L.) are all part of the Grossulariaceae family in the Saxifragales order. These plants are an excellent source of SDA. Piskernik et al. (2018) reported the fatty-acid composition of these plants and found that jostaberry contains the

highest amount of SDA (5.6% of total fatty acids) and total n−3 PUFA (33.4%). Jostaberry also has the lowest ratio of n−6/n−3 (1.17) PUFAs (Piskernik et al.2018).

Mertensia maritima (L.) Gray from the Boraginaceae family, grown in vitro, has foliage containing a significant amount of oil (10.9% DW). This oil is rich in SDA (6.0% of total lipids) and ALA (30.4%). The fatty-acid composition of various *Mertensia sp.* seeds, *M. alpine* (Torr.) G. Don was found to have the highest levels of SDA (9.3% of total lipids) and ALA (12.9%) (Park et al. 2019).

2.5.3 Genetically modified crop sources of n-3 PUFA

Food products containing GM oilseed crops that produce GLA and SDA have been approved for consumption. One example is the GM soybean line MON87769, which has been authorized for food and feed use by various countries such as Australia, the European Union, Japan, Canada, and Korea. This soybean line expresses the Δ15-desaturase (from *Neurospora crassa*) enzyme, which converts LA to ALA, and the Δ6-desaturase (*Primula juliae*) enzyme, responsible for the conversion of ALA to SDA (Petrie et al. 2014; Usher et al. 2017).

Australia has granted authorization for the use of GM canola that produces DHA, with the inclusion of Δ12-desaturase from *Lachancea kluyveri*, Δ15-desaturase from *Pichia pastoris*, D6-desaturase from *Micromonas pusilla*, Δ6-elongase from *Pyramimonas cordata*, Δ5-desaturase from *Pavlova salina*, Δ5-elongase from *Pyramimonas cordata*, and Δ4-desaturase from *Pavlova salina,* in food and feed production (Han et al. 2020).

Yarrowia lipolytica, a type of food-grade yeast that has been metabolically engineered, has been found to produce a remarkable amount of EPA. Studies have shown that it can produce over 25% of its dry cell weight of EPA during commercial-scale fermentation.

The demand for omega-3-PUFAs is increasing, leading to the creation of transgenic plants that can produce EPA and DHA. The successfully engineered traditional oilseed crops, like transgenic camelina, to produce these beneficial fatty acids, which expressed various

combinations of desaturases and elongases from plants and microalgae, showed that they can accumulate 15–20% of EPA + DHA (Xie et al. 2015).

2.5.4 Thraustochytrids, microalgae, and macroalgae source of n-3 PUFA

Thraustochytrids, a type of fungus-like *Stramenopiles*, are a valuable source of dietary EPA and DHA. It is also referred to as algae. Thraustochytrids, particularly species of *Schizochytriumm, Aurantiochytrium, Crypthecodinium,* and *Ulkenia*, are commonly used in producing vegan EPA and DHA. *Schizochytriumm sp.* can accumulate notable amounts of both EPA and DHA (16.18% and 33.72%, respectively). In comparison, DHA is mainly concentrated in most *Schizochytriumm sp.* (37.10–63.1%), *Aurantiochytrium* (30–40%), *Crypthecodinium* (40–45%), and *Ulkenia sp.* (45%) with trace amounts of EPA present (Gray et al. 2017; Huang et al. 2012; Fsanz and Arasco, 2003; Kiy et al. 2021).

The microalgae are the key source of n-3 PUFA; they also have a high percentage of total lipids (up to 37–60% of dry weight), and microalgae such as *Nannochloropsis sp.* can accumulate up to 37.8% EPA (Ma et al. 2017).

According to Pereira et al. (2012), macroalgae from chlorophytes, rhodophytes, and phaeophytes contain varying amounts of n−3 PUFAs, ranging from 9.5% to 18.0%, 2.90% to 27.26%, and 6.57% to 15.37%, respectively. Rhodophytes had the most favorable n−6/n−3 PUFAs ratio of 0.60–1.92, while chlorophytes and phaeophytes had ratios of 0.31–31.25 and 2.28–3.89, respectively.

Table 2.4 Plants, microalgae, GM crop sources of n-3 PUFA

Sources	ALA (%)	SDA (%)	EPA (%)	DHA (%)	References
Seeds					
Brassica napus sp. *oleifera* L. (rapeseed/canola)	8.7-9.5				Beyzi et al. 2018
Buglossoides arvensis (L.) I.M. Johnst. (Corn gromwell; Ahiflower®)*	49.6	21			Sreedhar et al. 2017
Camelina sativa (L.) Crtz. (Camelina)*	19.1-43.1				Vollmann and Eynck, 2015
Echium canatabricum	33.6	14.7			Guil-Guerrero et al. 2014
Echium plantagineum (Purple viper's bugloss)	34.5	11			Rincón-Cervera et al. 2020
Eucommia ulmoides Oliver	61.36				Zhang et al. 2018
Lappula patula	40	13.6			Guil-Guerrero et al. 2014
Lepidium sativum (Garden cress)	30.34				Umesha et al. 2013
Linum usitatissimum (Flax)	53.4				Zamani et al. 2020
Mertensia alpine (Torr.) G.Don.	12.9	9.3			Layshenko et al. 2021

Mertensia ciliata (James ex Torr.) G. Don	11.8	6.4			Layshenko et al. 2021
Ocimum basilicum (Basil)	63.8				Zamani et al. 2020
Paeonia section *Moutan* DC. (Tree peony)	26.1-54.7				Li et al. 2015
Perilla frutescens (Perilla)	65.6				Zamani et al. 2020
Plukenetia volubilis L. (Sacha inchi)	37.3-44.2				Chirinos et al. 2013
Ribes nidrigolaria Bauer (Jostaberry)	28.01	5.45			Piskernik et al. 2018
Ribes nigrum L. (Blackcurrant)	14.89	2.86			Piskernik et al. 2018
Ribes rubrum L. (Redcurrant)	24.40	3.35			Piskernik et al. 2018
Ribes uva-crispa L. (Gooseberry)	20.54	4.32			Piskernik et al. 2018
Salvia hispanica L. (Chia)*	54.5-64.7				Melo et al. 2019
Herbs					
Mertensia maritima (L.) Gray	30.4	6			Park et al. 2019
Portulaca oleracea L. (Purslane)	45.3-51.2				Nemzer et al.2020
Microalage					

Isochrysis galbana	3.1			11.8	Lim et al. 2012
Nannochloropsis salina *			25-30		Saini et al. 2021
Nannochloropsis sp.	0.1-17.5		4.7-33.7		Scott et al. 2013
Nannochloropsis sp. CCNM 1081			27.6		Ma et al. 2016
Nannochloropsis sp. BR2	0.4		18.8		Lim et al. 2012
Pavlova lutheri	0.1		21.8		Lim et al. 2012
Phaeodactylum tricornutum Bohlin	0.38-0.40	0.87-1.14	22.8-30.7	0.98-1.70	Qiao et al. 2016
Thraustochytrid					
Aurantiochytrium limacinum SR21				30-40	Huang et al. 2012
Crypthecodinium cohnii *				40-45	Fsanz and Arasco, 2003
Schizochytrium sp.		0.07	16.2	33.7	Gray, 2017
Schizochytrium limacinum SR21				66.7	Patel et al. 2020
Thraustochytrium sp. ONC T18				37.8	Scott et al. 2011
Schizochytrium sp. FCC-3204	0.1	0.3-0.4	0.5-0.9	59.8-63.1	Saini et al. 2021
Schizochytrium sp. ONC-Tl8 *		0.20-0.32		37.10-42.47	Saini et al. 2021

Ulkenia sp. SAM 2179 *				45	Kiy et al. 2021
Seaweeds					
Codium fragile (Suhr) Hariot (Chlorophyta)	14.2-19.9		3-4.4		Schmid et al. 2014
Laminaria digitata (Hudson) J.V. Lamouroux (Phaeophyceae)	5-5.5		12.51-13.1		Schmid et al. 2014
Palmaria palmata	0.8		32.1		Maehre et al. 2014
Palmaria palmata (L.) O. Kuntze (Rhodophyta)	1.5		36.8-41.2		Schmid et al. 2014
Vertebrata lanosa			34.3		Maehre et al. 2014

(Adapted from Saini et al. 2021).

Table 2.5 Microencapsulated vegan n−3 fatty acids−based commercial products

Company	Ingredient Brand	Major n-3 fatty acids
FrieslandCampina N.V. (Amersfoort, Netherlands)	Vana®-Sana algae DHA 11 IF	Microalgal derived DHA
Algarithm Ingredients, Inc. (Saskatoon, Saskatchewan)	Betamega3	Microalgal oil powder (120 mg DHA
	Gamma3	Microalgal DHA emulsions (400 mg DHA/g emulsion)

Cubiq Foods (Granollers. Barcelona)	Go!Mega3®	Microalgal DHA+EPA (2% *w*/*w*)
Seanova (Finistère, Brittany)	Algal DHA powder H100	100 mg/g DHA from *Schizochytrium* sp
	Chia powder-125	60 mg/g ALA from chia seeds
	Chia powder-435	55 mg/g ALA from chia seeds

(Adapted from Saini et al. 2021).

2.6 OXIDATION OF LIPIDS

The rancidity of fats and oils is an important problem in food industry. Lipids oxidation is the deteriorative reaction occurring during the processing and storage of oils and fats. Oxidation produces undesirable off-flavored compounds and decreases the nutritional quality and shelf life of the desired product (Andersson and Lingnert, 1997). Lipid oxidation is influenced by several factors (light, heat, oxygen, and metals) that react and initiate rancidity in products by producing primary and secondary oxidation compounds. Oxidation occurs in lipids by various mechanisms such as auto-oxidation, photo-oxidation, thermal oxidation, and enzymatic oxidation. There are several methods for measurement of the extent of oxidation in fats and oils. Peroxide value, *para*-anisidine value, conjugated diene, and 2-thiobarbituric acid (TBA) value are some of the most common methods used in industry.

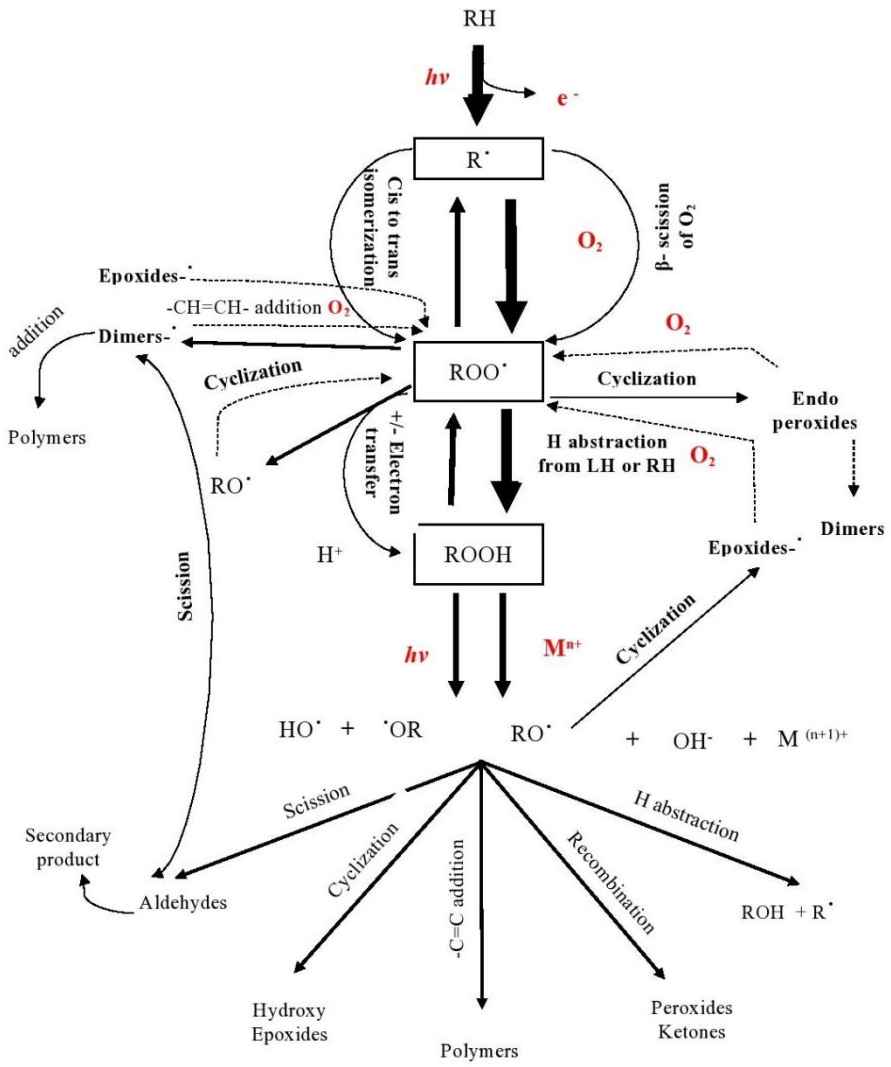


Figure 2.2 Reaction scheme that integrates alternate pathways of lipid oxidation with the traditional chain reactions driven by hydrogen abstractions (adapted from Schaich 2012)

2.6.1 Factors affecting oxidation of lipids

Lipid oxidation is influenced by several factors and these factors decrease the oxidative stability, nutritional value, and shelf-life by forming primary and secondary oxidized products in fats and oils. Unsaturated fatty acids, moisture, heat, light, oxygen, trace metal, free fatty acids and some enzymes are some of the most important factors that hasten the oxidation process.

2.6.1.1 Lipid composition

Oxidative susceptibility of lipids depends primarily on their fatty acids composition. The polyunsaturated fatty acids contain the highest number of bisallylic methylene positions that are most susceptible to oxidation. The stereospecific positional distribution of fatty acids in the triacylglycerol (TAG) molecules is also involved in the susceptibility of lipids oxidation. Shen and Wijesundera, (2009) reported that the oxidative stability of two regio-isomer TAG molecules SSD (1,2-distearoyl-3-docosaenoyl glycerol) and SDS (1,2-distearoyl- 3-docosaenoyl glycerol), it expressed that DHA is stable to oxidation when it located at the sn-2 position of the TAG. The marine sources of oil contain a high amount of PUFA. As the degree of unsaturation increases, the formation of primary oxidation compounds accumulation increases at the end of the induction period (Martin-Polvillo et. al., 2004). Free fatty acids are also susceptible to initiating oxidation and produce primary and secondary oxidation products in the presence of trace metals and environmental conditions. Phospholipid role in oxidative stability varies, depending on the fatty acids profile and the nature of the polar head group, which influences the alignment of the phospholipid molecule. Pikul and Kummerow, (1990) reported that phosphatidylinositol (PI), with the highest PUFA percentage, presented the highest oxidation rate.

2.6.1.2 Temperature and light

Elevated temperatures can accelerate the rate of lipid oxidation due to the breakdown of hydroperoxides, which leads to the production of free radicals. To control the formation of oxidation products during the induction period, it is recommended to lower the storage temperature. The refined fish oil should be stored at a temperature range of +4°C to −18°C to prevent oxidation to slow the production of primary and secondary lipid oxidation products and increases the shelf life (Velasco and Dobarganes 2002).

In some cases, lower temperature also degrades the product by solidification that causes changes in appearance, inability to pour, and disruption of oil-in-water emulsions (McClements and Decker 2008). Aidos et al., (2002) reported that crude herring oil stored

at 50°C under darkness increases the rate of hydroperoxide formation, and in the reverse phenomenon, stored at 0 to 20°C under darkness decreases the rate of hydroperoxide formation.

Light accelerates the singlet oxygen oxidation. The shorter wavelength had more detrimental effects on the oil oxidation compared to the longer wavelength. The transparent bottles of fats and oils showed singlet oxygen oxidation in the presence of light. The Tinuvin 234 or Tinuvin 326 is the UV absorber, which is incorporated in the transparent bottle to improve oxidative and sensory stability under the presence of light.

2.6.1.3 Oxygen

Oxygen is an important factor in lipid oxidation. Abiding by Hund's Rule, triplet oxygen ($^3O_2$) exists as a diradical form in its ground state with two unpaired electrons with parallel spins (Halliwell and Gutteridge 1990). The high oxygen partial pressure in the headspace of the oil increases the oxygen concentration and decreases the oxidation stability. Light, high temperatures and metal (iron, copper) contents increase the oxygen concentration. Oxygen is reduced during the oxidation of other molecules, forming reactive oxygen species (ROS) that can accelerate lipid oxidation in fats and oils. Hydroxyl (HO•), peroxyl (ROO•), alkoxyl (RO•), and hydroperoxyl (HOO•) radicals and superoxide anion (O_2^-) act as ROS. The degradation of H_2O_2 by UV light and metals also produces ROS.

Singlet oxygen is a highly reactive, electrophilic, and non-radical molecule. The singlet oxygen has a low activation energy of 22.4 kcal, increasing the reaction with fats and oil and initiating lipid oxidation. Active singlet oxygen reacts with lipids to form hydroperoxides, which produce free radicals that can initiate a free radical chain reaction to decrease the nutritional value and shelf-life of the product.

2.6.2 Various mechanisms of lipid oxidation

Various oxidation mechanisms initiate the oxidation process in fats and oils during processing, storage, and cooking. The autoxidation, photooxidation, thermal oxidation, and enzymatic oxidation mechanism describe in detail:

2.6.2.1 Autoxidation

Lipids are non-radical singlet states that auto-oxidize by heat, metals, or light. Autoxidation is the direct reaction with organic compounds under mild conditions. Low bond dissociation enthalpies easily remove the allylic hydrogen because it is attached to the carbon between two double bonds (Min and Boff 2002; Choe and Min 2005). The carbon and hydrogen dissociation enthalpies are the lowest at the bis-allylic methylene position (Wagner and others 1994). In linoleic acid, bis-allylic hydrogen at C11 is removed at 75 to 80 kcal/mol. The allylic hydrogen at C8 or C14 of linoleic acid is removed at 88 kcal/mol, and 101 kcal/mol is essential to eliminate alkyl hydrogen from C17 or C18 (Wagner and others 1994; Min and Boff 2002; Choe and Min 2005). Auto-oxidation occurs in three stages as follows:

Initiation: -Free radicals react with O_2, and the resultant peroxy radical invades several other lipids. Also, they may remove hydrogen (H) that would form hydroperoxide (intermediate condition), and another free radical is produced: -

$$RH + O_2 \rightarrow R\bullet + RO_2\bullet + OH\bullet + H_2O$$

Propagation: - Two free radicals (alkoxide and hydroxyl) formed by the break of hydroperoxides or it provides peroxy radical, hydroxyl free radical, and water. Further, the autocatalytic reaction occurs where free radical proliferation (branching steps) that encored propagation (it is called autocatalytic reaction) happens as follows: -

$$R\bullet + O_2 \rightarrow RO_2\bullet$$

$$RO_2\bullet + RH \rightarrow ROOH + R\bullet$$

Branching: -

$$ROOH \rightarrow RO\bullet + OH\bullet$$

$$2ROOH \rightarrow ROO\bullet + RO\bullet + H_2O$$

Termination: -Interaction between two free radical species produce non-radical species due to the collision of two high potential radical at the substantial concentration of radical species (Hamilton *et al.* 1997 and Pullen, Saeed 2012).

Terminated at low oxygen environment

$$2R\bullet \rightarrow R\bullet\ R$$

Terminated at high oxygen environment

$$R\bullet + RO_2\bullet \rightarrow ROOH$$

$$RO_2\bullet + RO_2\bullet \rightarrow ROOH + O_2$$

2.6.2.2 Photo-oxidation

Lipid oxidation is accelerated by light in the presence of photosensitizers (e.g., chlorophylls). Chlorophylls in the singlet state become excited upon absorption of light energy causing the triplet excited state of chlorophylls. The excited triplet state chlorophylls react with $^3O_2$, producing $^1O_2$ (Choe and Min 2006).

The molecule's spin multiplicity developed the triplet and singlet electronic states, defined as 2S+1, where S is the total spin number. Depending on the angular direction of the electronic spin, the S value can be either +1/2 or –1/2. The oxygen's two unpaired electrons have parallel spins, and the spin multiplicity will be 2 (1/2 + 1/2) + 1 = 3, which is the triplet state. The electrons with antiparallel spin values within the same orbital will cancel each other out, resulting in the singlet state. The oxidation of edible oils requires free radicals in order to react with oxygen.

Photosensitizers that produce high-energy oxygen species called singlet oxygen radical, which will in turn abstract hydrogen atoms from unsaturated fatty acids, without forming a carbon-based free radical. The chlorophyll, flavins (e.g., riboflavin), and myoglobin photosensitizer absorb light in the visible or UV (<270 nm) wavelength and become electronically excited (Frankel 2005). There are two distinct mechanisms: the electronically excited sensitizer can cause photosensitized oxidation in the presence of oxygen. In the type I mechanism, lower singlet oxygen concentrations react with the photosensitizer possessing enough energy to abstract a hydrogen atom from a fatty acid generating a free radical (Min and Boff 2002). In the type II mechanism, singlet oxygen directly reacts with high-electron-density double bonds via a 6-membered ring without lipid radical formation (Gollnick 1978; Choe and Min 2005). Hydroperoxide formed by singlet oxygen is conjugated and nonconjugated in the oxidation process. The nonconjugated hydroperoxides do not occur in autoxidation. The linoleic acid oxidation by singlet oxygen produces C9-, C10, C12, and C13 hydroperoxides.

2.6.2.3 Thermal oxidation

The chemical changes in thermal oxidation are the same as in autoxidation. Heat energy in the edible oil create chemical changes reaction that is faster than the autoxidation; it produces unstable primary oxidation products (hydroperoxides) that are decomposed rapidly into secondary oxidation products such as volatiles (aldehydes, ketones, short-chain hydrocarbons, lactones, alcohols, and esters) (Choe and Min 2007).

Radical reactions also produce nonvolatile polar compounds, triacylglycerol dimers, and polymers in thermally oxidized edible oil. Dimerization and polymerization are important reactions in the thermal oxidation of oil. Dimers and polymers are large molecules with 692 to 1600 Daltons molecular weight and formed by a combination of –C–C–, –C–O–C–, and –C–O–O–C– bonds (Kim and others 1999). Polymerization is present more easily in oil with high linoleic acid than in high oleic acid oil contents (Bastida and Sanchez-Muniz 2001). The C–C bonds are formed between two acyl groups to produce acyclic dimers in heated oil under low oxygen (Nawar 1996).

2.6.2.4 Enzymatic oxidation

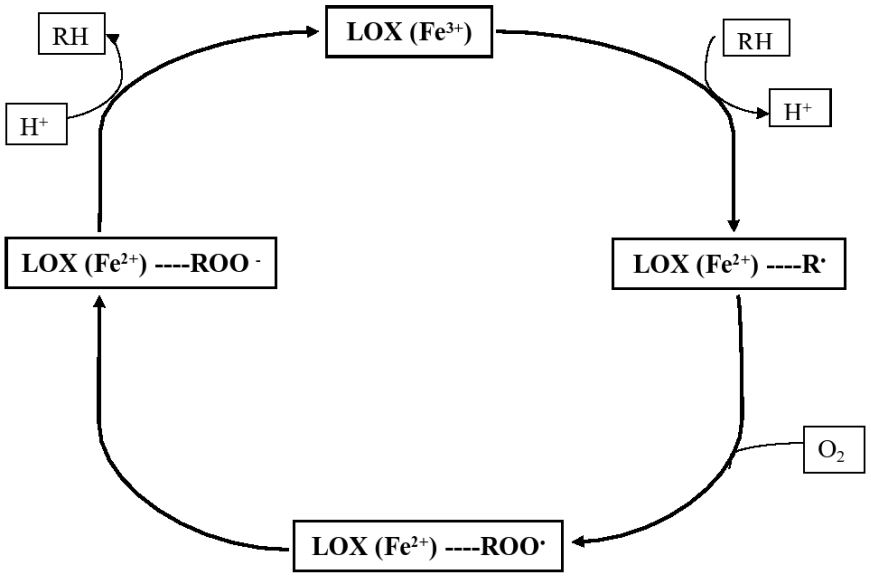


Figure 2.3 Oxidation of linoleic acid by lipoxygenase (LOX) (adapter from Min and Choe 2009).

The non-radical mechanism for lipid oxidation is catalysed by lipoxygenase (Niki 2004). This is an iron-bound enzyme with Fe in its active centre. Unsaturated fatty acids with 1-cis, 4-cis-pentadiene system oxidizes by lipoxygenase, resulting in oil deterioration (Engeseth et al., 1987; Hsieh and Kinsella 1986). The stereospecific complex forms between lipoxygenase in the ferric state with iron (LOX-Fe^{3+}) and unsaturated fatty acid with 1,4-pentadienyl system (RH) abstracts hydrogens from interrupted methylenes in the fatty acids. Lipoxygenase binds to pentadienyl radical, which is rearranged into a conjugated diene system, followed by the reaction with oxygen to produce lipid peroxy radicals ($ROO^{\bullet}$). The iron in the enzyme is reduced to the ferrous state (LOX-Fe^{2+}). Lipid peroxy radicals are reduced to ROO^{-} by lipoxygenase with iron in a ferric state again, and the attachment of a proton, produced by the oxidation of hydrogen abstracted from fats and oils by lipoxygenase, results in the release of hydroperoxides (Belitz and Grosch 1999).

2.7 Refining of sardine oil

Edible oils are important constituents of foods containing essential human health components like acylglycerols, vitamins, phytosterols, tocopherols, and polyphenols. Crude oil includes both essential components, which elicit a positive effect on the quality and stability of the oil and undesirable components. Undesirable components like unsaponifiable matters, waxes, pigments, phospholipids, solid impurities (fibers), and oxidation products (peroxides, aldehydes, ketones, alcohols, and oxidized fatty acids) generate adverse effects and degrade the oil stability. (Chandrasekar et al. 2014).

The spoilage in crude oil depends on lipid profile, the concentration of undesirable compounds, and prevailing environmental conditions that present oxidative and hydrolytic instability during storage and transhipment. Fish oil also contains polychlorinated biphenyls (PCBs) organic pollutants and the high level of polycyclic aromatic hydrocarbons (PAHs) due to pollution (Ortiz et al., 2011). These compounds have carcinogenic and mutagenic properties (Hua et al., 2016).

Every edible oil industry strives to produce flavour-neutral, light-coloured, and physically and oxidatively stable oil, thereby meeting consumer acceptance and industry standards. Crude edible oils do not meet these criteria as they often contain large amount of non-glyceride compounds, olive oil being the exception though. These numerous non-glycerides are removed through operations collectively known as refining. Hence, it is mandatory to know the composition of crude oil before refining to preserve essential components while eliminating undesirable compounds.

2.7.1 Composition of sardine oil

The composition of fat and oils varied based on environmental conditions, species, and strain. The extraction procedure of fat and oil also creates changes in composition. The bulk raw material for oil processing at large-scale operations varied at different seasons with varying freshness and composition.

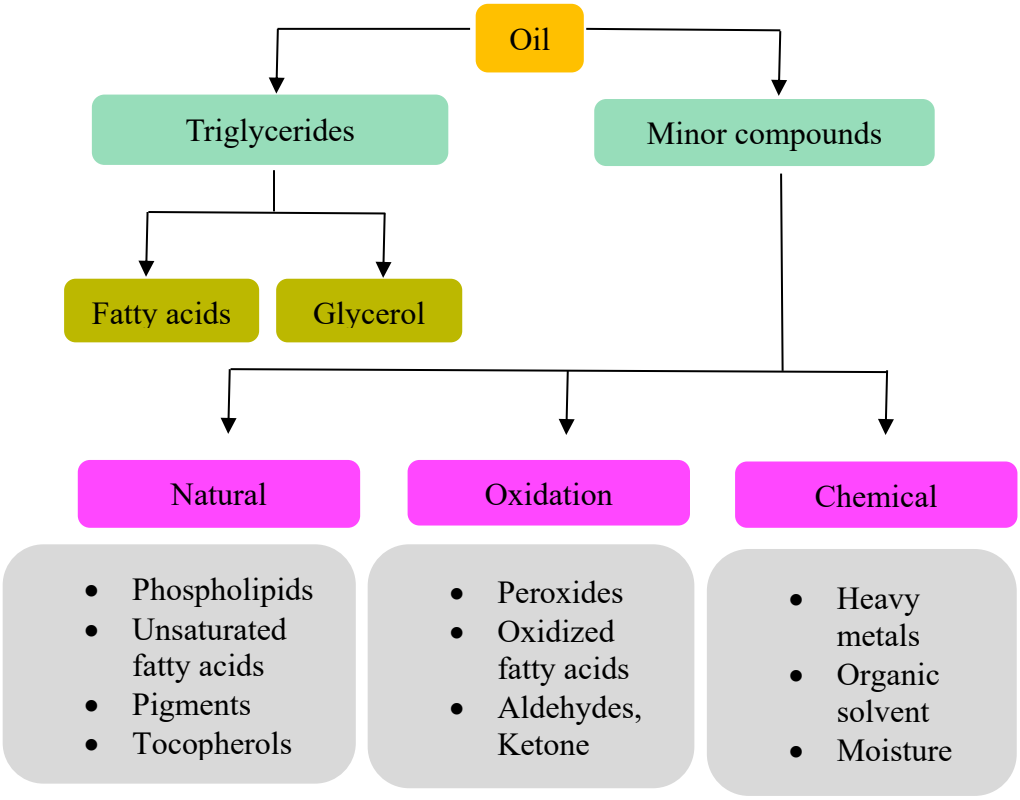


Figure 2.4 General overview of chemical composition and contaminants of edible oils. (Adapted from Chandrasekar et al. 2015)

2.7.2 Essential Compounds

The essential fatty acids play an important role in different catabolic and anabolic reactions in the human body. In fish oil, n-3 PUFA (EPA 20:5 n-3 and DHA 22:6 n-3) is present in a higher amount with nutritional and medical significance. The n-3 PUFA are chemically reactive with heat, light, atmospheric oxygen, and other impurities that cause oxidation problems during storage and decrease stability. The EPA and DHA are readily available in fish oil and vegetable oil α-linoleic acid and act as a precursor for producing EPA and DHA in the human body. The other vital non-fatty acids compound like squalene, tocopherols, fatty alcohols, and sterols in fish oil contribute to the unsaponifiable matter and have

beneficiary effects. It should ensure minimal loss of these vital compounds during the refining process.

2.7.3 Undesirable compounds

In lipids, free fatty acids are formed by cleavage of ester bonds due to heat, moisture, and enzyme (lipase) involved in the oil deterioration. In the oxidation mechanism, free fatty acids act as pro-oxidants, decreasing the smoke point of cooking oils. Edible oil has a higher amount of phospholipid that also involve in rancidity. It also leads to colour deepening when exposed to air or sunshine, which hinders the distillation and deodorization process during oil refining (Dijkstra, 2009). The other minor components such as metal ions and volatile matter, decrease the quality and stability. Removing these impurities via the refining process is necessary to delay oxidation.

2.8 Various process steps of refining

The edible oil industry faces challenges during the refining process to retain nutritionally essential compounds from vegetable and marine sources. The refining process is designed according to the composition of crude oil that minimize the removal of desirable compounds and eliminate the maximum amount of undesirable compounds to increase the preservation for several months and maintain the nutritional quality of edible oil for customer uses. Various techniques are used to refine oil. There are two primary processes for crude oil refining: chemical and physical.

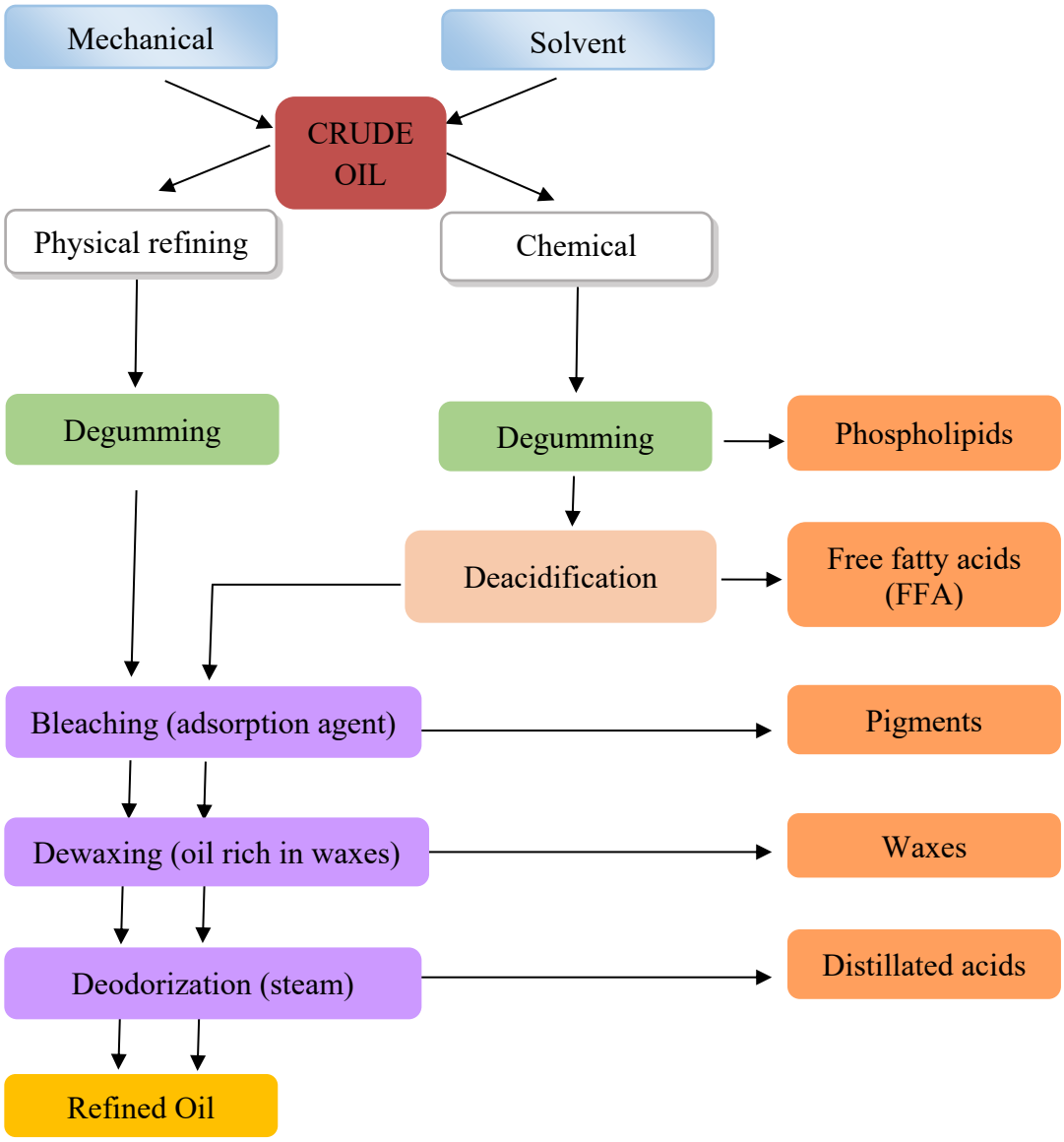


Figure 2.5 General overview of the chemical and physical refining process of crude oil. (Adapted from Chandrasekar et al. 2015)

2.8.1 Chemical refining process

In the chemical refining process, particular chemicals are used in oil to remove undesirable compounds. There are five steps in the chemical refining process:

- Degumming aim to eliminate phospholipids and mucilaginous gums.
- Deacidification allows the elimination of free fatty acids, phospholipids, and metals.
- Bleaching aims to remove pigments, peroxides, and residuals of fatty acids.
- Dewaxing to remove the waxes from oils rich in waxes.
- Deodorizing eliminates volatiles and free fatty acids.

2.8.1.1 Degumming

It is the initial step of refining edible crude oil to remove phospholipid and some trace metals with mucilaginous substances. Phospholipids have several types: Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI), which are naturally present in oils. The presence of these compounds in crude oils poses many problems for storage and processing. Phospholipids linked with heavy metal act as catalysts in an oxidation reaction and behave as prooxidants in edible oil. The ideal phospholipids concentration after refining is < 5mg/kg (Yang et al., 2008). There are several types of degumming processes: acid degumming, dry degumming, membrane degumming, and enzymatic degumming.

2.8.1.1.1 Acid degumming

The acid-degumming process has a lower residual phosphorous content by food-grade and sufficient phosphoric solid and citric acids that chelate divalent metal ions. The degumming acid conditions the non-hydratable phospholipids to hydratable phospholipids. Citric acid is preferably used in degumming because it does not increase the phosphoric content of oils. The acids dispersion is mandatory for maximal contact with the non-hydratable

phosphatide complexes. The isolated gums after acid degumming process are not used as standard lecithin because they contain degumming acid.

2.8.1.1.2 Dry degumming

The dry degumming process combines concentrated acid (phosphoric or citric) (0.05 to 1.2 g/100 g) and bleaching earth (1 to 3 g/100 g) and dispersing in oil at 353°K (80°C) for low phospholipid content. The non-hydratable phosphatides dissociated into phosphatidic acid by these acid combinations, and bleaching earth is then eliminated through centrifugation. This degumming process was developed for palm and coconut-type oils containing minimal phospholipids.

2.8.1.1.3 Enzymatic degumming

The phospholipase C enzyme is used for the enzymatic degumming process to convert non-hydratable phospholipids into lysophospholipids. These compounds are easily separated by centrifugation because of insolubility in oil. The enzyme is used to hydrolyze non-hydratable phospholipids into hydratable form. In this process, crude is initially treated with a combination of citric acid and caustic soda, and it is mixed with water and an enzyme (Lecitase Ultra) using a high-shear mixer. This is then centrifuges to break the stable emulsion and separate the phospholipids and mucilaginous materials from the oil.

2.8.1.1.4 Membrane degumming

This process removes phospholipids from crude oil with organic solvent mixtures or without adding organic solvent. The different varieties of microfiltration (MF) and ultrafiltration (UF) membranes used for oil degumming based on membrane material, size and shape of the components in oil, and the interaction of solutes with the membrane surface (Manjula and Subramanian, 2006). The ultrafiltration membrane separation efficiency by molecular size exclusion and reduction of phospholipids from the non-porous membrane is due to the interaction between the solute and the membrane material (Coutinho et al., 2009).

Some drawbacks to the membrane degumming process in crude oil include pore blocking, drastic flux drop, cake formation, and concentration polarization (Pagliero et al., 2005; DeSouza et al., 2008).

2.8.1.2 Deacidification

The deacidification removes free fatty acids from the crude oil. There are several approaches for deacidification, such as membrane process, solvent extraction, deep eutectic solvent, chemical catalyst re-esterification, and enzymatic re-esterification.

2.8.1.2.1 Deacidification by membrane processes

The membrane process separates hydrophilic free fatty acids from hydrophobic glycerides. The free fatty acids have a hydroxyl group, and the membrane has a carboxyl group that could show high selectivity and affinity for FFA in the oil due to the hydroxyl and carboxyl group interactions. Polar solvents have lower flow through the hydrophobic membrane, which facilitates efficient separation of free fatty acids with the solvents (retentate) from triglycerides in the oil (permeate), and it also involves the reduction of solvent content for permeate through the membrane (Bhanushali et al., 2001). In crude palm oil, the PVDF membrane removes free fatty acids (Azmi et al., 2015).

2.8.1.2.2 Deacidification by solvent extraction

The solubility of FFA in polar solvents, as against nonpolar glycerides, removes FFA from the oil. Short-chain alcohols (e.g., ethanol, methanol, and acetone) are preferred to efficiently extract FFA from oil. Long-chain alcohols are not preferred for separating FFA from the oil because they are hydrophobic (Rodrigues et al., 2007). Hamm (1992) has claimed that short-chain alcohols are an efficient solvent deacidification method for fish oil to enrich the EPA and DHA.

2.8.1.2.3 Deacidification by deep eutectic solvents

The deep eutectic solvent (DESs) has desirable characteristics in terms of biodegradability, biocompatibility, and green designer solvent. These can be effective deacidification

solvents for elimination of FFA. The solvent system consists of hydrogen bond acceptor (HBA) that forms hydrogen bonds with one or more hydrogen bond donors (HBD). Choline chloride (ChCl)-based DESs with ethylene glycol and other organic acids (acetic, propionic, lactic, malic, oxalic, citric, malonic, or phenylacetic acids) as HBDs have been successfully applied as green solvents for extraction of natural compounds from plants.

2.8.1.3 Re-esterification using a chemical catalyst

The re-esterification of free fatty acids in the oil is catalysed with a chemical catalyst with added glycerol or other glycerides with free hydroxyl groups. This reaction forms monoglycerides, diglycerides, and triglycerides with the generation of water molecules. Water should be removed by the influence of inert gas and by maintaining the reaction mixture under a vacuum. The main factor affecting the re-esterification process is the reaction temperature, quantity, type of catalyst, and amount of glycerol. Zinc dust, zinc chloride, and naphthalene-beta-sulphonic acid are various deacidified catalysts (Ebewele et al., 2010).

2.8.1.4 Enzymatic re-esterification

The unique enzymatic-esterification process uses microbial lipases to synthesize a glyceride from free fatty acids, and glycerol has been exploited to deacidify edible oils, and it is invariably carried out at higher temperatures (180–200°C) (Bhosle and Subramanian, 2005). Various microbial enzymes, such as 1,3-specific *Mucor miehei* lipase, and Candida sp. lipase, are mentioned in a few reports with effective re-esterification processes for removal of free fatty acids.

2.8.1.5 Bleaching

This process is used to improve the overall quality of the oil by removing traces of metals, pigments, and oxidized glycerides. The bleaching agent also eliminates some amount of phospholipid and free fatty acids. Bleaching adsorbents are usually neutral clays, activated earth, synthetic silicates, silica gel, and activated carbon (Chandrasekar et al. 2016). These adsorbents form complex interactions between the bleaching clay and the impurities. It

may be a physical reaction where impurities get trapped inside the pores of clay due to Van der Waals forces (Sabah et al., 2007), or it can be the chemical reaction between the bleaching agent and particles occurring via ionic bonds (Ahmad et al., 2009; Kuuluvainen et al., 2015; Aachary et al., 2016). This process is carried out under vacuum to avoid causing oxidative instability to the oil where adsorbent is mixed with hot oil at around 80–110°C for 15–30 min.

In marine oils, bleaching is critical for eliminating residual free fatty acids, phospholipids, metal ions, and other contaminants such as PAH and PCBs. In vegetable oil, bleaching adsorbent removes unwanted color, Polycyclic aromatic hydrocarbons (PAHs), and some organic contaminants such as polycyclic aromatic hydrocarbons, benzo(a)pyrene, benzo(a)anthracene, benzo(b) fluoranthene, and chrysene.

2.8.1.6 Dewaxing

Waxes present as alcohols and mono-ester of long-chain fatty acids in edible oil. The wax formation is spontaneous and is affected by storage conditions (e.g., temperature, time). The presence of waxes does not affect the oil functionality, but it creates a cloudy appearance in oil, especially during the winter. The fish also contain waxes due to a high degree of unsaturation. The dewaxing process for edible oil has three steps: First, liquid-bleached oil is heated to 328°K (55°C). In the second step, slowly cool down 283–288 K (10–15°C) oil and chill it for several hours. In the final step, they pumped the cooled oil into the filter machine to separate the waxes from the oil.

2.8.1.7 Deodorization

It is a vacuum steam distillation process to remove undesirable compounds such as free fatty acids, ketones, alcohols, dioxins, odors, and flavors. The negative effect of this process is that it may remove some essential bioactive compounds like tocopherols, squalene, sterols, and polyphenols. This process destroys essential nutrients, conjugation, polymerization, and unwanted side reactions like *cis-trans* isomerization (double bond isomerizes from *cis* to *trans*). The distillation process proceeds with a simple water vapor

injection into the oil. Using high-pressure steam boiler heating at a high temperature of 453–513 K (180–240°C) with a vacuum between 2 to 8mmHg this creates a passage of steam through the layer of oil and removes undesirable compounds.

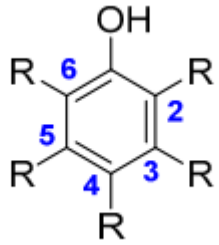
2.8.2 Physical Refining

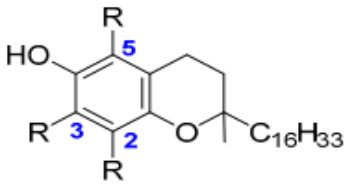
By steam distillation under a vacuum, physical refining removes free fatty acids, impurities, and unsaponifiable matter from the oil. The operating conditions are designed to remove some odor substances. Physical refining is economical, eco-friendly, and minimizes liquid effluent generation. This process is implemented at a high temperature unsuitable for heat-sensitive oil because it degrades the oil and form *trans*-isomers.

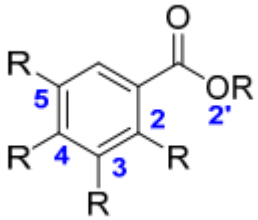
2.9 Antioxidants

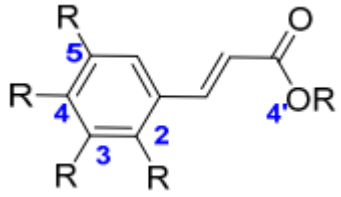
Antioxidants are molecules which can delay oxidation and maintain the nutritional value and shelf life of lipids. The daily intake of natural antioxidants in the human diet reduces the risk of degenerative diseases. Synthetic antioxidants are vital in food and pharmaceutical industries at limited concentrations. Antioxidants suppress the oxidation with several mechanisms: (i) sequestration of free radicals from the medium; (ii) metal chelation ions; (iii) production of enzyme for inhibition of free radicals; (iv) endogenous antioxidant enzymes activation; (v) prevention of lipid peroxidation; (vi) prevention of DNA damage; (vii) prevention of protein modification and sugar destruction. The antioxidants are classified as a primary and secondary antioxidant depending on their role in lipid systems. The primary antioxidant is a chain-breaking antioxidant that donates hydrogen to the lipid alkyl radicals. Secondary antioxidants suppress the oxidation by metal chelation, quenching the singlet oxygen, photosensitizing, and inactivating lipoxygenase. Synergism is the cooperative behaviour of antioxidants to retard oxidation.

Table 2.6 List of Antioxidants, their structure and bond dissociation enthalpies (BDE)

Synthetic antioxidants						
						
Name	**R(2)**	**R(3)**	**R(4)**	**R(5)**	**R(6)**	**BDE (kcal.mol^{-1})**
5-*Tert*-butylpyrogallol	OH	H	$C(CH_3)_3$	H	OH	66.6
Pyrogallol	OH	H	H	H	OH	68.0
Hydroxyquinol	H	H	OH	H	OH	69.1
Propyl gallate	OH	H	$C(O)OC_3H_7$	H	OH	69.6
BHA	$C(CH_3)_3$	H	OCH_3	H	H	72.3
4-*Tert*-butylcatechol	H	H	$C(CH_3)_3$	H	OH	72.3
BHT	$C(CH_3)_3$	H	CH_3	H	$C(CH_3)_3$	72.4
TBHQ	H	H	OH	H	$C(CH_3)_3$	74.3
o-*Tert*-butyl-*p*-cresol	H	H	CH_3	H	$C(CH_3)_3$	77.4
Phloroglucinol	H	H	H	OH	H	83.0

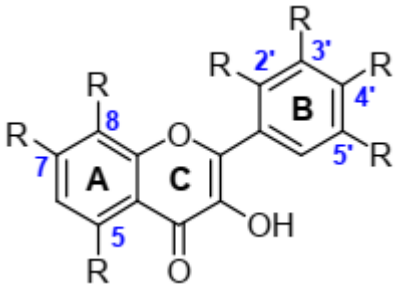
Tocopherols				
				
Name	**R(2)**	**R(3)**	**R(5)**	**BDE (kcal.mol^{-1})**
α-Tocopherol	CH_3	CH_3	CH_3	69.1
β-Tocopherol	CH_3	H	CH_3	73.4
γ-Tocopherol	CH_3	CH_3	H	73.5
δ-Tocopherol	CH_3	H	H	75.4

Hydroxybenzoic acids						
						
Name	**R(2)**	**R(3)**	**R(4)**	**R(5)**	**R(2')**	**BDE (kcal.mol^{-1})**
Gallic acid	H	OH	OH	OH	H	70.2
Protocatehuic acid	H	OH	OH	H	H	75.5
Syringic acid	H	OCH_3	OH	OCH_3	H	78.1
Ellagic acid	H	OH	OH	OC(O)-	- $C_6H(OH)_3$	78.4
Gentisic acid	OH	H	H	OH	H	79.5
Vanillic acid	H	OCH_3	OH	H	H	83.1
PHBA	H	H	OH	H	H	84.7
Salicylic acid	OH	H	H	H	H	95.2

Hydroxycinnamic acids						
						
Name	**R(2)**	**R(3)**	**R(4)**	**R(5)**	**R(4')**	**BDE (kcal.mol^{-1})**
Rosmarinic acid	H	OH	OH	H	$C_9O_4H_{10}$	69.2
Caffeic acid	H	OH	OH	H	H	72.1

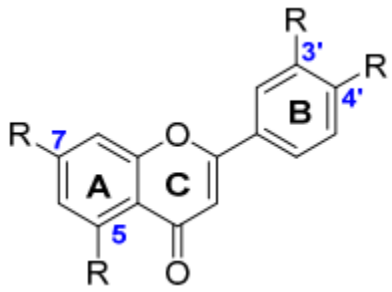
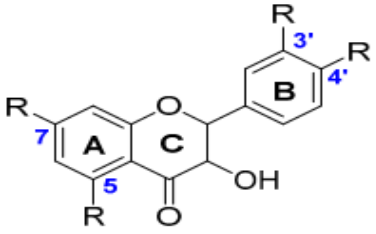
Chlorogenic acid	H	OH	OH	H	$C_6H_2(OH)_3CO_2H$	73.4
Sinapic acid	H	OCH_3	OH	OCH_3	H	75.4
Ferulic acid	H	OCH_3	OH	H	H	79.7
o-Coumaric acid	OH	H	H	H	H	80.1
p-Coumaric acid	H	H	OH	H	H	80.5
m-Coumaric acid	H	OH	H	H	H	84.4

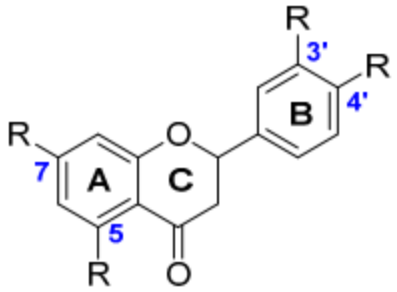
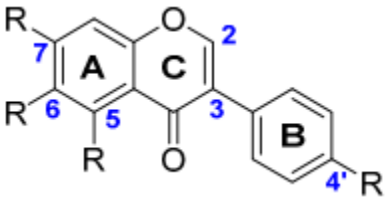
Flavonols

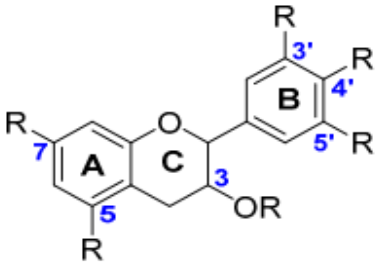


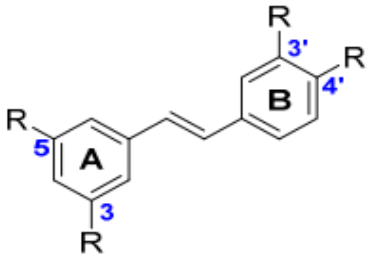
Name	R(2')	R(3')	R(4')	R(5')	R(5)	R(7)	R(8)	BDE (kcal.mol^{-1})
Gossypetin	H	OH	OH	H	OH	OH	OH	66.6
Myricetin	H	OH	OH	OH	OH	OH	H	67.4
Azaleatin	H	OH	OH	H	OCH_3	OH	H	71.1
Quercetin	H	OH	OH	H	OH	OH	H	71.8
Fisetin	H	H	OH	OH	H	OH	H	72.3
Laricitrin	H	OCH_3	OH	OH	OH	OH	H	72.5
Syringetin	H	OCH_3	OH	OCH_3	OH	OH	H	75.7
Rhamnazin	H	OCH_3	OH	H	OH	OCH_3	H	79.6
Kaempferide	H	H	OCH_3	H	OH	OH	H	79.8

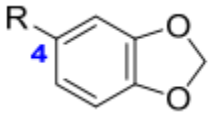
Flavonols								
Name	**R(2')**	**R(3')**	**R(4')**	**R(5')**	**R(5)**	**R(7)**	**R(8)**	**BDE (kcal.mol^{-1})**
Isorhamnetin	H	OCH_3	OH	H	OH	OH	H	79.8
Morin	OH	H	OH	H	OH	OH	H	79.8
Kaempferol	H	H	OH	H	OH	OH	H	80.1
Galagin	H	H	H	H	OH	OH	H	81.2
Rutin**	H	OH	OH	H	OH	OH	H	82.15

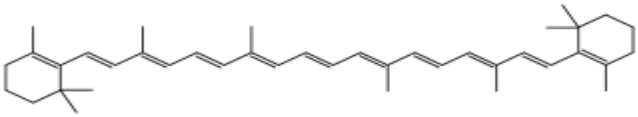
Flavones					
					
Name	**R(3')**	**R(4')**	**R(5)**	**R(7)**	**BDE (kcal.mol^{-1})**
Luteolin	OH	OH	OH	OH	73.1
Apigenin	H	OH	OH	OH	82.1
Flavanonols					
					
Name	**R(3')**	**R(4')**	**R(5)**	**R(7)**	**BDE (kcal.mol^{-1})**
Taxifolin	OH	OH	OH	OH	73.2
Aromadedrin	H	OH	OH	OH	82.3

Flavanones					
					
Name	**R(3')**	**R(4')**	**R(5)**	**R(7)**	**BDE (kcal.mol^{-1})**
Eriodictyol	OH	OH	OH	OH	73.6
Homoeriodictyol	OCH_3	OH	OH	OH	83.8
Hesperetin	OH	OCH_3	OH	OH	82.2
Naringenin	H	OH	OH	OH	82.4
Isoflavones					
					
Name	**R(3')**	**R(4')**	**R(5)**	**R(7)**	**BDE (kcal.mol^{-1})**
Glycitein	OH	H	OCH_3	OH	80.1
Genistein	OH	OH	H	OH	81.0
Daidzein	OH	H	H	OH	81.9

Flavanol							
							
Name	**R(3')**	**R(4')**	**R(5')**	**R(3)**	**R(5)**	**R(7)**	**BDE (kcal.mol[1])**
Epigallocatechin gallate	OH	OH	OH	$C(O)C_6H_2(OH)_3$	OH	OH	66.5
Gallocatechin	OH	OH	OH	H	OH	OH	68.5
Catechin	OH	OH	H	H	OH	OH	74.4

Stilbenes					
					
Name	**R(3')**	**R(4')**	**R(3)**	**R(5)**	**BDE (kcal.mol^{-1})**
Piceatannol	OH	OH	OH	OH	68.7
Resveratrol	H	OH	OH	OH	76.7

Lignans		
		
Name	**R(4)**	**BDE (kcal.mol^{-1})**
Sesamol	OH	75.1

Carotenoid		
		
Name		**BDE (kcal.mol^{-1})**
β-Carotene*		73.85

(Adapted from Guitard et al. 2016).

Note: - *indicated the BDEs observed in micelles, **indicated the estimated C-H BDE, and other antioxidants BDEs from DFT (Density functional theory) methods.

2.9.1 Classification of antioxidants

Antioxidants are classified into two forms: natural antioxidants and synthetic antioxidants.

2.9.1.1 Natural antioxidants

Antioxidants from natural sources of plant products have the potential to protect against various diseases. Natural antioxidants are stable hydroxyl derivatives vital in suppressing oxidation (Hou et al., 2003). These antioxidants scavenge free radicals, inactivate metal catalysts by chelation and reduces hydroperoxides (Frankel and Finley, 2008). Several classes of the natural antioxidant compound are phenolic acids, flavonoids, tocopherols, stilbenes, lignans, and tannins. Fruits, vegetables, nuts, seeds, flour, root, leaves, bark, or other sources fulfil the need for natural antioxidants with beneficial effects.

2.9.1.1.1 Phenolic acids

They are the multipurpose bioactive compounds plays a vital role in the human diet. They have several health-protective effects, including anti-mutagenic, anti-carcinogenic, anti-inflammatory, antimicrobial, and several biological properties. Phenolic acids have antioxidant activity and hence protect food from oxidation (Xu et al., 2008). Substituted hydroxybenzoic and hydroxycinnamic acid derivatives are predominant phenolic acids in plants. Precisely, benzoic acid derivatives are phenolic acids, and cinnamic acid derivatives are phenylpropanoids. The pattern of hydroxylation and methoxylation in their aromatic rings differs in these derivatives (Mattila and Hellström, 2007; Shahidi and Naczk, 2004). In hydroxycinnamic acid, rosmaric acid has the lowest BDE value (69.2 kcal.mol^{-1}), and hydroxybenzoic acid and gallic acid have the lowest BDE value 70.2 kcal.mol^{-1}.

The antioxidant activity of phenolic acid is due to the hydroxyl substituent on the aromatic ring that affects the radical-quenching ability to suppress oxidation (Shahidi and Wanasundara, 1992). The estimated consumption of phenolic acid in the human diet is 25mg to 1g, which can be fulfilled by fruits, vegetables, grains, tea, coffee, and spices (Clifford, 1999).

2.9.1.1.2 Tocopherols and tocotrienols

Both are monophenolic compounds with eight chromanol homologs and possess vitamin E activity in the human diet (Blekas et al., 1995). Tocopherols contain a 6-chromanol group and an apolar phytal chain. It is present in α-, β-, γ-, or δ-form that depends on the position and number of methyl groups attached to the chrome rings (Kiokias et al., 2008). The tocotrienols contain the unsaturated side chains at 3′, 7′, and 11′ positions (Seppanen et al., 2010). In plant, α-tocopherol is exhibited in chloroplasts of the plant cell, and β-, γ- and δ- is available outside these organelles. Tocotrienols are present in certain seeds' gran, cereals, and germ fractions (Kamal-Eldin and Appelqvist, 1996). Tocopherols and tocotrienols have antioxidant activity to donate the phenolic hydrogen to lipid free radicals to suppress oxidation. The potent antioxidant activity of tocopherols with hydrogen donating ability in the form of increasing order are; α > β > γ > δ. Tocopherol also shows a synergistic effect

with ascorbic acid, β-carotene, and glutathione, and it regenerates the tocopherol and forms tocopheroxyl radical (Torres and Medina, 2012).

2.9.1.1.3 Carotenoids

Carotenoids contain eight isoprenoid units joined together to form a conjugated double-bond system. The α-, β-, γ-Carotene, and lycopene forms of carotenoids contain isoprene units. The xanthophylls or oxygenated carotenoids (lutein and zeaxanthin) have oxygen (present in hydroxy, epoxy, or keto group) in addition to the hydrocarbon chain. Carotenoid molecules' ionone ring with hydroxyl groups can be esterified with long-chain fatty acids to form esterified carotenoids. β-Cryptoxanthin is another form of carotenoid that possess vitamin A activity and involve to play an essential role in vision.

Carotenoids have antioxidant activity to retard oxidation by quenching the effective $^1O_2$ (singlet oxygen) and scavenging the free radical (reactive oxygen species) to maintain the stability, flavor, and quality of the product (Ramel et al., 2012). In the singlet oxygen quenching mechanism, carotene converts the singlet oxygen excited state to the lowest excited triplet state (Schmidt, 2004). β-Carotene is a lipid-soluble provitamin with two retinyl groups. The breakdown of the retinyl group in the mucosa of the human small intestine forms retinal (vitamin A form) involved in the role of vision (Weber and Grune, 2012). Lycopene having eleven conjugated and two non-conjugated double bonds is sufficient to suppress the oxidation caused by singlet oxygen (Perretti et al., 2013).

2.9.1.1.4 Flavonoids

Flavonoids are cyclized diphenylpropanes usually present in plants and plant foods (Cao et al., 1997). Flavanones affecting the heterocyclic carbon ring undergo a series of transformations to give rise to anthocyanins and catechins (Das, 1994). All derivatives of flavonoids have C6-C3-C6 carbon skeleton characteristics, and the variation in their structure is around the heterocyclic oxygen ring (Shahidi and Naczk, 2004; Yao et al., 2004). The 2-phenylchromone is a parent compound composed of three phenolic rings (A, B, and C), which present several levels of hydroxylation and methoxylation.

Isoflavonoids consist of a phenyl ring (A-ring) fused with the six-membered heterocyclic C-ring and another phenyl ring (B-ring) at the C-3 position. In flavonoids, the B-ring is substituted to the C-2 position. After minor structural differences, Isoflavonoids have higher antioxidant activity than flavonoids (Han et al., 2009).

In food, flavones and flavonols are present as aglycones - the double bond between C-2 and C-3 position in these compounds. Flavonols can regard as 3-deoxyflavonols because of the hydroxyl group in the 3-position. The flavonols (gossypetin 66.6 kcal.mol-1, myricetin 67.4 kcal.mol^{-1}, and quercetin 71.8 kcal.mol^{-1}) have the lowest BDE. The flavonones and flavononols may be referred to as dihydroflavonesare because of a saturated C2-C3 bond and an oxygen atom (carbonyl group) in the 4-position.

Flavononols having a hydroxyl group in the 3-position are called 3-hydroxyflavonones (Shahidi and Naczk, 2004). In flavonoids, anthocyanins and catechins are flavan-3-ols and flavan-3,4-diols because of the lack of the carbonyl group in the 3-position. (Shahidi and Naczk, 2004).

The antioxidant activity of flavonoids is dependent on three factors (1) metal chelating potential that depends on the arrangement of hydroxyls and carbonyl group around the molecule (ii) It also depends on free radical scavenging activity by hydrogen donation, (iii) flavonoids can delocalize the unpaired electron for the formation of stable phenoxy radical. Musialik et al., 2009; Rice-Evans et al., 1996; Zhou et al., 2005 showed that the antioxidant activity of flavonoids is due to catechol hydroxyl groups in the B ring. The 3′,4′- dihydroxy configuration of all flavonoids possess antioxidant activity.

2.9.1.1.5 Tannins

It is a secondary metabolite produced by plants with 30,000 Dalton molecular mass. Tannins are divided into two classes of macromolecules; hydrolyzable tannins and condensed (proanthocyanidins) tannins (Shahidi and Naczk, 2004). The hydrolyzable tannins are glycosylated gallic acid and their molecular mass range between 500 to 5000 Da. The primary sources are berries, legumes, and leafy vegetable is beneficial for human

health. It also has antioxidant activity. The condensed tannins are flavan-3-ols with a molecular mass range of 30000 Da. It is generally found in wine, beer, tea, cocoa, and other natural nutritional products. It also showed radical scavenging activity against oxidation (Khanbabaee and van Ree, 2001).

The anti-nutritional nature of tannins is due to the binding of the phenolic group with –NH groups of peptides and proteins to prevent hydrolysis and digestion in the stomach (Shahidi and Naczk, 2004). The *in vitro* antioxidant activity of tannins retards lipid oxidation and lipoxygenases. In the cellular prooxidant state, tannins can scavenge the hydroxyl, peroxyl, and superoxide radical oxidation (Gyamfi and Aniya, 2002). Tannins also affect bacterial growth by various mechanisms, including extracellular microbial enzyme inhibition and inhibition of oxidative phosphorylation for the action on microbial metabolism (Scalbert, 1991). Yoshida et al. (1999) reported that raspberry extracts present anti-tumor activity because it contains ellagitannins sanguiin H6 and lambertianin. Propyl gallate is a white crystalline powder prepared by esterifying gallic acid with propyl alcohol via a distillation process to remove the excess alcohol. It is used in various food packaging and fat-containing food to stabilize oxidation. It is also added in mayonnaise baked products, pressure-sensitive adhesives, lubricating oil, and transforming oil (Zurita et al., 2007). Shahidi and Naczk, 2004 reported that propyl gallate chelates with citric acid to eliminate pro-oxidative iron and copper catalysts. Shahidi and Wanasundara, 1992 showed that propyl gallate also effectively responds against oxidation in combination with BHA and BHT. PG has shown liver toxicity and enhanced carcinogenesis, and it inhibits respiration and nucleic acid synthesis to suppress the growth of microorganisms (Eler, Peralta, and Bracht, 2009; Kim, Kang, Lee, Lee, and Lee, 2008). It also inhibits the activity of redox enzymes by decreasing hepatic microsomal hydrolase and demethylase activities (Han and Park, 2009).

2.9.1.1.6 Stilbenes

It presents two aromatic rings linked with an ethane bridge, and it is available in monomer (resveratrol, oxyresveratrol) and oligomer form (dimmers, trimmers, or polymer of

resveratrol). The other varities of stilbenes are ε-viniferin, pallidol, etc (Kostadinović et al., 2012; Charles, 2013). One prominent member of this group, resveratrol (3,5,4′-trihydroxystilbene), is an antimicrobial, deterrent, or repellent compound in plants. Adrian and Jeandet (2012) reported that grapevines produce resveratrol to protect the plant from *Botrytis* fungal infection. It also protects the grape vines from bacteria, nematodes, or herbivore attack. Resveratrol is found to elicit several health benefits to humans, including cardioprotective, neuroprotective, anti-tumor, and antioxidant activity. Resveratrol can exist in *trans*- and *cis*- isomeric forms which is influenced by light. Sources of resveratrol for human daily intake are red wine, peanuts, pistachio, berries, red cabbage, spinach, and a few herbs (Jaganath and Crozier, 2010). Trans-resveratrol-3-O-β-D-glucoside can inhibit cardiovascular disease and cancer (Jaganath and Crozier, 2010). Pterostilbene with one hydroxyl group protects the oxidation and inhibits cancer growth by *in vitro* inhibition of metastasis, cell cycle alteration, and apoptosis induction. In human breast cancer, pterostilbene retard VEGF (vascular endothelial growth factor) production.

2.9.1.1.7 Lignans

It is a bioactive polyphenolic compound formed by two coniferyl alcohol residues through the shikimic acid or the phenylpropanoid pathway. It has potent antioxidant and anti-inflammatory activities (Landete, 2012). Varities of lignans present in certain plants including isolariciresinol, secoisolariciresinol diglucoside, lariciresinol, and matairesinol etc. Flaxseed, grains, leaves, roots, fruits, and vascular plant's woody parts contain high concentrations of lignans (Ekiert et al., 2013). Sesame oil also have lignan compounds such as sesamin, sesamol, sesamolin, sesaminol and sesamolinol. These compounds maintain the oxidation stability and nutritional quality of the oil. Sesamol has a vigorous antioxidant activity to suppress the oxidation of n-3 PUFA-rich fish oil at different storage (30 and 50 °C) conditions.

2.9.1.2 Synthetic antioxidant

Currently permitted synthetic antioxidants for use in foods industries are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tertiary-butylhydroquinone (TBHQ) (Makahleh, Saad, and Bari, 2015). Some other synthetic antioxidant are; 5-*tert*-butylpyrogallol, pyrogallol, hydroxyquinol, 4-*tert*-butylcatechol, *o*-*tert*-butyl-p-cresol, phloroglucinol. These antioxidants are deliberately added with limited concentration (200mg/kg) to products to maintain oxidation stability while processing and storing fats and oils and preserving their nutritional value. Some reports mentioned that high concentrations of BHA and BHT are responsible for liver damage and carcinogenesis in animal laboratories. The synthetic antioxidant creates a molecular complex with nucleic acid structure and damages the DNA (Dolatabadi and Kashanian, 2010).

2.9.1.2.1 Butylated hydroxyanisole (BHA)

BHA is a white waxy flake monophenolic antioxidant. It is a mixture of two isomers, 3-tertiary-butyl-4-hydroxyanisole (90%) and 2-tertiary-butyl-4-hydroxyanisole (10%). BHA effectively suppresses the oxidation of short-chain fatty acids. The coconut and palm kernel oils contain short-chain fatty acids generally used in confectionery products (Shahidi and Wanasundara, 1992). The BHA synergized with some BHT, TBHQ, or PG regenerators. Nanditha and Prabhasankar, 2009 reported that the combination of BHA (0.01%) and dodecyl gallate (0.005%) protects the oxidation of margarine more effectively than BHA alone. Dolatabadi and Kashanian, 2010; Makahleh et al., 2015 showed that BHA is added to beverages, dessert mixtures, potato products, and baked/fried foods.

2.9.1.2.2 Butylated hydroxytoluene (BHT)

BHT is also a monophenol commercially available as a white crystalline compound. However, BHT is not as effective as BHA mainly because of the presence of two *tert*-butyl groups, which offer more significant steric hindrance than BHA to the molecule (Nanditha and Prabhasankar, 2009). BHT is soluble in fats and oils and insoluble in water (Table 2) and more effective in suppressing the oxidation of animal fats than vegetable oils (Dziezak,

1986). Shahidi and Naczk (2004) reported that BHT could produce radical intermediates with moderate resonance delocalization. The tertiary butyl groups of BHT do not generally allow the involvement of the radical formed from it after hydrogen abstraction in other reactions. Thus, a lipid peroxyl radical may join the molecule of BHT in the para position to the phenoxy group.

BHT can act synergistically with BHA. A combination of 3-BHA and BHT showed a higher antioxidant activity than using singly in soybean oil, lard, and methyl oleate (Nanditha and Prabhasankar, 2009).

2.9.1.2.3 Tertiary-butylhydroquinone (TBHQ)

TBHQ is formed from 3-tert-butyl-4-hydroxyanisole (BHA). It is also used as a food additive by O-demethylation. This synthetic antioxidant prevents the oxidation of fat and oils products and does not cause discoloration, even in the presence of iron. It also sustains flavour, odour, and nutritional quality. Shahidi and Wanasundara, (1992) reported that TBHQ might be used as a substitute for increasing oxidative stability through hydrogenation. TBHQ enhanced lipid oxidation stability with chelating agents (citric acid and monoacylglycerol citrate), mainly used in vegetable oils, not extensively for animal fats (Shahidi and Wanasundara, 1992). TBHQ has potent antioxidant activity by two para-hydroxyl groups (Nanditha and Prabhasankar, 2009). TBHQ damages the DNA at high doses and is a precursor to stomach tumours.

2.9.1.2.4 Propyl gallate (PG)

Propyl gallate is a white crystalline powder prepared by esterifying gallic acid with propyl alcohol via a distillation process to remove the excess alcohol. It is used in various food packaging and fat-containing food to stabilize oxidation. It is also added in mayonnaise baked products, pressure-sensitive adhesives, lubricating oil, and transforming oil (Zurita et al., 2007). Shahidi and Naczk, 2004 reported that propyl gallate chelates with citric acid to eliminate pro-oxidative iron and copper catalysts. Shahidi and Wanasundara, 1992 showed that propyl gallate also effectively responds against oxidation in combination with

BHA and BHT. PG has shown liver toxicity and enhanced carcinogenesis, and it inhibits respiration and nucleic acid synthesis to suppress the growth of microorganisms (Eler, Peralta, and Bracht, 2009; Kim, Kang, Lee, Lee, and Lee, 2008). It also inhibits the activity of redox enzymes by decreasing hepatic microsomal hydrolase and demethylase activities (Han and Park, 2009).

2.10 Mechanism of actions of antioxidants

Antioxidants are molecules that prevent or delay the oxidation in lipids when present in low concentrations (Halliwell and Gutteridge 2007). The addition of antioxidants to oil can minimise rancidity and prevent the formation of oxidation products. Various antioxidants exhibit diverse mechanisms of action while retarding the oxidation in lipids.

2.10.1 Free radical scavenging

Antioxidants scavenge the free radicals from foods by donating hydrogen and producing relatively stable antioxidant radicals. Hydrogen transfer from antioxidants to the peroxy or alkyl radicals of foods is more thermodynamically favourable (Choe and Min 2005).

The ability to scavenge free radicals by a phenolic compound is due to the presence of phenolic hydroxyl groups. The primary mechanism of free radical scavenging could be through hydrogen atom transfer (HAT), single electron transfer followed by proton transfer (SETPT), or sequential proton loss electron transfer (SPLET). The HAT mechanism is characterized by the BDE values corresponding to the ability of an XH (X=C,O) moiety to donate its hydrogen atom and consequently form a radical.

In the case of SETPT mechanism, ionization of the antioxidant molecule is the first step, and thus ionization energies (IE) is used to describe the electron donor ability. The lower the IE value, the easier the electron transfer and the higher the antioxidant activity. The last step of the SETPT mechanism is the loss of a proton from the cation radical formed in the first step; thus, it is characterized by the PDE that determines the thermodynamically preferred X-H (X=C, O) group for deprotonation.

SPLET mechanism starts with dissociating the acidic moiety, which can be characterized by the proton affinity. An electron transfer follows this to the free radical at the cost of the electron transfer energy. Lower proton affinities (PA) are characteristic of higher antioxidant capacity via this mechanism. The PA value and ETE (electron transfer enthalpies) value are the enthalpies of the reaction related to the SPLET mechanism. The PA value represents the degree of difficulty of phenolic hydroxyl dephosphorization, while the ETE value represents the electron donation ability of corresponding polyphenol ions. Metal chelation decreases oxidation by preventing metal redox cycling, forming insoluble metal complexes, or providing stearic hindrance between metals, food components, or their oxidation intermediates. Singlet oxygen quenching leads to the formation of ground state triplet oxygen by energy or charge transfer (Quan et al. 2018).

2.10.2 Singlet oxygen quenching

$^1O_2$ has high energy of 93.6 KJ above the ground state $^3O_2$, and they react with lipids at a higher rate than $^3O_2$. Singlet oxygen produces undesirable compounds in foods during processing and storage. Singlet oxygen quenching can be achieved by physical and chemical methods (Korycka-Dahl and Richardson 1978; Girotti 1998). Physical quenching leads to the deactivation of singlet oxygen to the ground state triplet oxygen by energy transfer or charge transfer. $^1O_2$ quenching by energy transfer occurs when the energy level of a quencher is very near and below that of singlet oxygen (Min et al. 1989). Carotene and lycopene with 11 conjugated double bonds are more effective singlet oxygen quencher than lutein with ten conjugated double bonds (Beutner et al. 2001). The presence of oxo and conjugated keto groups or cyclopentane rings in the structure confer the singlet oxygen quenching ability.

Chemical quenching of singlet oxygen is a reaction involving the oxidation of a quencher rather than quenching, thus producing breakdown or oxidation products of a quencher. Tocopherol reacts irreversibly with singlet oxygen and produces tocopherol hydroperoxydienone, tocopherylquinone, and quinine epoxide (Halliwell and Gutteridge 2001).

2.10.3 Metal chelation

The metal-chelating antioxidant is a class of secondary antioxidants to suppress lipid oxidation by sequestration of free metals through chelation (iron, zinc, copper, etc.). In the redox reaction, free metal catalyzes non-enzymatic oxidation by an electron from hydrogen or lipid hydroperoxide, generating lipid radicals and reactive oxygen species. In oxidation, ferrous iron (II) converts into ferric iron (III). In the redox cycle, ferric ion reduces into ferrous ions via a reaction with superoxide anion or several reducing agents. It is the reduction of hydrogen peroxide into hydroxide anion and hydroxyl radical by the Fenton reaction (Pratt and Hudson, 1990).

Antioxidants with metal chelation activity inhibit metal-catalysed reactions by various properties; steric hindrance between metals and lipid oxidation intermediates, formation of insoluble metal complex, prevention of metal redox reaction, and occupation of all metal coordination sites. Metal chelator antioxidants with an *o-quinol* group at the B-ring tend to stabilize iron and copper in their oxidized or noncatalytic form. In a food matrix, metal chelator efficacy depends on the environmental conditions, in which chelators bind with metals through charged acid groups, then the pH is more significant than pKa (Miller et al., 1990). Metal chelators bolster food quality by enhancing antimicrobial activity and increasing water-holding capacity within muscle foods.

Phytate has been shown to retard lipid oxidation by decreasing iron, calcium, and zinc absorption (Pratt and Hudson, 1990). Flavonoids also have metal chelation activity to bind with metal ions. It is related to the structural feature: 3', 4'-dihydroxy group in the B ring, the 4-carbonyl and 3-hydroxy group in the C ring, or the 4-carbonyl group in the C ring together with the 5-hydroxy group in the A ring (Hudson and Lewis, 1983; Feralli et al., 1997). Some antioxidants with metal-chelating antioxidant activity are catechin, sesamol, ascorbic acid, and in amino acids, carnosine and histidine (Decker et al., 2001).

2.10.4 Synergism

Improvement of oxidation stability with a combination of antioxidants that have various mechanisms and show the combined effect to regenerate the primary antioxidant is referred to as synergism. The oxidation process degrades the product in several ways, and a combination of antioxidants can retard the oxidation for a long duration. In the cooperative effect of antioxidants, bond dissociation enthalpy (BDE) and the reduction potential value play vital roles.

Based on the mechanism, there are two types of synergism; Homo synergism comprises two similar antioxidants exhibiting same mechanism of action against lipid oxidation. In this, the free-radical scavenging antioxidants donate hydrogen to reactive free radicals during propagation. The primary antioxidant possesses a lower BDE value, and hence releases hydrogen bonds to free-radical too fast. Another antioxidant with a higher BDE value acts as a regenerator to donate hydrogen bonds to the primary antioxidant and thus inhibit the rancidity of oil for a long duration. Hetero-synergism is a combined effect of antioxidants with different mechanisms. One antioxidant has a free-radical scavenging mechanism, and another has a metal-chelating mechanism. It shows effect synergism to inhibit the oxidation of food components, mainly due to chelators' sparing action of free radical scavengers. Metal chelator mainly acts during the initiation process to inhibit metal-catalyzed oxidation that reduces the amount of free-radical production, and free-radical scavengers efficiently suppress it.

2.11 Oxidative stability study of various bulk oils with antioxidants

Numerous reports on the study of oxidative stability of edible oils in the presence of various natural and synthetic antioxidants are available. Pure components of various herbal extracts and the complex mixture of antioxidants and herbal extracts have been used to study the oxidative stability during storage of oil. A comprehensive list of those studies is presented in Table 2.7 to 2.11.

Table 2.7 Effect of phenolic compounds and their components in edible oils.

Concentration	Oil	Conditions	Conclusions	Authors
200 ppm of natural antioxidants [apigenin, chrysin, quercetin, myricetin, morin, kaempferol, rutin, naringin, naringenin, taxifolin, and [-] epicatechin] and 200 ppm of synthetic antioxidants [BHA, BHT]	Purified canola oil	Accelerated oxidation at 65°C	Myricetin, quercetin, and rutin, as well as (-) epicatechin, exhibited superior oxidative stability than synthetic antioxidants.	Shahidi and Wanasundara, (1995)
Myricetin compared with α-tocopherol and TBHQ (100, 500 & 1000 ppm)	Soybean oil methyl esters	At room temperature under darkness for 90 days	Myricetin (100 ppm) possessed greater antioxidant activity than α-tocopherol, but was inferior to TBHQ at 500 ppm.	Moser, (2008)

100 ppm of catechin, quercetin, rutin, ferulic acid, caffeic acid and sinapic acid	Refined n-3 PUFA rich sardine oil	Stored at 37°C in the dark in contact with atmospheric air for 14 days.	Quercetin and rutin followed by caffeic acid were effective in restraining sardine oil oxidation.	Chandrasekar et al. (2016)
Quercetin (120 ppm) and rutin (244 ppm)	Purified soybean oils containing α-tocopherol and α-tocopherol stripped oil	Stored in sealed bottle with headspace, incubated at 25°C for 45 days.	Both acted as antioxidant in α-tocopherol stripped soybean oils and as a pro-oxidant in oil containing α-tocopherol. Rutin fared better compared to quercetin in exhibiting antioxidant effects in the bulk oil.	Lee et al. (2016)
Commercial rosemary extract and its active constituents (carnosol, carnosic acid, and rosmarinic acid)	tocopherol stripped corn oil	Kept in shaker oven at 60°C	Best antioxidant activity was obtained with 50 ppm of rosmarinic acid, 50 ppm of carnosic acid and 500 ppm of rosemary extract.	Frankel et al. (1996)

Carnosic acid (100 ppm and 1000 ppm)	Virgin olive oil	Accelerated oxidation conditions at 60°C	Dose-dependent inhibition in the formation of primary and secondary oxidation products	Zunin et al. (2010)
Carnosic acid (100,200,300 ppm) , tocopherols (300 ppm), TBHQ (300 ppm)	n-3 PUFA rich fish oil	4 and 30°C for 66 days.	Dose-dependent enhancement of oxidative stability and the antioxidant activity of 300 ppm of carnosic acid was stronger than that of tocopherols but still weaker than that of TBHQ.	Wang et al. (2011)
200 ppm of various phenolic compounds and synthetic antioxidants (tannic acid, ferulic acid, caffeic acid, sinapic acid, catechin, quercetin, rutin, sesamol,	Cold-pressed pecan oil	Aerated condition in dark at 60°C	Caffeic acid inhibited oxidation of pecan oil effectively, and its performance was superior to BHT and BHA and was close to TBHQ.	Zhang et al. (2017)

TBHQ, BHT, and BHA).				
Green tea extracts, α-tocopherol and BHT.	Soybean oil and fish oil	Accelerated oxidation conditions at 98°C	60 ppm of green tea polyphenols showed superior performance than 200 ppm of α-tocopherol in soybean oil. 250 ppm of green tea polyphenols showed almost the same antioxidative activity as 500 ppm of BHT in fish oil.	Koketsu and Satoh, (1997)
Black tea theaflavins and catechin derivatives (200 ppm)	Canola oil	Accelerated storage conditions at 95°C	Both catechins and theaflavins were more effective than BHT, and catechins were more potent as the antioxidant than theaflavins in heated canola oil.	Su et al. (2004)

(Adapted from Mishra et al. 2021).

Table 2.8 Effect of tocopherols in edible oils.

Concentration	Oil	Conditions	Conclusions	Authors
50, 125, 250 ppm	Purified triacylglycerols of sunflower oil	Accelerated oxidation conditions under	Performance of α-tocopherol showed positive correlation with concentration	Marinova et al. (2008)

		darkness for 20 hours		
50, 100, 250, 500 ppm	Purified n-3 PUFA rich mackerel oil	30, 4, -40°C for 66 days	Superior performance of 50 ppm at all the conditions	Zuta et al. (2007)
5, 10, 50, 100, 500 ppm	Purified rapeseed oil triacylglycerols	40°C for 16 days	100 ppm showed superior antioxidant activity	Lampi et al. (1999)
50,100, 250, 500 ppm	Purified triacylglycerol of menhaden oil and purified menhaden oil	30°C in the dark for 6 days	100 ppm showed the highest antioxidant effect throughout the study period in both the oils	Kulås and Ackman, (2001)
1 mM	Mixture of olive and perilla oil (6:4 w/w) having 6.02 ppm of γ-tocopherol and 62.05 ppm polyphenols	10 days at 60°C under darkness.	Pro-oxidant effect of α-tocopherol at all the concentrations	Jung et al. (2014)
20, 200, 2000, 20000 and 50000 ppm	Soybean oil having 130.0 ppm of α-, 21.9 ppm of β-, 781.7 ppm of γ-, and 316.1 ppm of δ-tocopherol	Accelerated oxidation condition at 70°C	Pro-oxidant effect of α-tocopherol in all the samples and production of toxic oxygenated α, β-unsaturated aldehydes and monoepoxides along with other oxidative degradation products.	Martin-Rubio et al. (2017)

50, 100, 200, 500 ppm	Unrefined flaxseed oil having significantly high amount of moisture (400 ± 2 ppm), different types of sterols, carotenoids (57 ppm), and γ-tocopherol (320 ppm)	Under dark at various temperatures (25, 40, 60°C) for 30 days, +Accelerated oxidation condition at 110°C.	Pro-oxidant effect of α-tocopherol under all the storage conditions	Mohanan et al. (2018)
800 ppm	Refined cod liver oil containing traces of free fatty acid	Under nitrogen environment under dark at 25°C for 14 weeks	Pro-oxidant effect	Zhu et al. (2014b)
10, 20, 42, and 84 ppm	Stripped corn oil	Exposed to humidity under accelerated oxidation condition at 60°C	Pro-oxidant behavior was evidenced at high concentration of α-tocopherol (84 ppm) compared to a low concentration (10 ppm)	Kim et al. (2015a)

(Adapted from Mishra et al. 2021).

Table 2.9 Effect of carotenoids in edible oils.

Concentration	Oil	Conditions	Conclusions	Authors
20 ppm of β-Carotene	Purified triacylglycerol fraction of rapeseed oil	Sealed sample stored under light at 25°C +Aerated sample stored	Exhibited the antioxidant effect under light.	Haila and Heinonen, (1994)

		under light at 25°C	Exhibited a pro-oxidant effect in the aerated sample stored under light	
20 ppm of β-Carotene	Purified olive oil	Sealed sample stored under light at 25°C	Exhibited the antioxidant effect	Fakourelis et al. (1987)
β-Carotene and Lycopene	Unrefined safflower seed oil	Accelerated oxidation conditions at 75°C.	Upto 500 ppm concentrations, both exhibited a slightly delayed onset of oxidation, and beyond 500 ppm concentrations, both carotenoids acted as pro-oxidants	Henry et al. (1998)
1000 ppm of Astaxanthin	Groundnut oil, gingelly oil, palm oil, sunflower oil, mustard oil, rice bran oil, coconut oil, and olive oil	Stored at 25 to 90°C	40-50% reduction in peroxide values compared to control	Rao et al. (2007)

Table 2.10 Effect of ascorbic acid and its derivatives in edible oils.

Concentration	Oil	Conditions	Conclusions	Authors
Linoleic acid with L-ascorbic acid or capryloyl, lauroyl, or palmitoyl L-ascorbate with various molar ratios (0.025, 0.05, 0.075, 0.1, or 0.2)	Linoleic acid	Stored under dark conditions at various temperatures (37, 50, 65, or 80°C	Positive correlation between molar concentration and antioxidant effect in both ascorbic acid and acyl L-ascorbates. capryloyl ascorbate had a greater antioxidative ability than ascorbic acid.	Watanabe et al. (2005)
Ascorbyl palmitate (200 ppm) and TBHQ (200 ppm)	Fresh walnut oil having 289 ppm of tocopherols, about 0.93 ppm of carotenoids, and 0.52 ppm of chlorophylls.	Light and dark conditions at room temperature for 6 months	Ascorbyl palmitate and TBHQ failed to contribute markedly in inhibiting photo-oxidative degradation. However, under the darkness-storage condition, they significantly reduced lipid oxidation.In both the conditions, TBHQ fared better than ascorbyl palmitate.	Martínez et al. (2013)

Linoleic acid with ascorbic acid, octanoyl, lauroyl or palmitoyl ascorbate at a molar ratio of 0.025, 0.05, 0.075, 0.1 or 0.2	Linoleic acid	In plastic container at a relative humidity of 12% at various temperatures (37, 50, 65 and 80°C) under darkness.	Positive correlation between the molar ratio of antioxidant and oxidative stability. All the ascorbates exhibited superior antioxidant effect than ascorbic acid.	Watanabe, (2015)
Ascorbic acid (10, 20, 42, and 84 ppm)	Stripped corn oil	Exposed to various relative humidity at 60°C	Positive correlation between ascorbic acid concentrations with the oxidative stability of corn oil irrespective of relative humidity	Kim et al. (2015b)
Ascorbyl palmitate, ascorbyl stearate, BHT, TBHQ, 2,5-di-*tert*-Butylhydroquinone, Propyl gallate, tocopherols, and 2,2'-methylene-bis (4 methyl-6-*tert*-butyl phenol))	Unrefined n-3 PUFA rich flaxseed oil having tocopherol (501 to 674 ppm), carotenoids (25 to 34 ppm), phytosterols	Accelerated oxidation at 100°C	Ascorbyl palmitate and ascorbyl stearate were inferior only to TBHQ and 2,5-di-*tert*-butylhydroquinone and exhibited excellent oxidative stability compared to all the other antioxidants.	Shadyro et al. (2017)

	(5274 to 5869 ppm), and minor amount of coenzyme Q10			
400 ppm of ascorbyl palmitate	Unrefined cold-pressed sesame oil, olive oil (extra virgin), sunflower oil, refined corn oil, and refined deodorized rapeseed oil	Accelerated oxidation at 100°C	oxidative stability exhibited by ascorbyl palmitate was different in different oils and it was most effective in flaxseed oil	Shadyro et al. (2017)

(Adapted from Mishra et al. 2021).

Table 2.11 Effect of plant extracts on oxidative stability of edible oils.

Antioxidant & its Concentration	**Oil**	**Conditions**	**Conclusions**	**Authors**
200 ppm of rosemary extract having carnosic acid concentration, 24.9, 60.5, 98.3 %	Refined sunflower oil	60°C for 21 days under darkness	Positive correlation between carnosic acid concentration and the oxidative stability of	Zhang et al. (2010)

(w/w) compared with 200 ppm of BHA, BHT and TBHQ			sunflower oil. 98.3 % carnosic acid exhibited more effectiveness than BHA and BHT and less effective than TBHQ	
Rosemary extract (2500 ppm)	Docosahexaenoic acid C22:6 (DHA) and Eicosapentaenoic acid C20:5 (EPA), in menhaden oil	60°C for 5 days	Protection of DHA and EPA was reported.	Bhale et al. (2007)
1000 ppm rosemary extract	Refined sunflower oil	Thermal oxidation conditions (180°C)	Extended the oxidative stability from 7.52 to 13.5 hours.	Ramalho and Jorge, (2008)
400 ppm of Rosemary extract & compared with 50:50 mixture of BHA & BHT (200 ppm)	Refined Soybean oil, rice bran oil, and cottonseed oil	Accelerated oxidation condition	Rosemary extract exhibited much better chemical stability, including higher induction period, antioxidant capacity, total phenolic content and lower peroxide value	Yang et al. (2015)

Rosemary powder and powder of black pepper, ginger, turmeric and oregano at 0.5 % (w/v) and BHT (100 ppm)	Tocopherol-stripped corn oil	Accelerated oxidation conditions	Rosemary powder was found to be the most effective one among all the natural ground herbs and BHT	Redondo-Cuevas et al. (2017)
Methanolic extract of sesame cake and compared with TBHQ and BHT (200 ppm).	Refined soybean, sunflower, and safflower oils	Accelerated storage conditions (60°C)	Comparable oxidative stability of oils having 100 ppm of sesame cake with BHT.	Suja et al. (2003)
Methanolic extract of sesame cake and compared with BHT, BHA and TBHQ (200 ppm).	Refined sunflower oil and soybean oil.	Accelerated storage conditions (70°C for 72 hours)	Sesame cake extract (at 200 ppm) exhibited stronger antioxidant activity in than BHT and BHA, while its antioxidant activity was less than that of TBHQ.	Mohdaly et al. (2011)

(Adapted from Mishra et al. 2021).

2.12 Effects of antioxidants on human health

The daily intake of antioxidants through diet is found to show dramatic effects on human health. The average daily intake (ADI) of 1 gram phenolic compounds through fruits,

vegetables, legumes, and beverages, among other sources, suffice the human body requirements. The hydroxycinnamic acids and flavonoid compounds have a wide range of antioxidant activity that protects against several diseases including cancer, cardiovascular, obesity, etc. (Rice-Evans, Miller, Bolwell, Bramley, and Pridham, 1995). The orange peel extract, black tea extract, and coffee extract which are rich in antioxidants showed anti-obesity effects by inhibiting adipose tissue and weight-gaining formation. Together they showed a synergistic effect against obesity (Huang et al., 2009).

The phenolic compounds confer various health benefits that include; anti-allergenic, anti-atherogenic, anti-inflammatory, antimicrobial, antioxidant, anti-thrombotic, cardioprotective, and vasodilatory effects (Mazur and Scalbert, 2005; Puupponen-Pimiä et al., 2001). The tea polyphenols show a protective effect against different stages of carcinogenesis diseases. The EGCG (epigallocatechin-3-gallate) antioxidant in green tea acts as a chemopreventive agent against various lung, liver, gastrointestinal tract, skin, and prostate cancers. It also performs as an anti-obesity and cardiovascular protective compound. Zhong et al. (2012) reported that the tetraester could restrain colon cancer, which is formed by EGCG (epigallocatechin gallate) enhancement via conjugation with DHA (docosahexaenoic acid). Orange peel contains polymethoxyflavones, an essential component with several physiological properties such as anti-inflammatory, anti-carcinogenic, anti-viral, anti-thrombogenic, and anti-atherogenic (Huang et al., 2009; Lai et al., 2007).

Caffeine also suppresses obesity by stimulating thermogenesis, extending sympathetic stimulation, and reducing adipose tissue (Kobayash et al., 2005). In olive oil, the phenolic constituent, hydroxytyrosol, effectively responded against coronary heart disease, platelet aggregation, arachidonic acid metabolism, and atherosclerosis (Tuck and Hayball, 2002). Isoflavones protect against cancer, osteoporosis, menopausal symptoms, and cardiovascular disease (Krischer, 2010; Rimbach et al., 2008). The daily intake of isoflavones reduces the prevalence of allergic rhinitis. Resveratrol has potent antioxidant activity and is effective against type 2 diabetes, cardiovascular disease, neurological

conditions, and cancer (Marques et al., 2009). The available sources of resveratrol are peanuts, grapes, berry fruit, pistachio etc. Rayalam et al., (2008) showed that resveratrol is a potential obesity treatment; it inhibits adipogenesis by decreasing fat mass. Resveratrol also affects the gene expression that modulates mitochondrial function by inducing adipocyte apoptosis.

2.13 Summary and research gaps

2.13.1 Summary

The Indian oil sardine is a crucial marine resource for India due to its nutritional and industrial value. It is a rich source of essential fatty acids (e.g. n-3 PUFA), making it a popular and affordable option for those seeking a healthy diet. However, the high number of double bonds in these fatty acids makes them prone to oxidation, which can lead to the production of hazardous compounds and the destruction of crucial fatty acids. To combat this, antioxidants can be used to enhance the oxidative stability of oils. Natural antioxidants are widely distributed in plant materials, animal tissues, and microorganisms, while synthetic antioxidants are chemically synthesized and toxicologically examined for safe use in the human diet. A combination of antioxidants can retard oxidation for a long duration and improve the product's shelf life. By utilizing antioxidants, the nutritional quality of edible oil can be maintained for customer use, making it a healthier and safer option for all.

2.13.2 Research gaps

There is a significant gap in research regarding the collective influence of natural antioxidants in sardine oil, especially from a statistical screening and optimization approach based on factorial and response surface methodology with kinetic and thermodynamic perspectives.

There is a clear need for more research on the combined effects of synthetic antioxidants in sardine oil. Specifically, a statistical screening and optimization approach based on factorial and response surface methodology with both kinetic and thermodynamic

perspectives would greatly benefit our understanding.It is worth noting that synthetic antioxidants, which are frequently present in food and pharmaceuticals, may raise concerns regarding their safety for consumption. Specifically, these antioxidants can potentially have a carcinogenic impact, even at limited concentrations. Nevertheless, the use of a combination of synthetic antioxidants can help to mitigate these concerns by minimizing the concentration used while simultaneously improving oxidation stability.

Combining different classes of natural antioxidants is a highly effective method of enhancing antioxidant activity against oxidation. The various mechanisms of each class of antioxidants work together to combat oxidation, providing a more holistic approach to defending against harmful oxidative effects. This approach is safe and has no adverse side effects, making it an ideal way for individuals to safeguard their health using natural antioxidants.

CHAPTER – 3

COMPARISON OF NATURAL AND SYNTHETIC ANTIOXIDANTS FOR IMPROVING OXIDATIVE STABILITY OF REFINED SARDINE OIL

CHAPTER 3

In this chapter, the Indian sardine oil was refined to enhance its quality and purity, as it contains a significant amount of n-3 PUFA. The crude and refined oil were then characterized to determine the impurities percentage after refining. Chemical tests were conducted to analyse peroxide, *p*-anisidine, conjugated diene, acid, phospholipid, and iodine values. Trace metals, including Zn, Cu, and Fe, were analysed by ICP-OES after acid digestion of the oil sample. Gas chromatography was also used to analyse n-3 PUFA (EPA and DHA) after esterifying the oil sample and converting it into FAME (fatty acid methyl ester). The stability of sardine oil was ensured by adding antioxidants individually in a calculated manner. Various types of antioxidants can be classified into distinct categories, such as natural hydroxybenzoic, hydroxycinnamic, flavanols, flavonols, stilbenes, carotenoids, and synthetic phenolic antioxidants that are used commercially in the food and pharmaceuticals industry. These selected antioxidants have different mechanisms to suppress the various route of oxidation. These selected antioxidants have free radical scavenging, metal chelating, and singlet oxygen quenching activity to retard the sardine oil oxidation for maintaining oxidation stability.

Choosing suitable antioxidants is a crucial and cost-effective approach to prevent oil oxidation. Antioxidants work to delay, prevent, or retard oxidation through different mechanisms, such as free radical scavenging, metal chelation, and inhibiting pro-oxidative enzymes (Choe and Min, 2009). Each class of antioxidants has different mechanisms to slow down the oxidative degradation of oil (Shahidi and Zhong, 2010). Extensive research has been done on the effectiveness of various antioxidant molecules such as tocopherols, carotenoids, flavonoids, phenolic acids, polyphenols, and lignan compounds of sesame oil, as well as synthetic antioxidants like BHA, BHT, TBHQ, and propyl gallate and their combinations in enhancing the oxidative stability of edible oil (Mishra et al., 2021). Studies have shown that certain antioxidants like caffeic acid and quercetin, and quercetin-3-O-glucoside have better efficacy than synthetic antioxidants like BHA and BHT in preventing oxidation and maintaining the quality of bulk fish oil (Leonardis and Macciola, 2003;

Huber et al., 2009). Sesamol, a natural antioxidant, has demonstrated better or comparable antioxidant activity to a commercial rosemary extract in fish oil during storage, and curcumin has also shown promising efficacy in bulk fish oil during storage for 70 days (Huang et al., 2017).

Many studies have summarized the efficacy of natural and synthetic antioxidants in stabilizing fish oils. Still, few have examined the simultaneous efficacy of carotenoids, phenolic acids, polyphenols, flavonoids, and prominent synthetic antioxidants in a single bulk oil system over a long storage period.

It is essential to evaluate the effectiveness of different antioxidants belonging to various categories to identify the best possible antioxidant for a specific system. The efficacy of antioxidants in lipids is influenced by several factors, including lipid composition, antioxidant hydrophilic-lipophilic balance, and interfacial interactions (Chang et al., 2003).

Therefore, a comprehensive study has been conducted to determine the most effective antioxidant for commercial fish oil containing n-3 PUFA. The study evaluated ten natural antioxidants belonging to four different classes (carotenoids, phenolic acid, polyphenols, and flavonoids) and three synthetic antioxidants (BHA, BHT, and TBHQ). Each antioxidant's efficacy in stabilizing the bulk fish oil during storage was studied and compared. This study is a step towards identifying the best possible antioxidant for a specific system and understanding how to maintain the quality of bulk fish oil during storage.

3.1 Material and Methods

3.1.1 Raw materials

Crude fish oil without synthetic antioxidants was bought from Raj Fishmeal and Oil company (Karnataka, India). The crude oil was refined in the lab as per the method developed earlier (Sampath et al., 2017). The composition and other chemical properties were determined and stored at -20ºC. Selected antioxidant belong to the different class and chemicals are; (+)-catechin hydrate (≥98%), resveratrol (≥ 99%), β-carotene (≥93%),

caffeic acid (≥98%), sinapic acid (≥98%), *trans*-ferulic acid (99%), ellagic acid (≥95%), vanillic acid (≥97%), quercetin (≥95%), rutin hydrate (≥94%), tert- butyl hydroquinone (TBHQ) (97%), butylated hydroxyanisole (BHA) (≥98%), *p*-anisidine (≥99%), eicosapentaenoic acid methyl ester, docosapentaenoic acid methyl ester, L-α-lecithin, soybean ,boron-trifluoride solution were purchased from Sigma-Aldrich, India. The butylated hydroxytoluene (BHT) was purchased from SRL, India. potassium iodide, starch was purchased from Merck, India. 2,2,4-trimethylpentane (99%), acetic acid glacial (99.5%), sodium thiosulphate anhydrous (97%), hexane, hydrochloric acid, nitric acid, *ortho*-phosphoric acid (85%), methanol, ethanol, wiji's solution, potassium hydroxide, granulated activated charcoal, chloroform, phenolphthalein indicator solution, diethyl ether was purchased from Loba Chemicals, India.

3.1.2 Refining of sardine oil

There are three steps (degumming, deacidification, and bleaching) of refining to remove the impurities of sardine oil and increase the oxidation stability.

3.1.2.1 Degumming

To refine sardine oil, the first step is degumming. This crucial stage involves removing phospholipids from the oil, which is done by adding a degumming agent, *ortho*-phosphoric acid 5% (w/w) to crude sardine oil. After stirring the mixture for 30 minutes, it is centrifuged at 6000 x g for 20 minutes. The resulting supernatant is clear and less viscous, making it perfect for further analysis and studies of its phospholipid content.

3.1.2.2 Deacidification

Free fatty acids were effectively removed from degummed sardine oil using liquid-liquid extraction using a 1:1 (w/w) ratio of methanol. The degummed oil was placed in a beaker and mixed with methanol for 60 minutes on a magnetic stirrer. The resulting mixture was then transferred to a separating funnel and allowed to separate overnight. The separated oil was collected with care, and the methanol layer was discarded.

3.1.2.3 Bleaching

To remove trace metals, the oil underwent a bleaching process by granulated activated charcoal 3% (w/w) were measured in a conical flask. The flask was sealed and placed on a magnetic stirrer with a heating mantle. Then, the oil was bleached at 80°C for 10 minutes under a vacuum of approximately 40-70 mmHg. After this, the activated carbon was removed from the oil by centrifugation at 10000 rpm for 15 minutes. The clear oil supernatant was collected and analysed for its physicochemical properties before being used for further studies.

3.1.3 Selected antioxidants characteristics

Various antioxidants, including hydroxybenzoic (vanillic acid, ellagic acid), hydroxycinnamic acids (caffeic acid, sinapic acid, ferulic acid), flavonols (quercetin), flavone (rutin), flavanol (catechin), stilbene (*trans*-resveratrol), carotenoids (β-carotene), and synthetic phenolic antioxidants (*tert*-butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), and 2,6-di-tert-butyl-4-methylphenol (BHT).) can maintain or inhibit oxidation in sardine oil. They work by neutralizing free radicals, chelating metal cations, or providing efficacy against reactive oxygen species.

3.1.4 Oxidative stability experiments

A specific amount of various antioxidants was dissolved in a small quantity of ethanol and placed in 15 ml amber glass vials. The solvent was then evaporated using nitrogen gas. Refined fish oil was added to each vial, resulting in a final concentration of 0.9mM of antioxidants, which was determined based on the reports of Chandrasekar et al. (2016); Maqsood and Benjakul, (2010); and Hopia et al. (1996) to prevent any potential pro-oxidant effect. The samples were homogenized thoroughly for 15 minutes, stored in an incubator at 25ºC, and exposed to atmospheric air for 50 days. Each oxidation experiment was conducted in triplicate, and the samples were withdrawn every fifth day to determine the peroxide, *p*-anisidine, conjugated diene and TOTOX values.

3.1.5 Characterization of the oil

3.1.5.1 Peroxide value

Peroxide value was analysed using AOCS Cd 8b-90, AOCS (2009) standard method. Peroxide value measures the primary oxidation product, e.g., hydroperoxide concentration based on milliequivalent peroxide per 1000 grams of oil.

$$LOOH + KI \rightarrow LOH + KOH + I_2$$

Hydroperoxide (LOOH) concentration was analysed by measuring the concentration of iodine formed, which was calculated through titration with sodium thiosulphate and using a starch indicator.

$$I_2 + (C_6H_{10}O_5)_n + 2Na_2S_2O_3\ (Voilet) \rightarrow (C_6H_{10}O_5)_n + Na_2S_4O_6(colorless)$$

Value calculated as follows;

$$Peroxide\ value\ (meq\ of\ peroxide/1000of\ oil)\ = ((S - B) \times M \times 1000)/m$$

S- Sample titer value, B- Blank titer value, M- molarity of thiosulphate solution, m- the mass of sardine oil in grams.

3.1.5.2 *p*-Anisidine value

p-Anisidine value was analysed according to AOCS Cd 18-90, AOCS (2009) standard method. *p*-anisidine value analyzes secondary oxidation compounds (e.g., 2-alkenals and 2,4 akladienals) from oil, which are created due to the hydroperoxide decomposition. The reaction was carried out with 1 gram of lipid in 2,2,4-Trimethylpentane (100 ml) with *p*-anisidine (0.25%) in acetic acid glacial. Furthermore, the generated product by this reaction with unsaturated 2-alkenals was analysed at 350nm wavelength. Value calculated as follows;

$$p - Anisidine\ value = 25 \times (1.2\ As - Ab)/m$$

As – Absorbance of oil solution after reaction with *p*-anisidine, Ab – Absorbance of oil solution, m – the mass of sardine oil in grams.

3.1.5.3 Conjugated diene value

The conjugated fatty acid contains a positional and geometric isomer of fatty acid with a conjugated double bond. The conjugated dienes are formed by electrophilic singlet oxygen, which directly reacts with high electron density double bonds and form hydroperoxide. The hydroperoxide and *trans* fatty acid formed were measured at a specific UV absorbance of 234nm (Hopia et al., 1996) by diluting the weighed oil sample with 2,2,4-Trimethylpentane. The increased absorbance corresponded to an increase in oxidation.

3.1.5.4 TOTOX value

The TOTOX value is the complete oxidation status of lipid, which increased linearly, an equal sum of *p*-anisidine plus twice peroxide value (Albert et al., 2015).

$$TOTOX\ value = (2 * Peroxide\ value) + p - Anisidine\ value$$

3.1.5.5 Acid value

The presence of free fatty acids in oil is more susceptible to autoxidation than esterified fatty acids. The free fatty acid act as pro-oxidants in edible oil. The structure of free fatty acid has a hydrophilic and hydrophobic group. The carbonyl group is the hydrophilic group, and the hydrocarbon chain is the hydrophobic group. The FFA carbonyl group is preferably concentrated on the surface of edible oil, decreasing the surface tension and increasing the oxygen diffusion rate from the headspace into the oil, accelerating oil oxidation. The acid value was analysed using AOCS Cd 3b-63, AOCS (2009) standard method. The acid value was calculated as potassium hydroxide required to neutralize the free fatty acid in one gram of sardine fish oil.

$$Acid\ value\ (mg\ KOH/g) = \frac{(S - B) \times\ N \times 56.1}{W}$$

S – standard alkali used in titration (ml), B – standard alkali used for titration of the blank (ml), N – normality of standard alkali, W – the weight of oil

3.1.5.6 Iodine value

The iodine value (IV) determines the relative degree of unsaturation in oil components, as determined by the uptake of halogen. The IV is proportional to the number of mono- and polyunsaturated bonds within the constituent fatty acids. The degree of unsaturation related to the melting point, oxidation stability, and the iodine value estimates these quality factors. The higher iodine value in oil indicates highly unsaturated fatty acids susceptible to fast degradation reactions such as autoxidation or polymerization. Iodine value was analysed according to AOCS Cd 1c-85, AOCS (2009) standard method. The iodine value was used to observe the degree of unsaturation of oil. It was measured as the amount of iodine absorbed by 100g of oil.

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

S- sample titrate value, B- Blank titrate value, N – (0.1N) normality of sodium thiosulphate solution, m – the mass of sample (g)

3.1.5.7 Density estimation

The substance's mass per volume at fixed temperature and pressure is defined as density. As the temperature increases, the fluid volume expands, so the density decreases. The specific gravity of a liquid is equal to its density. The density of sardine fish oil was measured by Density Meter (Rudolph Research Analytical) in g/cm^3 with specific gravity.

3.1.5.8 Viscosity estimation

Viscosity measures a fluid's resistance to deformation under shear stress. Dynamic viscosity is the resistance of fluid movement from one layer over another. The instrument measures the dynamic viscosity via the concentric tubes whose annular space can be filled with the fluid. The tube is rotated, and the force it takes to rotate at a fixed angular velocity is proportional to the dynamic viscosity. The kinematic viscosity describes as fluid resistance. It is a physical property related to the chain length and degree of unsaturation associated with oxidation stability. In kinematic viscosity, its density at the same

temperature divides the absolute viscosity of a liquid. Rolling Ball Microviscometer (Anton Paar) measured the viscosity of sardine fish oil. The measured data was presented in dynamic viscosity (m Pa.s) and kinematic viscosity (mm^2/s).

3.1.5.9 Moisture content estimation

Moisture content effect on the stability and quality of fats and oils. It deteriorates the flavor, texture of food. It increases the oxidation reaction and affects the shelf life. The trace water content of sardine fish was quantified by gravimetric method. The oil was weighed (g) in the ceramic crucible (w_1) and kept in the oven at 100ºC for 30 minutes. After 30 minutes, the oil was weighed (w_2), and the moisture content was estimated as follows;

$$\%\ Moisture = \frac{W_1 - W_2}{W_1} \times 100$$

3.1.5.10 Trace metal analysis

For trace metal analysis, the oil was weighed (g) and digested by acid (e.g., hydrochloric acid, nitric acid), and the necessary metal content (Fe, Zn, and Cu) that initiates oxidation was measured by ICP-OES (Agilent technologies). In ICP-OES, the acid solutions sample is introduced and then forms fine aerosol through nebulized that is transported into the plasma where it undergoes desiccation, vaporization to molecular gases, and dissociation into atoms that can be ionized. The atoms and ions become excited in the plasma, reverting to their ground state with light emission, measured using an optical spectrometer. All elements present in the radiation source emit their characteristic spectra at the same time. Thus, from the principles of OES, it is clear that it is a multielement method that can be operated in simultaneous or sequential mode.

3.1.5.11 Phospholipid estimation

The oil contains a gum-like substance produced during traditional mechanical pressing and solvent extraction. Gum-like substances are phospholipids, which are polar lipids that absorb trace amounts of water content. Phospholipids can decompose, cause dark colour during heating, and produce foaming during frying. Standard phospholipid content in oil

should be less than 5mg/kg. The method reported by Hundreiser et al. (1985) was followed with minor modifications.

Preparation of chromogenic solution –

Solution I: 8g ammonium molybdate A.R. dissolved in 60 ml distilled water.

Solution II: Filtered mixture of 40 mL solution I, 5 mL concentrated HCl, and 5 mL mercury was prepared and mixed for 30 minutes to achieve solution II. The 100ml sulphuric acid diluted to the leftover solution I.

Solution III: It is a collective mixture of solution I diluted with sulphuric acid and solution II.

The chromogenic solution was prepared with 12.5 mL of solution III with 22.5 mL of methanol and 2.5 mL chl oroform, and 10 mL of distilled water. This solution was then stored at 4°C for further analysis.

The phospholipid content in oil was analysed as follows;

- The oil samples were weighed and taken in glass vials. The oil sample was dissolved in chloroform, and the solvent was evaporated by heating in the water bath.
- Then 0.4 mL chloroform and 0.1 mL of chromogenic reagent were added to the residue and mixed gently.
- Then the mixture was vortexed and placed in a boiling water bath for 1.25 minutes.
- Then samples were cooled at room temperature, and 4 mL of chloroform was added.
- Then the mixture was vortexed, and the absorbance of the chloroform portion was read at 730 nm using a UV-Vis spectrophotometer against a blank that does not contain an oil sample.

The standard graph of soy lecithin was plotted to optimize the concentration of the sample phospholipid.

3.1.5.12 Fatty acid composition estimation

The fatty acid composition of sardine fish oil was estimated through aatty acid methyl esters (FAME) using gas chromatography (Thermo Fisher Scientific) with flame ionization detector (FID) and DB-5 column from Agilent Technologies. Nitrogen as a carrier gas, hydrogen, and zero air as a fuel gas was used to detect fatty acid composition. The parameter for analysis was as follows: Injector temperature - 280 °C; Detector temperature - 300 °C. Column over gradient: initial time 0-1 minute; 160°C of oven temperature; the temperature was ramped at 5°C/min to 185 °C. The temperature was maintained at 185 °C, for 10 minutes. The temperature was further ramped at 8 °C/min to 240 °C. The temperature was further maintained at 240 °C for another 10 minutes.

The preparation of FAME was according to the conventional method explained by Ichihara and Fukubayashi (2010) with slight modifications. Trans-esterification process involves the conversion of fatty acids to their respective methyl ester. Oil (100mg) was weighed in screw-capped glass vial and dissolved in 200µl of diethyl ether. Later, 2ml of 0.5M of methanolic NaOH solution was added and mixed well. Then the mixture was hydrolysed for 1 hour at 70ºC under a vacuum oven. Then 0.6 ml of 2M HCl was added to the hydrolysed mixture. The mixture was mixed thoroughly to extract the FFA resulting from the hydrolysis reaction, then 1ml hexane was added to the mixture. The hexane phase was evaporated by heating in the water bath. Then 2.175ml of (10%) boron trifluoride in methanol for methylated FFA was added. The mixture was heated for 20 minutes at 37ºC and then cooled at room temperature. The methylated fatty acids were extracted by solvent extraction with hexane and water (1:1 v/v) and vortexed. The upper phase of the hexane fraction was collected for analysis. Appendix II and III feature the standard graph of EPA methyl ester and DHA methyl ester, respectively. The regression value of 0.99 indicates a high level of accuracy. The graph exhibits the concentration range of 0.4 - 1 mg/ml and 0.6 - 2 mg/ml for EPA methyl ester and DHA methyl ester, respectively. Both compounds have a

retention time of 21 and 24 minutes. This graph serves as a tool to determine the concentration of EPA and DHA present in sardine fish oil.

3.1.5.13 Statistical analysis

The data were compared and examined by using Origin 19b software. The significance of the data ($p < 0.05$) was analysed using one and two-way analysis of variance (ANOVA).

3.2 Results and discussion

Locally sourced sardine fish oil underwent a refined process that utilized an optimized method, removing impurities while retaining essential components. Then various parameters are used to analyse and compare the oil quality before and after refining and evaluate the efficacy of various natural and synthetic antioxidants in stabilizing the fish oil. The selected antioxidants possess multiple mechanisms to suppress oil oxidation, ensuring the highest quality possible.

3.2.1 Characterization of crude sardine oil

The commercial crude sardine fish oil obtained from a native fishmeal industry was taken for the study. It was subjected to the optimized refining process reported elsewhere (Sampath et al., 2017). Table 3.1 mentions peroxide value, *p*-anisidine value, phospholipids, metal ions, and free fatty acids in refined and crude oil.

The sardine oil had essential n-3 PUFA in abundant amounts, and during the refining process, some quantity of PUFA was lost. The concentration of EPA and DHA in n-3 PUFA-rich sardine oil was detected using gas chromatography. The oil sample was trans-esterified and converted into fatty acid methyl ester (FAME). However, approximately 3.73 g of n-3 PUFA (EPA+DHA) per kg of oil will be lost during the refining process.

The degree of unsaturation in oil components was determined by the uptake of halogen observed through the iodine value. The iodine value 127, while that of the refined oil was 130. This indicates the presence of a large number of unsaturated fatty acids other than n-

3 PUFA. The higher iodine value in the oil indicated the presence of highly unsaturated fatty acids that are susceptible to fast degradation reactions such as autoxidation or polymerization.

Phospholipids are polar lipids that absorb trace amounts of water content. The phospholipid decomposition causes dark colour during heating and foaming during frying. After refining, there was a significant reduction in the phospholipid content (1.35 µg/kg), which is determined using the standard plot of phospholipid (soy lecithin). The crude oil contained 15.31µg/kg phospholipid content.

The oxidation stability of the oil was also decreased due to the excessive amount of metal ions. The sample was digested with nitric acid and hydrochloric acid with a 3:1 ratio and was through ICP-OES. The concentration of ferric ions is higher in crude oil (15.31 ppm) as compared to refined oil (1.19 ppm). Other metal ions (Cu, Zn, and Fe) also decreased after refining. FFA are more susceptible to autoxidation; they act as pro-oxidants.

The crude oil had a higher FFA content (4.15 mgKOH/g); after refining, it decreased to 1.68 mgKOH/g. The moisture content deteriorates the flavour and texture of food. It increases the oxidation reaction and affects the shelf life. The gravimetric method examined the trace amount of water in refined oil. Refined oil contains high amounts of moisture (414.06 mg/kg) compared to crude oil (328.36mg/kg).

The peroxide value indicates the primary oxidation product (e.g., hydroperoxide) that oxidized the sardine oil. Crude oil had a higher peroxide value (41.5 meq/kg) because of high impurities. After refining, the oil contained less undesirable content that suppresses the oxidation process and decreases the peroxide value (10 meq/kg) in refined oil.

The anisidine value tested secondary oxidation products (e.g., aldehyde, ketone) that showed rancidity in flavour and texture. The *p*-anisidine value in crude was 1.31, and the refined oil is 0.8075.

The conjugated value examined conjugated dienes formed by electrophilic singlet oxygen, which directly react with high electron density double bonds and form hydroperoxide, and the minor changes (1.05 to 1.025) after refining was observed in sardine oil.

The TOTOX value, which indicates the overall oxidation state of the oil, was also reduced to a great extent. The TOTOX value of crude (84.32) and refined oil (20.80) decreased substantially, indicating a substantial reduction in the primary and secondary oxidation products in the oil.

The viscosity measures a fluid's resistance to deformation under shear stress. Dynamic viscosity is the resistance of fluid movement from one layer over another. Kinematic viscosity is the physical property related to the chain length and degree of unsaturation, which is associated with oxidation stability. Refined oil's dynamic and kinematic viscosity at ambient conditions was 41.25mPa.s and 44.74mm^2/s., respectively. At an ambient temperature, the density of refined fish oil exhibits slight variations and is measured at 0.92 g/cm^3.

Nevertheless, the TOTOX value indicates that some primary and secondary oxidation products are still left in the oil. Along with that, a small amount of moisture, metal ions, and phospholipids are also present in the oil. The density and viscosity indicated minor changes after refining. The residual ferric ions, moisture, phospholipids, and free fatty acids in the oil tend to increase oxidative instability. Furthermore, the n-3 PUFA present in the oil also contributes to the oxidation of the oil during storage (Sampath et al., 2019).

Table 3.1 Parameter of crude and refined sardine Oil

Parameter	**Crude Indian sardine oil**	**Refined Indian sardine oil**
EPA (g/kg)	0.61	0.47
DHA (g/kg)	4.82	1.23
Peroxide value (meq/kg)	41.5	10.00
Conjugated diene	1.05	1.02
p-Anisidine value	1.31	0.80
TOTOX value	84.31	20.80
Viscosity 25ºC (mPa.s)/(mm^2/s)	40.27/43.68	41.25/44.74
Moisture (mg/kg)	328.36	414.06
Phospholipid value (µg/kg)	15.18	1.35
Density 25ºC (g/cm^3)	0.92	0.92
Iodine value	127.76	130.81
FFA (mgKOH/g)	4.15	1.68
Cu (327.395 nm) (ppm)	0.13	0.03
Zn (213.857 nm) (ppm)	1.63	1.31
Fe (238.204 nm) (ppm)	15.31	1.19
Ni (231.604 nm) (ppm)	0.77	0.19

3.2.2 Antioxidants analysis in sardine oil

A study was conducted on 13 different class antioxidants to suppress oil oxidation. This was done under dark conditions with atmospheric air at 25°C for 50 days. To ensure safety, the FDA and European Food Safety Authority have set an acceptable limit of 0.9mM for the addition of synthetic antioxidants. Thus, all antioxidants were tested at this concentration for their oxidation stability. Primary oxidation products were analysed

through the peroxide test, while secondary oxidation products were analysed through the *p*-anisidine test. The overall oxidation was analysed through the TOTOX value.

3.2.2.1 Peroxide value

In assessing the initial oxidation of sardine oil, the peroxide value was measured alongside various antioxidants. When hydroperoxides break down, they can produce secondary products that are often colourless and odourless. To gauge their effectiveness, the peroxide value of sardine oil was compared to a "Control" sample in the presence of thirteen different antioxidants, as depicted in Figure 3.1.

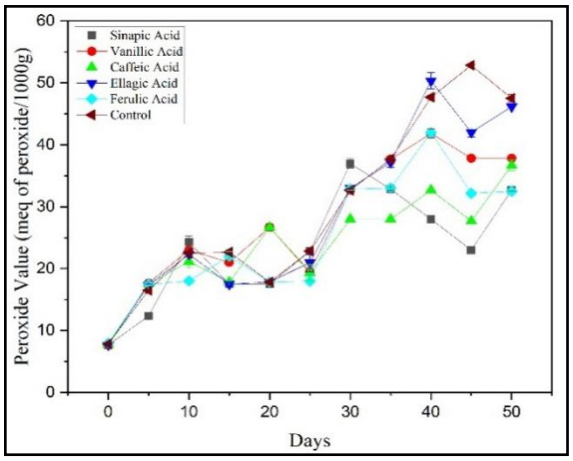
During the first 25 days, it was observed that catechin had the lowest peroxide value of 17.6 meq/kg among natural antioxidants. ferulic acid, caffeic acid, and vanillic acid also effectively fought against oxidation, with peroxide values of 18 meq/kg, 19.3 meq/kg, and 19.5 meq/kg, respectively. However, β-carotene had the highest peroxide value of 33 meq/kg and displayed a pro-oxidant effect, resulting in a higher peroxide value than the "Control" sample. On the other hand, sinapic acid, resveratrol, rutin, and quercetin did not perform significantly against oxidation, with peroxide values averaging around 23 meq/kg, similar to the "Control" sample.

After 50 days of storage, catechin (flavan-3-ol) showed significant performance against oxidation from 25-40 days. The percentage (%) decrease in the oxidation of sardine oil at 40^{th} days of catechin was 50.67%. The hydroxycinnamic acid antioxidants like, caffeic and Sinapic acid retarded oxidation from 25-45 days. Sinapic acidshowed a decrease in oxidation by after 40 days by 40.42%, and caffeic acid was 31.45%. Ferulic acid showed a decrease in oxidation by 11.90%. Resveratrol (stilbene) presented antioxidant activity in sardine oil from 25-40 days, and the decreased percentage oxidation by 20.61% at 40^{th} days. The levels of flavonoid antioxidants, quercetin and rutin, in sardine oil, do not significantly impact in the lipid oxidation. Quercetin showed a decrease in oxidation of 18.81%, and rutin showed a decrease of 14.02% on the 40^{th} day. However, regarding hydroxybenzoic acid antioxidants, vanillic and ellagic acid, their effectiveness in sardine oil was insufficient after 25 days, as oxidation increased. Vanillic acid decreased oxidation

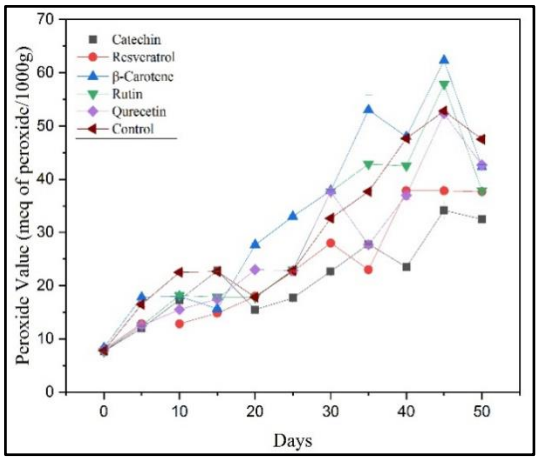
by 11.90%, whereas ellagic acid was ineffective due to high oxidation values compared to the "Control" on the 40th day. Additionally, β-carotene (carotenoids) can act as a pro-oxidant for up to 20 days, with the peroxide value continuously increasing and the oxidation.

Regarding synthetic phenolic antioxidants, TBHQ stood out as the most effective option and surpassed other synthetic and natural antioxidants to retard the oxidation of sardine oil. Sardine oil treated with TBHQ maintained the lowest peroxide value of 12.6 meq/kg until the 25th day. In comparison, oil samples treated with BHT and BHA showed peroxide values of 17.8 meq/kg and 18 meq/kg. These results demonstrated the unparalleled effectiveness of TBHQ in preserving the quality of oils and other essential products. Following a storage period of 50 days, it was observed from the peroxide values that synthetic phenolic antioxidants (TBHQ, BHT, and BHA) could inhibit sardine oil oxidation. TBHQ (62.58%) was the most effective on the 40th day. The BHA and BHT performed significantly, and the decreased oxidation by 52.09% and 51.74%, respectively in sardine fish oil on the 40th day.

Based on the findings of this peroxide analysis, it was evident that catechin displayed a decrease in oxidation percentage, which was quite similar to BHA and BHT. These results were observed over a period of 40 days, indicating that catechin may be effective against oxidation and could be a viable option to explore further.



(a)



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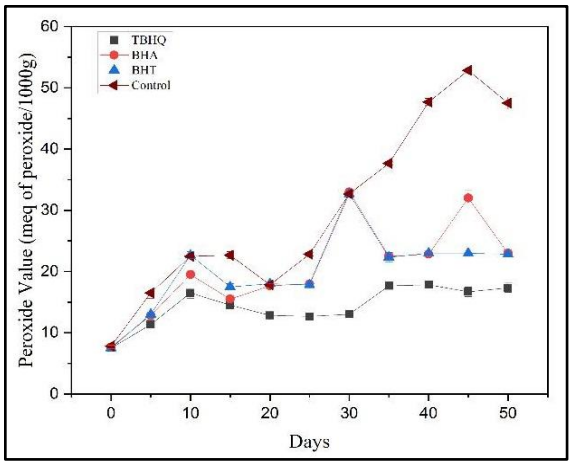


(c)

Figure 3.1 The peroxide value of bulk oil samples added with (a) various phenolic acids, (b) polyphenols, flavonoids, β-carotene, and (c) synthetic antioxidants compared to the control. Bars represent standard deviation (n = 3).

3.2.2.2 *p*-Anisidine value

For an accurate assessment of oxidation progress in sardine oil, it is crucial to simultaneously measure both primary and secondary oxidation products. The *p*-anisidine value (*p*-AV) technique evaluates secondary oil oxidation. The *p*-AV determines the amount of secondary oxidation products, such as aldehydes, ketones, alcohols, acids, and hydrocarbons, formed during the breakdown of hydroperoxides. This method is based on the reaction between the aldehyde carbonyl bond and the *p*-anisidine amine group, which creates a Schiff's base that absorbs at 350nm wavelength. Figure 3.2 shows the anisidine value of sardine oil in the presence of antioxidants at 0.9 mM.

In the initial 25 days, sardine oil benefits from catechin's natural antioxidant properties, which effectively inhibited the formation of secondary oxidation products. This resulted in a remarkably low *p*-anisidine value of 23.28. The *p*-anisidine values for caffeic acid (48.93), resveratrol (50.06), quercetin (51.95), and sinapic acid (54.6) indicate their ability to inhibit the formation of secondary oxidation products. The rutin, vanillic acid (74.64),

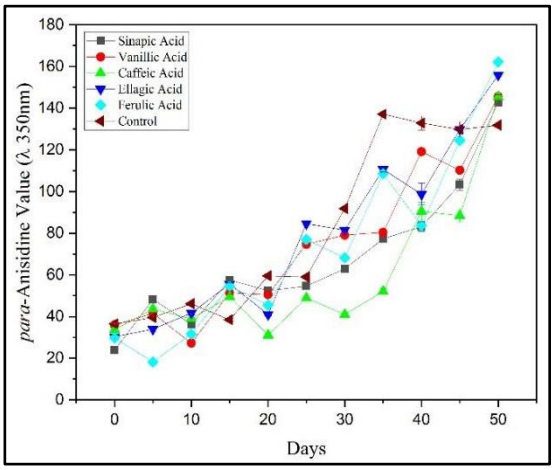
ferulic acid (76.88), β-carotene (77.37), and ellagic acid (84.5) *p*-anisidine values indicated the oxidation process on the 25th day, and the *p*-anisidine value was higher than "Control."

After 50 days of storage, catechin (flavan-3-ol) showed significant performance against oxidation from 25-40 days. The 40th day decreased percentage (%) of secondary oxidation of *p*-anisidine value was 50.08%; it performs more effectively than other natural antioxidants. The hydroxycinnamic antioxidants, like caffeic and sinapic acid, retarded the oxidation for 25-45 days. The decreased percentage of oxidation at 40th days by sinapic acid was 37.39%, and caffeic acid was 31.66%. The ferulic acid oxidation stability is ineffective, and the *p*-anisidine value continuously increases. On the 40th day, 36.90% oxidation decreased, but the oxidation value was again too high after a few days. The findings indicate that vanillic and ellagic acid, two hydroxycinnamic acid antioxidants, can effectively suppress oxidation for up to 15-25 days. However, after that time period, the oxidation tends to increase. In the case of *p*-anisidine analysis, vanillic acid was observed to decrease oxidation by 10.16%, while Ellagic acid exhibited a decrease of 25.78% on the 40th day. Similarly, resveratrol (stilbene) demonstrated oxidation stability in sardine oil until the 30th day, following which oxidation increased. The percentage of oxidation decrease was 26.88% on the 40th day. Furthermore, flavonoid antioxidants quercetin and rutin were found to inhibit oxidation until the 30th day, after which the oxidation value increased. The percentage of oxidation decrease for qurecetin was 33.49% on the 40th day. However, the rutin *p*-anisidine value was higher than the "Control," and oxidation increased. Finally, β-carotene (carotenoids) acted as a pro-oxidant for 15 days, and the *p*-anisidine value increased. The oxidation decreased percentage was "Nil" because the oxidation value was higher than the "Control."

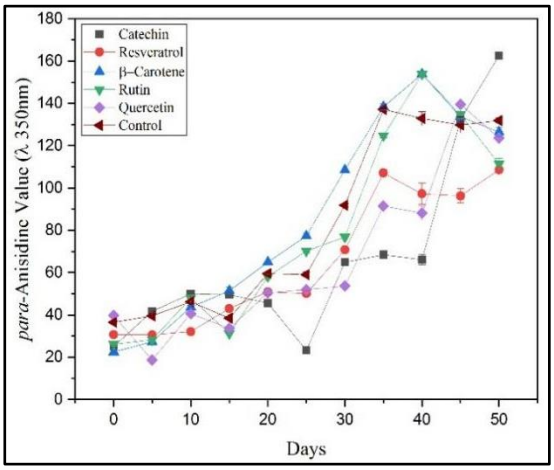
In the realm of synthetic phenolic antioxidants, TBHQ, BHA, and BHT have proven to be effective in combating secondary oxidation and reducing the *p*-anisidine value. TBHQ was particularly potent until the 50th day and could reduce oxidation by 85.04% on the 40th day. BHA and BHT also exhibited antioxidant properties against secondary oxidation and have

been found to be effective until the 45th day. On the 40th day, BHA decreased oxidation by 46.73%, while BHT decreased it by 53.22%.

When examining the effects of *p*-anisidine, it was found that catechin had a higher percentage of oxidation decrease (50.08%) compared to BHA (46.73%) but was very close to BHT (53.22%) in terms of preventing oxidation for up to 40 days.



(a)



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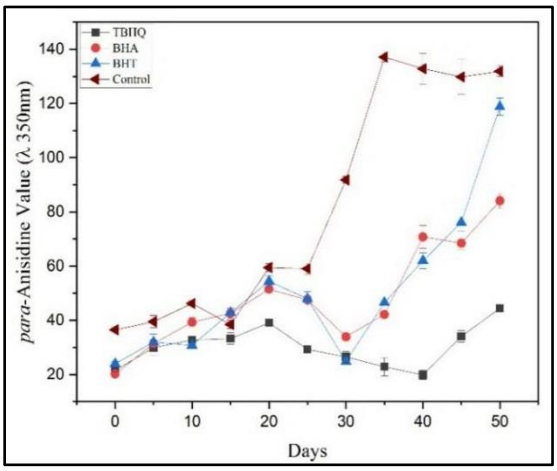


(c)

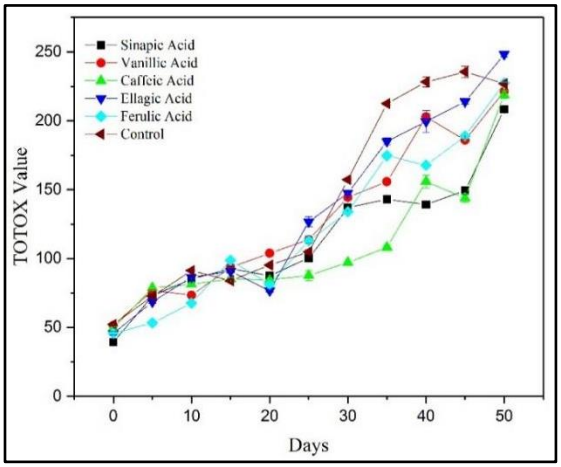
Figure 3.2 The *p-anisidine* value of bulk oil samples added with (a) various phenolic acids, (b) polyphenols, flavonoids, β-carotene, and (c) synthetic antioxidants compared to the control. Bars represent standard deviation (n = 3).

3.2.2.3 TOTOX value

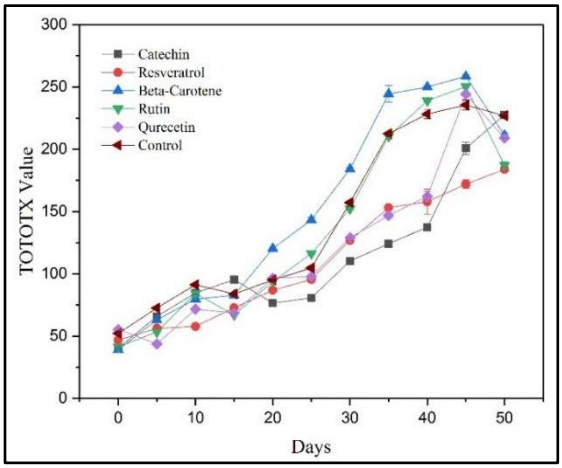
The TOTOX value represents the full oxidation status of lipids and increases linearly. It is calculated by adding the sum of *p*-anisidine and twice the peroxide value. Figure 3.3 displays the comprehensive oxidation effect in the sardine oil sample, including the individual antioxidants.

In the study of five phenolic acids, caffeic acid showed the highest oxidative stability compared to the others. The rate of primary and secondary oxidation was similar in all cases, except for ferulic acid in the first 15 days, where it provided better protection against oxidative instability. However, during the storage period of 15-45 days, caffeic acid performed better than all other phenolic acids. Resveratrol exhibited superior oxidative stability among two flavonoids (quercetin and rutin), two polyphenols catechin and resveratrol), and β-carotene tested in the study. Although catechin performed better during the storage period of 15-45 days, its overall performance was not as consistent and superior compared to resveratrol. Interestingly, β-carotene showed pro-oxidant activity during the 15-45 day storage period. Among the three synthetic antioxidants tested, TBHQ exhibited superior oxidative stability in the bulk oil system compared to BHA and BHT. BHA and BHT showed comparable and similar oxidative stability until the 45th day, but their performance was far inferior to TBHQ. The TOTOX value of the oil during 50 days of storage ranged from 44.72 to 114.03 in the case of TBHQ. In contrast, it steadily increased to 164.44 and 130.04 in the case of BHT and BHA, respectively. TBHQ showed the least change in TOTOX value (115.03%), while BHT had the highest (321.98%) among the synthetic antioxidants studied, indicating a 297.68% change in its TOTOX value by the end of the 50-day storage period. Among all the natural antioxidants, resveratrol was the only one that offered comparable oxidative stability to the oil, with a TOTOX value increasing to 184.36 by the end of the 50-day storage period. Caffeic acid also offered

comparable stability, with a TOTOX value increasing to 218.84 by the end of the 50-day storage period, signifying a 343% increase in its TOTOX value.



(a)



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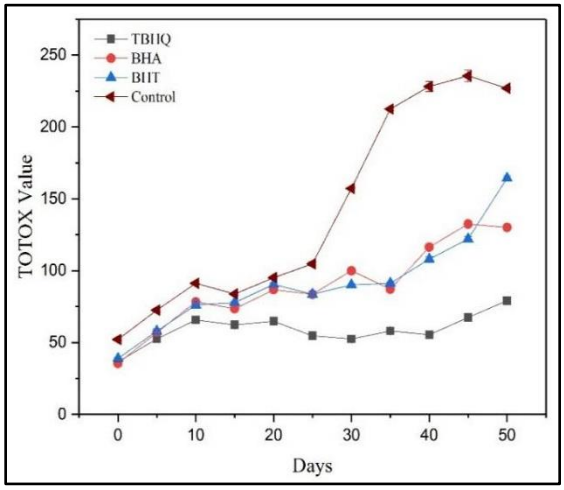


(c)

Figure 3.3 The TOTOX value of bulk oil samples added with (a) various phenolic acids, (b) polyphenols, flavonoids, β-carotene, and (c) synthetic antioxidants compared to the control. Bars represent standard deviation (n = 3).

Based on Figure 3.4, it can be determined that the various antioxidants have a significant difference in TOTOX value through the Two-way analysis of variance (ANOVA). The statistical analysis revealed a *p*-value of less than 0.05 in the two-way ANOVA.

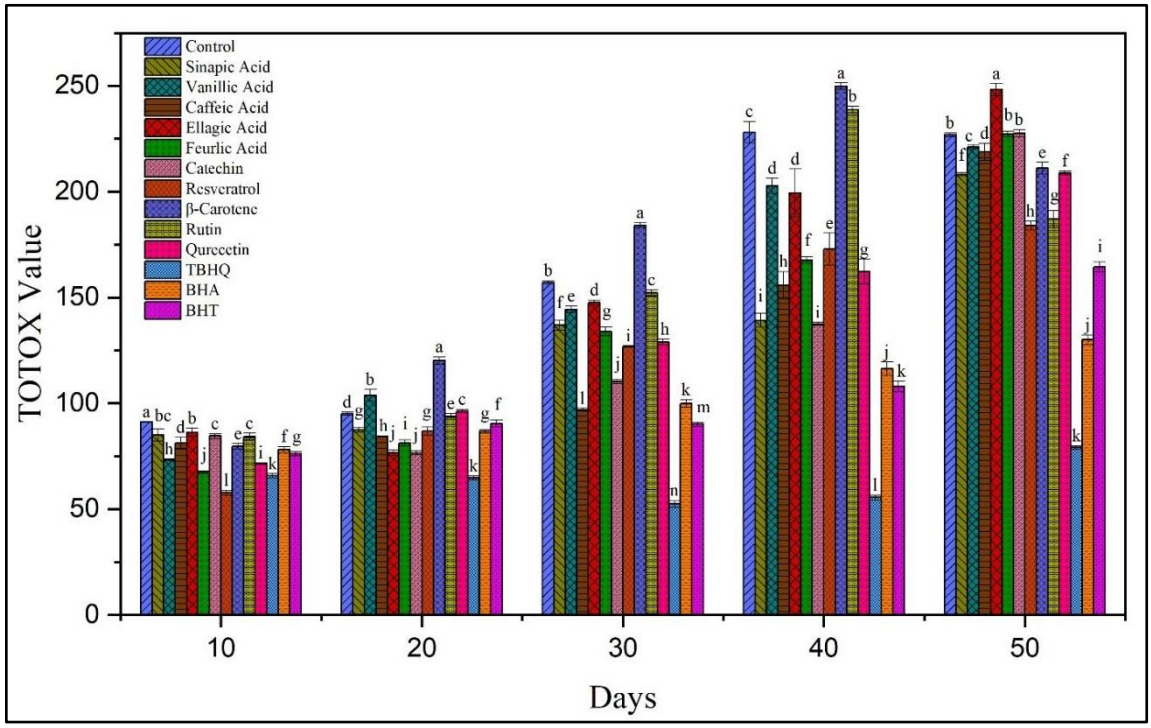


Figure 3.4 Comparison of the TOTOX values of the bulk oil samples having various natural and synthetic antioxidants during 50 days of storage. ^{a,b,c,d,e,f,g,i,j,k,l,m,n}. Means with different superscripts letters within each cluster are significantly different at $p < 0.05$

According to Table 3.2, the study evaluated the antioxidant activity of natural and synthetic antioxidants over an extended period. The results showed that the percentage of oxidation decreased until the 40th day, after which all the natural antioxidants' degraded against oxidation. Catechin was only effective against oxidation for up to 40 days. In contrast, TBHQ, one of the synthetic antioxidants, exhibited significant inhibition activity against oxidation after the 50th day. Selva et. al, 2024 mentioned the TBHQ reduction percentage of TOTOX value is 85.6%. Furthermore, BHA and BHT were also effective in suppressing oxidation until the 45th day.

Table 3.2 Effectiveness of each antioxidant on improving the oxidative stability of sardine oil at the end of the 40th day storage period.

Antioxidants	% Decrease in the oxidation of sardine oil		
	Peroxide value	***p*-Anisidine value**	**TOTOX value**
Hydrocinnamic acid			
Sinapic acid	40.42±0.00^{c}	37.39±0.75^{c,d}	39±0.26^{c}
Caffeic acid	31.45±0.83^{d}	31.66±4.80^{d}	31.62±2.99^{c,d}
Ferulic acid	11.90±1.00^{f}	36.90±1.42^{c,d}	26.47±0.65^{d}
Hydrobenzoic acid			
Vanillic acid	12.24±0.99^{f}	10.16±2.59^{e}	11.07±1.33^{e}
Ellagic acid	Nil	25.78±2.29^{d}	12.68±2.10^{e}
Flavan-3-ol			
Catechin	50.67±1.40^{b}	50.08±2.96^{b}	50.36±2.10^{b}
Stilbene			
Resveratrol	20.61±0.79^{e}	26.88±1.96^{d}	24.24±1.18^{d}
Flavonoids			
Rutin	14.02±2.63^{f}	Nil	Nil
Qurecetin	18.81±4.85^{e}	33.49±3.27^{d}	28.87±2.20^{d}
Carotenoids			
β-Carotene	Nil	Nil	Nil
Synthetic Phenolic			
TBHQ	62.58±0.53^{a}	85.04±0.78^{a}	75.66±0.33^{a}
BHA	52.09±0.59^{b}	46.73±1.04^{b,c}	48.96±0.76^{b}
BHT	51.74±0.33^{b}	53.22±2.21^{b}	52.62±1.27^{b}

Percentage decrease was calculated by considering the control to be 100% oxidized. $^{a, b, c, d, e, f}$ values with the same letter in each column were not significantly different

($p<0.05$). *Nil – Insignificant (Control value:- Peroxide – 47.66, Anisidine – 132.82, TOTOX – 228).

3.2.2.4 Discussion

The oxidation inhibition analysis of n-3 PUFA-rich sardine oil with various antioxidants independently through peroxide and *p*-anisidine value. The thirteen antioxidants were selected from different classes to retard the oxidation for 50 days studied at 25°C under darkness. As a natural antioxidant, catechin showed long-duration oxidation stability in sardine oil because catechin has an adjacent hydroxyl group at the B-ring to increase the antioxidant chelating ability. The hydroxyl attached to the 3′, 4′, 3, and 5 positions are the potential chelating sites for increasing antioxidant activity. Catechin also has free-radical scavenging activity because it also has a –OH group at the A-ring and C-ring. In the study, catechin was more effective than BHA and almost similar with BHT till the 40^{th} day. Nain et al. (2022) reported the superior performance of green tea catechins extract (GTE1) in DHA-rich oil with 250ppm stored at 30°C for 21 days. Wanasundara and Shahidi (1996) studied the efficacy of green tea catechins (200ppm) in seal blubber, and menhaden oil is more effective than TBHQ, BHA, and BHT. In hydrocinnamic acid, antioxidants, like caffeic and sinapic acid, are significant against oxidation from 15-35 days. Caffeic acid also has an adjacent hydroxyl group at the C-ring that expresses the metal chelating ability with free-radical scavenging activity to inhibit oxidation. Zhang et al. (2018) studied the various phenolic antioxidants in cold-pressed pecan oil under aerated conditions in the dark at 60°C for 20 days. Caffeic acid performed exceptionally against oxidation among all the natural antioxidants tested. Chandrasekar et al. (2016) studied 100ppm caffeic acid in sardine oil at 37°C for 15 days of analysis, indicating significant performance in maintaining oxidation stability. Sinapic acid is also significant in inhibiting the total TOTOX value. Thiyam et al. (2006) presented a higher concentration of sinapic acid (500 µmol/kg oil) was performed effectively in stripped rapeseed oil at 40°C for 21 days, and inhibited the oxidation by 80%. Resveratrol expressed the antioxidant activity against oxidation in sardine oil to the initial 20-30 days in peroxide and *p*-anisidine analysis.

Medina et al. (2010) reported that the high resveratrol (100-200ppm) concentration effectively suppressed the oxidation of fish oil-in-water emulsions in 11 days at 40°C analysis. Rutin and quercetin antioxidant was not show effectiveness in sardine oil. Lee et al. (2016) presented that 0.4mM concentration of quercetin, and rutin is effective in stripped soybean oil but acts as a pro-oxidant in non-stripped soybean oil in 45 days analysis at 25°C. Chandrasekar et al. (2016) showed an effective response by quercetin and rutin with 100ppm against oxidation in sardine oil stored at 37°C for 14 days under darkness. In hydroxybenzoic acid antioxidants, vanillic acid influenced the significant response against oxidation from 20-30 days; after that, oxidation increased. Ellagic acid was also ineffective against oxidation, β-carotene acted as pro-oxidant in sardine fish oil. The oxidation value is higher than among antioxidants as well as "Control." The ability of β-Carotene to impart oxidative stability is due to light filtering, singlet oxygen quenching, photosensitizer inactivation, and free radical scavenging (Choe and Min, 2006). Many authors have reported the efficacy of carotenoids in bulk oil systems in retarding oxidation. However, quite a few authors have reported the pro-oxidant behavior of β-carotene in bulk oil systems (Haila and Heinonen, 1994; Henry et al., 1998). Synthetic phenolic antioxidants (TBHQ, BHA, and BHT) have superior efficacy against oxidation and inhibit oxidation percentage for a long duration. These antioxidants also maintain oxidation stability at limited concentrations. These antioxidants have less BDE value which increases the H-bond donating ability. TBHQ expressed high efficacy in these synthetic antioxidants in these synthetic antioxidants in these synthetic antioxidants to sustain the sardine oil oxidation in 50 days of analysis. The oxidation inhibition percentage in the TOTOX value was 75%, proving the antioxidant activity is too high. TBHQ is an approved antioxidant as per European Food Safety Authority and Food and Drug Administration, USA.

3.2.2.5 Summary and conclusion

Sardine fish oil has a significant amount of n-3 polyunsaturated fatty acids (n-3 PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). As n-3 PUFA contains two or more 1,4-diene systems, loss of hydrogen atoms occurs in those structures, producing primary and secondary oxidation products. The oxidation product production is

influenced by various factors (light, metal, heat) that create several oxidation routes. Antioxidants can inhibit the different pathways of oxidation by various antioxidant mechanisms. Thirteen antioxidants from different classes and different antioxidant mechanisms were used to retard the oxidation of sardine oil, and the following conclusions were derived;

- Analysis of ten natural antioxidants in sardine oil, the catechin, caffeic acid, sinapic acid, resveratrol, and vanillic acid presented high efficacy against oxidation.
- Catechin was more effective than BHA and comparable to BHT in the inhibition oxidation percentage. Catechin has both free-radical scavenging and metal-chelating activity and so is effective in maintaining the oxidation stability of oil compared to other antioxidants.
- Caffeic acid also has metal-chelating sites, such as the hydroxyl group at the B-ring, which increases the antioxidant capability. These abilities serve to retard oxidation for a long duration.
- Sinapic acid and vanillic acid also showed effective responses till the 35^{th} day to suppress oxidation in sardine oil.
- Resveratrol is an effective antioxidant, and it suppresses oxidation from 20-35 days in sardine oil.
- Ellagic acid, ferulic acid, rutin, and qurecetin are not too effective in long-term inhibiting oxidation in sardine oil.
- β-Carotene act as a pro-oxidant and increases the oxidation value in sardine oil
- The three synthetic antioxidants (TBHQ, BHA, and BHT) significantly suppressed the oxidation for a long storage period. TBHQ was a superior antioxidant in the 50 days studied.

CHAPTER – 4

SCREENING AND OPTIMIZATION OF SELECTED ANTIOXIDANTS FOR THE OXIDATIVE STABILITY OF SARDINE OIL

CHAPTER 4

This chapter describes the methods of screening and optimization for an appropriate combination of antioxidants concentration to inhibit oxidation for a long duration and maintain the quality of PUFA contents. The Factorial design of experiments, a method to minimize the number of experiments yet explains the effect of each factor on the response as well as how the effect of each factor varies with the change in the level of the other factors, was employed. The studies using experimental designs showed the relevance of this methodology. The full factorial design was used to envisage the effective antioxidants and their combination by analysing the main and interactive effect of antioxidants using the resulting oxidation parameter, TOTOX value. The statistical significance ($p<0.05$) of the antioxidants and TOTOX value were the basis for identifying the suitable antioxidants and their synergistic effect on oil oxidation.

The natural antioxidant combination was studied to maintain oxidation stability for a long duration. Statistical analysis provides an efficient combination against oxidation to find the significant value. Catechin and resveratrol are an effective combination ($p<0.05$) to suppress the oxidation of sardine oil. Skroza et al. (2015) had analysed the synergism effect of resveratrol with other binary phenolic antioxidants by FRAP, DPPH, and Briggs-Rauscher (BR) reaction. Resveratrol and catechin combination showed effect synergism effect in all three reactions. Caffeic and resveratrol synergistic effects in FRAP provided 10% better activity than individual compounds. Catechin and Resveratrol in the initial stage (4 min.) act as antagonists; in another stage, it performs the synergistic effect. Quercetin and Gallic acid act as pro-oxidant. In DPPH analysis, Resveratrol presented a synergistic effect with Catechin and Gallic acid. The other antioxidants (caffeic, quercetin) are expressed as antagonistic. In BR reaction, Catechin combination with resveratrol expressed more effective antioxidant activity than other antioxidants. Yin et al. (2020), studied the sodium caseinate particle with co-encapsulated epigallocatechin-3-gallate (EGCG) and resveratrol combination to suppress the fish oil oxidation for 30 days at room temperature. The protective effect of co-encapsulated resveratrol and EGCG on fish oil oxidation was similar to that of EGCG alone with 23 days, but was better thereafter indicating that EGCG

played a protective role before resveratrol against the oxidation of fish oil. Huang et al. (2020) had reported the synergistic effect of resveratrol (0.06%) v/v and sesamol (0.08%) v/v in high oleic acid peanut oil through the Rancimat method at 130°C till 10.60 hours. Cheng et al. (2020) had analysed the co-encapsulation of α-tocopherol and resveratrol through sodium caseinate to maintain the oxidation stability in sunflower oil. Almajano et al. (2007) had evaluated BSA in combination with EC, EGCG, EGC, and ECG in refined sunflower oil for 45 days at 30°C show strong antioxidant activity against oil oxidation.
A combination of natural antioxidants delivers the combined effect and suppress oxidation for a long duration by synergism compared to individual antioxidant effects. The plant or herbal extract also presents an effective response against oxidation compared to synthetic antioxidants. Chen et al. (2022) studied the seven antioxidants in walnut and DHA algae oil. The six antioxidants (e.g., ascorbyl palmitate (AP), phytic acid (PA), vitamin E (VE), antioxidant of bamboo leaves (AOB), rosemary extract, tea polyphenols (TP)) were each combined with tea polyphenol palmitate (TPP) antioxidant in different combination in dark containers. They were stored in an air-circulating oven (60°C) for three days. The combination of TPP (480mg/kg) and TP (80mg/kg) was reported to be effective in DHA algae oil. The combination of TPP (450mg/kg) and TP 100mg/kg showed high efficacy against oxidation in walnut oil.

In the herbal extract, Zhang et al. (2010) had studied rosemary extract (200ppm) in refined sunflower oil at 60°C for 21 days in darkness. The rosemary extract presented an effective response against oxidation compared to BHA and BHT but was less effective against TBHQ. The combination of antioxidants expresses the various antioxidant mechanism that retard several oxidation routes. These synergistic effects of supportive antioxidants to primary antioxidants are significant to maintain product quality for a long duration.

4.1 Factorial design of experiments

As the natural and synthetic antioxidants had significantly different effects on the retardation of oil oxidation, the factorial design of experiments and subsequent analysis were performed separately for four natural antioxidants and three synthetic antioxidants.

The 2^4 full factorial experimental designs used for natural antioxidants, which are selected from different classes of antioxidants viz, catechin (flavan-3-ol), resveratrol (stilbene), sinapic acid (hydrocinnamic acid), and vanillic acid (hydroxybenzoic acid), by considering the oxidative stability in sardine oil. The main and interactive effect of four factors (antioxidants) were analysed, and the combination of antioxidants offering synergistic effect on the oxdative stability was identified. Similarly, 2^3 full factorial experimental designs were used for synthetic phenolic antioxidants, TBHQ, BHA, and BHT, and the effects of these three factors were considered to identify the combination among them to offer the synergistic effect.

4.2 Response Surface Methodology

Response Surface Methodology (RSM) is a powerful approach that combines the design and analysis of experiments, modeling techniques, and optimization methods. By integrating these three components, RSM achieves higher statistical and mathematical sophistication. It allows for equating process results based on a few control parameters before production starts. Moreover, optimal responses with minimum variance can be obtained by assigning specific setups to control parameters. Whether it is a new or known process, RSM offers a consistent approach using experimental designs and empirical data. The optimal concentrations of individual natural antioxidants for offering synergistic effects on oxidative stability were obtained by RSM. The experiments were designed using central composite design (CCD) methadology. Central composite design (CCD) is a powerful tool for generating appropriate experimental designs. This method involves three experimental points: factorial, central, and axial. The factorial points are located at the vertices of a hypercubic design and allow data generation for 2-sample t-hypothesis tests. Central points enable the curvature assessment in the region of interest, while axial points can estimate the quadratic effects observed in the determinate region. Additionally, CCD can accurately calculate the value of the lack-of-fit parameter, making it an invaluable resource in experimental design.

Catechin and resveratrol were selected the basis on full factorial analysis to evaluate the optimized concentration to suppress the oxidation of sardine oil by the TOTOX value that acts as a response. Using 2^2 CCD, three sets of experiments were developed at different concentration ranges of catechin and resveratrol combinations to perform the RSM and identify the optimal/appropriate concentrations for their synergistic effect. Similarly, synthetic antioxidants TBHQ and BHT, selected from the full factorial analysis, are considered to estimate the optimized concentration using the experimental design developed with 2^2 CCD, and the responses, TOTOX values were analysed RSM to retard the oxidation of sardine oil.

4.3 Material and Methods

4.3.1 Oxidative stability of oil

A specific amount of various antioxidants was mixed with ethanol and placed in amber glass vials. Nitrogen gas evaporated the solvent, and refined fish oil was added to each vial to achieve a final concentration of antioxidants. This concentration was chosen based on research by Chandrasekar et al. (2016), Maqsood and Benjakul (2010), and Hopia et al. (1996) to prevent any potential pro-oxidant effect. The samples were carefully mixed for 15 minutes, stored at 25ºC under darkness, and exposed to atmospheric air for 50 days. The oxidation experiment was conducted in triplicate, with samples taken every fifth day to determine the peroxide and *p*-anisidine values.

4.3.2 Full factorial design (FFD) of experimental analysis

A (2^4) full factorial design taking four independent variables of natural antioxidants at two levels, augmented with three central point experiments, was implemented in 19 experimental runs (Table 4.1). A factorial model comprises a list of coefficients multiplied by an associated factor. In a 2^k factorial experimental design, *k* factors are varied over two levels. More than one test can be performed for a given combination of the *k* factors. These are referred to as replicates, r. Therefore, the total number of trials is given as follows:

$$N = r \times 2^k + C \quad (4.1)$$

Where, C represents the number of central point measurements used to test for quadratic terms in the low-to-high range. Central points are used to estimate pure error and curvature in the model.

The polynomial equation based on the first-order model with four parameters (X_i, X_j, X_k, and X_l) and their interaction terms can be given in the form of the following expression:

$$Y_{TOTOX} = b_0 + b_iX_i + b_jX_j + b_kX_k + b_lX_l + b_{ij}X_iX_j + b_{ik}X_iX_k + b_{il}X_iX_l + b_{jk}X_jX_k + b_{jl}X_jX_l + b_{kl}X_kX_l \quad (4.2)$$

where b_0 is the average value of the result; b_i, b_j, b_k and b_l are the linear coefficients; and b_{ij}, b_{ik}, b_{il}, b_{jk}, b_{jl}, and b_{kl}, represent the interactions coefficients. The letters X_i, X_j, X_k, and X_l represent the factors in the model. Combinations of factors (such as X_i X_j) represent interactions between the individual factors in that term.

4.3.2.1 Natural antioxidants

Based on our preliminary screening studies comprising ten natural antioxidants (Mishra, Belur, and Iyyaswami, 2022), four were selected and investigated: Catechin (X_1), resveratrol (X_2), sinapic acid (X_3), and vanillic acid (X_4). The oxidation degradation efficiency of antioxidants is analysed by TOTOX value, which was considered a response or dependent factor. Table 4.1 illustrates the four parameters and their chosen levels for the experiment. The factor levels were coded as -1 (low), 0 (central point), and +1 (high). Sixteen experiments were carried out, and three were in the central of the experimental field. The results were analysed with 95% confidence intervals using the Design Expert 11.1.2 (Stat-Ease, Minneapolis, MN, USA) software. In this study N = 19 (r = 1, *k* = 4, C = 3).

Table 4.1 Levels of different natural antioxidants used in a full factorial design

Independent Variables	Coded Symbol	Variable Levels		
		Low (-1)	Central (0)	High (+1)
Catechin (mM)	X_1	0.2	0.35	0.5

Resveratrol (mM)	X_2	0.2	0.35	0.5
Sinapic acid (mM)	X_3	0.2	0.35	0.5
Vanillic acid (mM)	X_4	0.2	0.35	0.5

4.3.2.2 Synthetic antioxidants

Three synthetic antioxidants (TBHQ (X_1), BHA (X_2), and BHT (X_3) were selected from preliminary screening studies (Mishra et al. 2022) and considered as main factors. TOTOX value has been considered as a response or dependent factor to quantitate the oxidation degradation efficiency of antioxidants. Table 4.2 illustrates the three parameters and their chosen levels for the experiment. The factor levels were coded as -1 (low), 0 (central point), and +1 (high). Eleven experiments were carried out, where three were the central point of the experimental field (N = 11 (r = 1, *k* = 3, C = 3). The results were analysed with 95% confidence intervals using the Design Expert 11.1.2 (Stat-Ease, Minneapolis, MN, USA) software.

Table 4.2 Levels of different synthetic antioxidants used in a full factorial design.

Independent Variables	**Coded Symbol**	**Variable Levels**		
		Low (-1)	**Middle (0)**	**High (+1)**
TBHQ (mM)	X_1	0.1	0.2	0.3
BHA (mM)	X_2	0.1	0.2	0.3
BHT (mM)	X_3	0.1	0.2	0.3

4.3.3 Response surface methodology of experimental analysis

RSM was used to determine the optimal concentration of antioxidants for suppressing the oxidation of sardine oil. The three 2^2 central composite design models with natural antioxidants studied with different concentrations. The two independent variables, Catechin (X_1) and resveratrol (X_2), were selected in the design model with a central and axial point. The central points are placed exactly in the design central and can be obtained

by the average values of the factors levels. The central point forms the second typology of points to be included in the CCD since they allow curvature analysis in the investigated region and reduce the prediction variance in the design central. When the curvature is identified, the axial points are finally added. The axial points, in turn, extrapolate the lower and upper levels of each factor and are placed outside the cuboidal experimental region, allowing a better estimation of the quadratic terms. It determines the total number of experiments runs of the CCD model is expressed as;

$$N = 2^k + 2k + n_c \quad \text{(4.3)}$$

where N is the total number of experiments, k is the number of factors, 2^k is the number of factorial points, $2k$ is the number of axial points, and n_c is the number of central points. The distance from the axial points to the design centre is given in the equation;

$$\propto = 2^{\frac{k}{4}} \quad \text{(4.4)}$$

where $\propto$ is the axial distance and k is the number of factors.

The CCD of experiment methodology was considered to develop the experimental runs with different combinations/ranges of independent variables coded in five levels of (−α, −1, 0, +1, +α) as illustrated in Table 4.3. The applicability and significance of the equation were analysed using the ANOVA test. The performance of the response surface was analysed with the second-order polynomial quadratic equation expressed as;

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{\substack{i=1 \\ j>1}}^{n=1} \sum_{j=2}^{n} \beta_{ij} X_i X_j + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \varepsilon \quad \text{(4.5)}$$

where, Y is the predicted response; β_0 represents the intercept or regression coefficient; β_i, β_{ii}, and β_{ij} represents the linear, quadratic, and interaction coefficients; X_i and X_j are the coded values of the process variables; and ε is the experimental/residual error. The quadratic equation was obtained by regression analysis, and the constant and coefficients of the equation were determined. The model was validated by conducting on given optimal medium setting and statistical *t*-test using; coefficient of determination (R^2), adjusted R^2

(R^2adj), and predicted R^2 (R^2pred). The results were analysed with 95% confidence intervals using the Design Expert 11.1.2 (Stat-Ease, Minneapolis, MN, USA) software.

The two-dimensional contour plot (2D) and the three-dimensional response surface diagram (3D) can be further developed using the evaluated polynomial equations. The contour plot displays the relationship between responses, mixture components, or numeric factors in a two-dimensional (2D) format. It can illustrate how these factors interact and clearly represent the response based on their combinations. The three-dimensional (3D) response surface diagram displays the interaction effects of independent variables of oxidation stability of antioxidants to suppress the TOTOX value of sardine oil. For 3D surface diagrams, two variables were changed, while the third variable remained constant at the zero-coded level. The 3D graphs could provide support for illustrating more information about the behaviour of the system and could describe the level and nature of interactions of independent variables as a function of factors on the degradation of TOTOX value. The shape of contour lines could describe the interactions among factors, and the elliptic contour lines indicated a significant interaction, while the circular contour lines indicated an insignificant interaction between corresponding variables.

4.3.3.1 Natural antioxidants

In this study, the 'CCD of experiment' method was utilized to develop three different experimental designs (Model-I, Model-II and Model-III) for natural antioxidants to identify the synergistic effect of antioxidants. 13 experimental runs were suggested by CCD with four cubic points (coded as ±1), four axial points (coded as ±α), and five replication of central points (coded as 0) for each model (Tables 4.8, 4.10, and 4.12).

Table 4.3 Levels of different natural antioxidants used in a central composite design for the RSM methodology

Independent Variables	Coded Symbol	Variable Levels				
		Low (-1)	Middle (0)	High (+1)	-α	+α
Model-I						
Catechin (mM)	X_1	1.2	2.4	3.6	0.702	4.097
Resveratrol (mM)	X_2	1.2	2.4	3.6	0.702	4.097
Model-II						
Catechin (mM)	X_1	0.25	0.5	0.75	0.146	0.853
Resveratrol (mM)	X_2	3	3.5	4	2.792	4.207
Model-III						
Catechin (mM)	X_1	0.25	0.5	0.75	0.146	0.853
Resveratrol (mM)	X_2	0.25	0.625	1	0.094	1.155

4.3.3.2 Synthetic antioxidants

Using a full factorial analysis (2^2), the TBHQ and BHT were identified as significant antioxidants, which provide an interactive effect and synergistically retard the oxidation of sardine oil. The TBHQ and BHT, were evaluated to determine the ideal concentration to effectively retard the oxidation using the RSM by analysing the response, TOTOX value. The experimental design details in five levels (−α, −1, 0, +1, +α) are illustrated in Table 4.4. For the RSM analysis, 13 experimental trials were designed based on the CCD for two factors (TBHQ and BHT) in Table 4.17.

Table 4.4 Levels of different synthetic antioxidants used in a central composite design.

Independent Variables	Coded Symbol	Variable Levels				
		Low (-1)	Middle (0)	High (+1)	-α	+α
TBHQ (mM)	X_1	0.06	0.18	0.3	0.0103	0.3497
BHT (mM)	X_2	0.06	0.18	0.3	0.0103	0.3497

4.4 Result and discussion

4.4.1 Development of a model equation to analyse the effects of natural antioxidants

The refined fish oil underwent testing to determine its oxidative stability after being mixed with antioxidants in a calculated amount based on the experimental design (Table 4.1). It is important to note that antioxidants can have both antioxidant and pro-oxidant effects when used in excessive amounts, but at appropriate concentrations, they can work together to produce a synergistic effect. The experimental design included four natural antioxidants, each at two different levels of concentration, and the results of the response studied are presented in Table 4.5.

Table 4.5 Experimental design matrix of the two-level full factorial experiments. The levels of each antioxidant were given in coded values.

	X_1	X_2	X_3	X_4	Response
Run	**Catechin (mM)**	**Resveratrol (mM)**	**Sinapic Acid (mM)**	**Vanillic Acid (mM)**	**TOTOX Value**
1	-1	-1	1	1	199.333
2	-1	1	1	1	221.600
3	-1	-1	-1	-1	203.667
4	-1	1	-1	-1	190.700
5	0	0	0	0	161.300
6	1	-1	-1	1	212.467
7	0	0	0	0	161.300
8	1	1	-1	1	138.333
9	-1	-1	1	-1	192.633
10	1	1	-1	-1	152.933
11	1	1	1	1	177.500
12	1	-1	1	1	173.367
13	1	1	1	-1	156.833

14	0	0	0	0	161.300
15	1	-1	-1	-1	210.300
16	1	-1	1	-1	190.333
17	-1	-1	-1	1	199.367
18	-1	1	-1	1	197.900
19	-1	1	1	-1	170.267

Control value:- TOTOX – 297.23

A quadratic equation (Eq. 4.6) was developed based on the effect of independent antioxidant concentrations (variables) on the TOTOX value (response). By examining these results and Eq. 4.6, we could estimate the main and interactive effects. The statistical significance of the main and interactive effects were analysed by considering the P value (P< 0.05), which is listed in Table 4.3.

$$Y_{TOTOX} = 186.72 - 10.21X_1 - 10.96X_2 - 1.49X_3 + 3.26X_4 - 9.15X_1X_2 - 0.5124X_1X_3 - 4.35X_1X_4 + 7.28X_2X_3 + 4.81X_2X_4 + 4.45X_3X_4 \quad \text{(4.6)}$$

Table 4.6 Main and interaction coefficients of the factors.

b_i	**coefficients**	***p*-value**
b_0	186.72	0.0346
b_1	-10.21	0.0157
b_2	-10.96	0.0114
b_3	-1.49	0.6582
b_4	3.26	0.3448
b_{12}	-9.15	0.0251
b_{13}	-0.5124	0.8781
b_{14}	-4.35	0.2185
b_{23}	7.28	0.0583
b_{24}	4.81	0.1788
b_{34}	4.45	0.2092

Table 4.7 The ANOVA of natural antioxidants from the full factorial model

Source	Sum of Squares	df	Mean Square	F-value	*p*-value	Remarks
Model	6979.00	10	697.90	4.21	0.0346	significant
X_1-Catechin	1668.74	1	1668.74	10.06	0.0157	significant
X_2-Resveratrol	1922.84	1	1922.84	11.59	0.0114	significant
X_3-Sinapic Acid	35.41	1	35.41	0.2133	0.6582	
X_4-Vanillic Acid	170.31	1	170.31	1.03	0.3448	
X_1 X_2	1338.37	1	1338.37	8.06	0.0251	significant
X_1 X_3	4.20	1	4.20	0.0253	0.8781	
X_1 X_4	303.33	1	303.33	1.83	0.2185	
X_2 X_3	847.81	1	847.81	5.11	0.0583	
X_2 X_4	370.55	1	370.55	2.23	0.1788	
X_3 X_4	317.44	1	317.44	1.91	0.2092	
Curvature	1632.55	1	1632.55	9.84	0.0165	
Residual	1161.73	7	165.96			
Lack of Fit	1161.73	5	232.35			
Pure Error	0.0000	2	0.0000			
Cor Total	9773.28	18				

4.4.2 Analysis of main and interactive effects

From the equation of the model with ANOVA (Table 4.7), it was noted that the coefficients of catechin (X_1) and resveratrol (X_2) could affirm the significant effect on the response (TOTOX value) with a statistically significant P value. This result means that oxidation degradation increases when these two factors change from low to high. The main effect factors, catechin (X_1) and resveratrol (X_2), having Prob < 0.05, are considered potentially significant. On the other sinapic acid (X_3) and vanillic acid (X_4) indicated a non-significant

effect against the response. This means oxidation degradation falls when these two factors pass from lower to high levels.

The coefficients of Xi Xj ($b_{i,j}$) and the corresponding p-values ($p<0.05$) indicate the interactive effects that exist between the antioxidants on oxidative stability. The equation model with ANOVA presented that the interaction of catechin (X_1) and resveratrol (X_2), was showed efficacy against oxidation. Other interactions, $X_1 X_3$, $X_1 X_4$, $X_2 X_3$, $X_2 X_4$, and $X_3 X_4$, showed a non-significant effect ($p>0.05$) on the response, and it indicated these interaction factors did not present an efficient role in suppressing the oxidation.

Half-normal probability plot of the effect estimates to assess the importance of effects in order to screen for the 'vital few' effects. Under the null hypothesis of no active effects, all contrasts follow a normal distribution with mean zero and constant variance. A half-normal probability plot is the scatter plot of the absolute values of the contrasts versus the half-normal quantiles of that contrast (Figure 4.1) half-normal probability plot of natural antioxidants analysis estimates the main and interactive effect. Catechin (X_1), resveratrol (X_2), and catechin-resveratrol (X_1X_2) are considered significant ($p<0.05$) given how these effects differ from the near-straight line of other effects are non-significant.

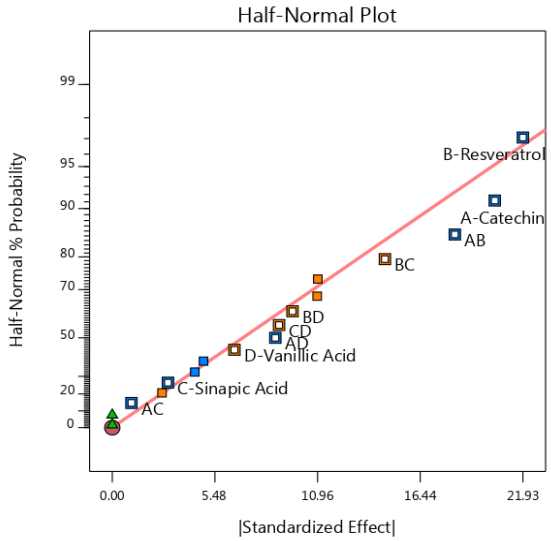


Figure 4.1 Half-Normal plot of the main and interactive effect of natural antioxidants factors.

4.4.3 Validity of the model

The fit of the model was further checked by the coefficient of determination R^2. The R^2 value is always between 0 and 1. The closer the R^2 value is to 1, the better the model predicts the response. Figure 4.1 presents the half-normal probability plot effect show the absolute value of standardized effect from the largest effect to smallest effect. It is found that there is a correlation between the two performances with a coefficient of about 0.8573. That is to say that the model explains 85% of the results. In addition, according to Table 4.7, the F-ratio (4.21) is significant, so the model adopted in this study (full factorial design) is acceptable and validated.

4.4.4 Determination of optimal concentration of natural antioxidants using RSM

4.4.4.1 RSM for Model – I

RSM uses to determine the optimal concentration of selected antioxidants [catechin (X_1) and resveratrol (X_2)], which shows the interactive/synergistic effect for suppressing the oxidation of sardine oil. After full factorial analysis, a CCD of experiments for the two selected antioxidants were developed (Table 4.3) and the resulting experimental response (TOTOX) was analysed by identifying the optimum concentration of catechin (X_1) and resveratrol (X_2) from the response surfaces developed at different conditions. The experimental runs along with the responses are summarized in Table 4.8.

Table 4.8 Experimental design matrix of the central composite design experiments. The levels of each natural antioxidant were given in coded values (Model-I).

	X_1	X_2	Response 1
Run	**A:Catechin**	**B:Resveratrol**	**TOTOX value**
	mM	**mM**	
1	0	0	260.622
2	1	-1	258.933
3	0	0	260.622
4	0	0	260.622
5	0	-1	254.422
6	0	0	260.622
7	0	0	260.622
8	-1	1	249.956
9	-1.414	0	249.178
10	0	1.414	250.222
11	1.414	0	256.667
12	1	1	252.222
13	-1	-1	258.356

Control value:- TOTOX – 336.62

The lowest TOTOX value, 249.178, was obtained at trial 9 (Table 4.6) with the condition of ratechin (0.702mM) and resveratrol (2.4mM) for 50 days. The experimental design matrix (variables, X_1 and X_2) for the degradation of TOTOX value from sardine oil was further used for the regression analysis and the quadratic equation was developed with coded values (Eq. 4.7). The lack of fit was conducted to determine the adequacy of various models.

$$Y_{TOTOX} = 260.62 + 1.68X_1 - 2.63X_2 + 0.4222X_1X_2 - 3.29X_1^2 - 3.59X_2^2 \quad (4.7)$$

4.4.4.2 ANOVA of natural antioxidants (Eq. 4.7)

The analysis of variance (ANOVA) was performed on the experimental results to determine the accuracy and significance of the model (Table 4.9) . The Fisher variation ratio (F-value), probability of error value (*p*-value), lack of fit and adequate precision were all evidence of ANOVA. Generally, the *p*-value (probability of error value) less than 0.05 suggested that the terms of the model were significant. In the ANOVA quadratic model, F-value is 11.19, and the *p*-value is 0.0031, implying that the model was significant and adequate in the oxidation stability process. The 0.7813% coefficient of variation (CV%) value suggested the acceptable variation and reproducibility of the model for further predicting TOTOX value reduction within the range of particular variables. The regression coefficient (R^2) value was 0.8888, indicating that the model obtained was significant. Generally, the R^2 value of the model should not be less than 0.75 for a suitable model. However, a large R^2 value does not necessarily mean the regression model is good, and the inference is only valid based on the similarly high value of adjusted R. In this study, the regression coefficient (R^2 = 0.8888) value was consistent with the adjusted R^2 value (0.8094), indicating that the regression model was significant and fitted well with the experimental data. The difference between the adjusted and predicted values was less than 20%, indicating that the adjusted R^2 was in reasonable agreement with the predicted R^2 (0.2094). Additionally, an appropriate regression model should have had a greater adequate precision (>4.0), which was used for representing the signal-to-noise ratio. In this study, the quadratic model had adequate precision with a high ratio of 8.5315. Therefore, it was concluded that this model could be used to navigate the design.

Table 4.9 The ANOVA of natural antioxidants from the central composite design (Model-I)

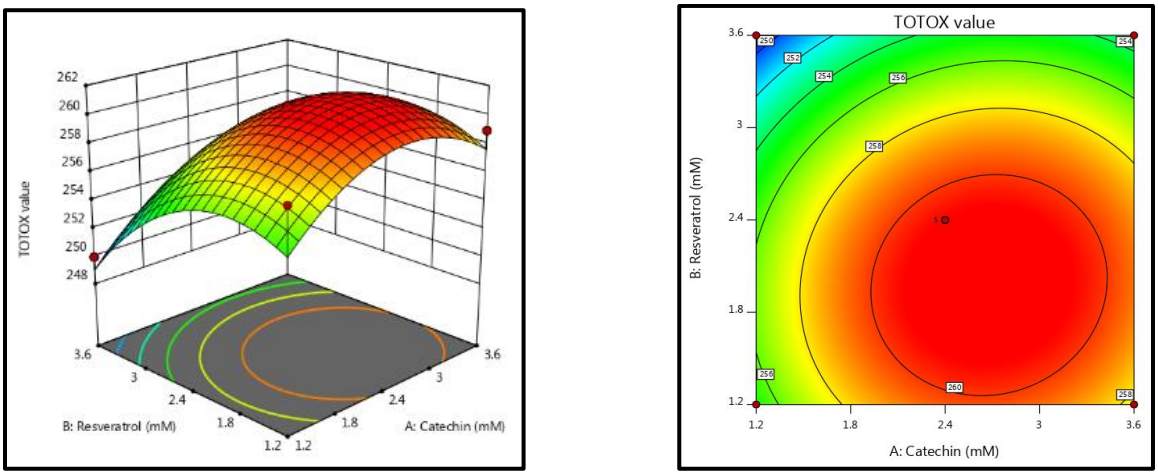
Source	Sum of Squares	df	Mean Square	F-value	*p*-value	
Model	224.58	5	44.92	11.19	0.0031	**significant**
X_1-Catechin	22.56	1	22.56	5.62	0.0495	
X_2-Resveratrol	55.39	1	55.39	13.80	0.0075	
X_1 X_2	0.7131	1	0.7131	0.1777	0.6860	
X_1^2	75.25	1	75.25	18.75	0.0034	
X_2^2	89.60	1	89.60	22.33	0.0021	
Residual	28.09	7	4.01			
Lack of Fit	28.09	3	9.36			
Pure Error	0.0000	4	0.0000			
Cor Total	252.67	12				
Adjusted R^2					0.8094	
Predicted R^2					0.2094	
Regression Coefficient R^2					0.8888	
Coefficient of variation (%)					0.7813	
Adeq. Precision					8.5315	

The positive sign of the terms in the equation indicates the proportional effect of variables on the response and conversely the negative sign indicates the inverse proportionality of variables on the response. The coefficient of linear terms, X_1, and coefficient of interactions term X_1X_2 positive sign indicated that the TOTOX value increases with increasing concentration of X_1. Whereas other terms were significant in the model to suppress the oxidation. The significant variable influencing the oxidation stability was the linear term of X_2, indicating that resveratrol (p-value 0.0075) was the most significant factor. The coefficient of a quadratic term X_1^2 and X_2^2 are efficient variables to maintain oxidation

stability with significant p-value 0.0034 and 0.0021 against oxidation in 50 days of analysis. The interaction term X_1X_2 are insignificant variable which unable to maintain the oxidation stability with p-value 0.6860. The linear term of X_1 (catechin) with p-value 0.0495 was a significant factor, but the positive sign showed the high TOTOX value.

4.4.4.3 Analysis of contour graph and surface plot (Model-I)

Figure 4.2, the contour and 3D-surface graph showed the red colour, which indicates a higher response (TOTOX) value. It means the catechin and resveratrol concentration presented an antagonistic effect against oxidation stability. The plots also indicated that the higher concentration of resveratrol and lower concentration of catechin provide significant efficacy in suppressing oxidation.



(a) (b)

Figure 4.2 The (a) 3-D surface and (b) Contour plots of TOTOX value of natural antioxidants (Model-I)

According to the analysis, the independent variables concentration range in model is not significant to decrease the TOTOX value. However, it was found from the countur and surface plots that the lower concentration of catechin is good enough to resist the oxidation of oil when compared to resveratrol. Accordingly, further analysis with modified concentration of resveratrol was planned to optimize for the minimum TOTOX value.

4.4.5 RSM for Model – II

Even though catechin and resveratrol individually able to provide the antioxidant effect, the experimental design (Model -I) failed to provide the synergistic effect of these two antioxidants combination with the selected concentration range on the oxidation of oil. Hence, a new CCD experimental design (Model- II) was developed with the modified concentration of Resveratrol. The Table 4.10 presents the findings from the 13 experimental runs conducted using the central composite design. This statistical design was utilized to establish the correlation between the response (TOTOX value) and the factors (catechin and resveratrol).

Table 4.10 Experimental design matrix of the central composite design experiments. The levels of each antioxidant were given in coded values (Model-II). Control TOTOX– 310.13

	X_1	X_2	Response TOTOX value	
Run	**Catechin (mM)**	**Resveratrol (mM)**		
			Experimental	**Predicted**
1	0	0	255.933	255.933
2	0	0	255.933	255.933
3	-1	1	256.000	240.05
4	0	0	255.933	255.933
5	-1.414	0	220.800	235.90
6	0	-1.414	218.667	205.89
7	1	-1	202.933	226.41
8	0	0	255.933	255.933
9	1	1	191.400	202.14
10	1.414	0	245.133	222.50
11	0	1.414	206.533	211.78
12	-1	-1	210.667	207.46
13	0	0	255.933	255.933

Based on the findings outlined in Table 4.10, trial 9 resulted in the lowest TOTOX value at 191.4. This was achieved by maintaining a concentration of 0.75mM for catechin and 4mM of resveratrol for 50 days via peroxide and anisidine value. In order to obtain the regression equation, all runs were tested using a quadratic experimental design matrix for the degradation of the TOTOX value from sardine oil. The second-order polynomial expression in terms of coded values from the ANOVA is expressed:

$$Y_{TOTOX} = 255.93 - 4.74X_1 + 2.08X_2 - 14.22X_1X_2 - 13.37X_1^2 - 23.55X_2^2 \quad (4.8)$$

The obtained regression coefficient (R^2) value of 0.7515 indicated the significance of the model. Nevertheless, it is essential to note that a high R^2 value does not necessarily equate to a good regression model. The validity of the inference is based on the adjusted R^2 value, which in this study was found to be 0.5739. This indicates that the regression model was non-significant with the experimental data, and the predicted R^2 was -0.7674. A negative Predicted R^2 suggests that the overall mean may better predict the response than the current model. An appropriate regression model should have had a greater adequate precision (>4.0), representing the signal-to-noise ratio. In this study, the quadratic model was found to have a precision ratio of 4.8544, which is acceptable. As a result, it was concluded that this model could be utilized for navigating the design.

4.4.5.1 ANOVA of natural antioxidants (Eq. 4.8) - Model II

Based on the analysis, the coefficient of the linear term X_1 (catechin), interaction terms X_1X_2, and quadratic terms X_1^2 negative sign indicate the lowest response, but the high p-value (0.4382, 0.1248 and 0.0675) expressed as non-significant factor. The coefficient of the quadratic terms X_2^2 negative sign showed lowest response with significant p-value 0.0066. The linear term of X_2 was the insignificant variable against oxidation stability, with p-value 0.7290, according to the ANOVA in the 50-day analysis.

Based on the ANOVA quadratic model (Table 4.11), it appears that the model was significant and adequate for utilization in the oxidation stability process, as indicated by the F-value of 4.23 and the *p*-value of 0.0431. However, the coefficient of variation (CV%)

value of 6.99% suggests that the model's variation may not be acceptable for predicting TOTOX value reduction within the specific range of variables.

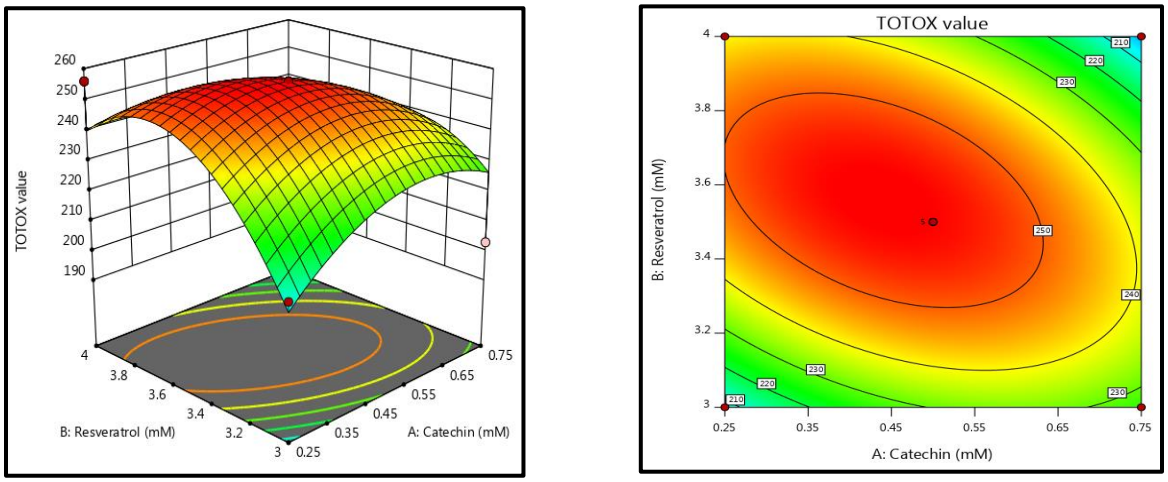
4.4.5.2 Analysis of contour graph and surface plot (Model-II)

Based on the findings shown in Figure 4.3, it appears that the colour red in the contour and 3D-surface graph indicates the higher response (TOTOX) value. This suggests that the concentration of catechin and resveratrol has an antagonistic effect on oxidation stability. Additionally, the plots suggest that varying the concentration of resveratrol and catechin could effectively suppress oxidation. However, the analysis shows that the concentration range of independent variables in model-II was not significant in decreasing the TOTOX value.

Table 4.11 The ANOVA of natural antioxidants from the central composite design (Model-II)

Source	Sum of Squares	df	Mean Square	F-value	*p*-value	Remark
Model	5630.97	5	1126.19	4.23	0.0431	**significant**
X_1-Catechin	179.75	1	179.75	0.6756	0.4382	
X_2-Resveratrol	34.61	1	34.61	0.1301	0.7290	
X_1 X_2	808.45	1	808.45	3.04	0.1248	
X_1^2	1242.91	1	1242.91	4.67	0.0675	
X_2^2	3858.10	1	3858.10	14.50	0.0066	
Residual	1862.44	7	266.06			
Lack of Fit	1862.44	3	620.81			
Pure Error	0.0000	4	0.0000			
Cor Total	7493.41	12				
Adjusted R^2					0.5739	
Predicted R^2					-0.7674	
Regression Coefficient R^2					0.7515	

Coefficient of variation (%)	6.99	
Adeq. Precision	4.8544	



(a) (b)

Figure 4.3 The (a) 3-D surface and (b) Contour plots of TOTOX value of natural antioxidants (Model-II).

4.4.6 RSM with Model – III

Even after reducing the catechin range from 1.2 - 3.6 mM (Model I) to 0.25 -0.75 mM (Model II), the contour and surface plots revealed that the optimal concentration of resveratrol for the synergistic effect may be in the lower concentration range than the concentration range consider in model II (3-4 mM). Accordingly, a new low concentration range at lower level (0.25 – 1mM) was considered for resveratrol along with the catechin concentration range of 0.25 - 0.75mM for the new CCD matrix (Table 4.3). The data in Table 4.12 showcase the outcomes of the 13 experimental trials conducted through the central composite design.

Table 4.12 Experimental design matrix of the central composite design experiments. The levels of each antioxidant were given in coded values.

	X_1	X_2	Response TOTOX value	
Run	**Catechin (mM)**	**Resveratrol (mM)**		
			Experimental	**Predicted**
1	0	0	218.933	218.933
2	0	0	218.933	218.933
3	0	0	218.933	218.933
4	0	0	218.933	218.933
5	+1.414	0	234.933	228.05
6	0	0	218.933	218.93
7	-1	-1	268.800	256.07
8	0	-1.414	247.933	256.03
9	+1	+1	220.400	228.85
10	-1	+1	238.200	233.89
11	0	+1.414	236.067	232.25
12	+1	-1	240.267	240.30
13	-1.414	0	231.600	242.77

Control value:- TOTOX – 310.13

According to Table 4.12, trial 1 had the lowest TOTOX value of 218.93. This was achieved by maintaining a concentration of 0.5mM for catechin and 0.625mM of resveratrol for 50 days using peroxide and anisidine values. A quadratic experimental design matrix was used to test all runs for the degradation of the TOTOX value from sardine oil to obtain the regression equation. The adequacy of different models was determined through a lack of fit analysis. The second-order polynomial expression in terms of coded values from the ANOVA is expressed:

$$Y_{TOTOX} = 218.93 - 5.20X_1 - 8.41X_2 + 2.68X_1X_2 + 8.24X_1^2 + 12.60X_2^2 \quad \text{(4.9)}$$

Based on the regression coefficient, it was determined that the quadratic model accurately described the experimental results with a regression (R^2) value of 0.8151. It is worth noting that the lack of fit *p*-value must be non-significant, which was the case with this model, as it had an insignificant lack of fit *p*-value >0.05. As shown in Table 4.13, the quadratic model was deemed suitable for analysing the experimental data, and as a result, it was chosen for further analysis.

4.4.6.1 ANOVA of natural antioxidants (Eq. 4.9) - Model III

Based on the ANOVA quadratic model (Table 4.13), the model seemed significant and adequate to use in the oxidation stability process. This is supported by the F-value of 6.17 and the *p*-value of 0.0167. The coefficient of variation (CV%) value of 3.66% indicated that the model has acceptable variation and reproducibility. With this in mind, the model can be used to confidently predict TOTOX value reduction within the range of particular variables.

Table 4.13 The ANOVA of natural antioxidants from the central composite design (Model-III)

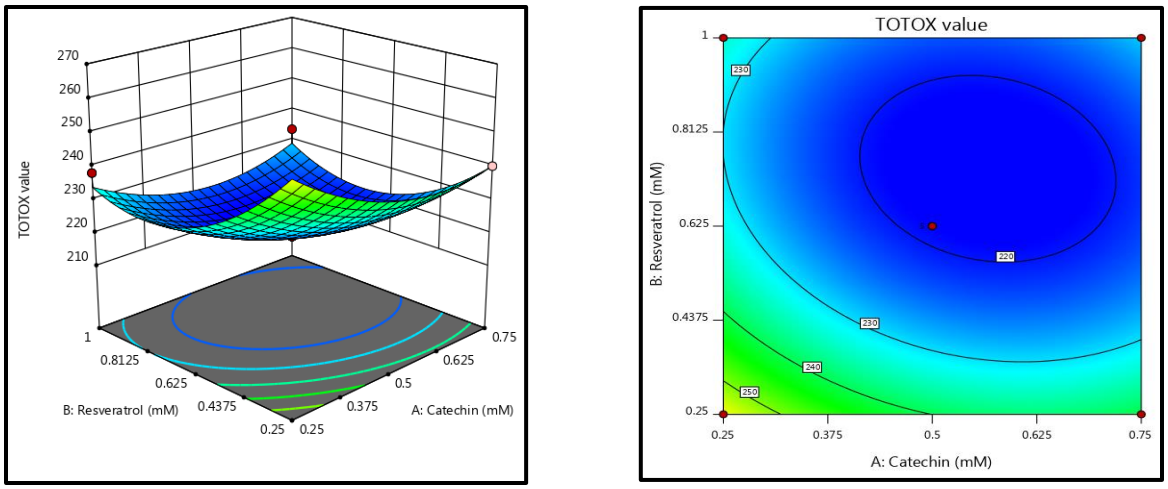
Source	Sum of Squares	df	Mean Square	F-value	*p*-value	Remark
Model	2223.43	5	444.69	6.17	0.0167	**Significant**
X_1-Catechin	216.52	1	216.52	3.01	0.1266	
X_2-Resveratrol	565.30	1	565.30	7.85	0.0265	
X_1 X_2	28.80	1	28.80	0.3998	0.5473	
X_1^2	472.04	1	472.04	6.55	0.0376	
X_2^2	1105.15	1	1105.15	15.34	0.0058	
Residual	504.26	7	72.04			
Lack of Fit	504.26	3	168.09			
Pure Error	0.0000	4	0.0000			
Cor Total	2727.68	12				

Adjusted R^2	0.6831	
Predicted R^2	-0.3146	
Regression Coefficient R^2	0.8151	
Coefficient of variation (%)	3.66	
Adeq. Precision	6.4400	

4.4.6.2 Analysis with 2D contour graph and 3D-surface plot (Model-III)

According to the analysis, the linear term X_2 (resveratrol) significantly influences the oxidation stability with p-value of 0.0265. The X_1 (catechin) negative sign exhibited lowest response, but act as non-significant factor with high p-value 0.1266. The coefficient of interaction term, X_1X_2 expressed non-significant effect against oxidation stability with p-value 0.1248 respectively. The quadratic terms X_1^2 and X_2^2 positive sign showed high response with p-value (0.0376 and 0.0058) respectively, in the 50-day analysis.

According to Figure 4.4, it seems that the colour blue on the contour and 3D-surface graph indicates a lower response value (TOTOX). This indicates that the concentration of catechin and resveratrol significantly affects oxidation stability. The combination of resveratrol and catechin, with a synergistic effect, is recommended to provide the minimum TOTOX value over 50 days at 25°C, which can effectively suppress oxidation. However, the analysis reveals that the concentration range of independent variables in model-III considerably reduces the TOTOX value at very low concentrations of resveratrol and catechin.



(a) (b)

Figure 4.4 The (a) 3-D surface and (b) Contour plots of TOTOX value of natural antioxidants (Model-III).

4.4.7 Development of a model equation to analyse the effects of synthetic antioxidants

Table 4.2 shows the experimental design matrix and the results of the response (TOTOX value) studied. The table indicates that response varied in important ways in the experimental domain. This could likely contain the required optimal zone. The exploitation of experimental results allowed us to estimate the main and interactive effects that are grouped in Table 4.14

Table 4.14 Experimental design matrix of the two-level full factorial experiments. The levels of each antioxidant were given in coded values.

Run	X_1 TBHQ (mM)	X_2 BHA (mM)	X_3 BHT (mM)	Response TOTOX Value
1	-1	1	1	83.6
2	1	-1	-1	73.0
3	-1	-1	-1	232.1
4	1	1	1	82.2
5	-1	1	-1	108.4
6	-1	-1	1	110.2
7	0	0	0	71.5
8	1	-1	1	60.7
9	0	0	0	71.5
10	1	1	-1	65.5
11	0.	0	0	71.5

Control value:- TOTOX – 297.23

Table 4.15 Main and interaction coefficients of the factors.

b_i	Interaction coefficients	*p*-value
b_0	101.983	
b_1	-31.6167	0.0076
b_2	-17.0333	0.0405
b_3	-17.7834	0.0363
b_{12}	20.5167	0.0251
b_{13}	18.8833	0.0311
b_{23}	15.7667	0.0491

By substituting the coefficients b_i in Eq. (4.4) by their values, we get:

$$Y_{TOTOX} = 101.98 - 31.62X_1 - 17.03X_2 - 17.78X_3 + 20.52X_1X_2 + 18.88X_1X_3 + 15.77X_2X_3 \quad (4.10)$$

4.4.7.1 ANOVA of synthetic antioxidants for Equation 4.10

ANOVA presents the variation of the observed efficiency depending on the predicted efficiency. It was found that there was a correlation between the two performances with a coefficient of about 0.9732. That is to say that the model explains 97% of the results. In addition, according to Table 4.16, the F-ratio (18.14) is significant, so the model adopted in this study (full factorial design) is acceptable and validated.

Table 4.16 The ANOVA of synthetic antioxidants from the full factorial model.

Source	Sum of Squares	df	Mean Square	F-value	*p*-value	Remarks
Model	21056.81	6	3509.47	18.14	0.0186	**significant**
X_1-TBHQ	7996.93	1	7996.93	41.34	0.0076	**significant**
X_2-BHA	2321.06	1	2321.06	12.00	0.0405	**significant**
X_3-BHT	2529.98	1	2529.98	13.08	0.0363	**significant**
X_1 X_2	3367.48	1	3367.48	17.41	0.0251	**significant**
X_1 X_3	2852.63	1	2852.63	14.75	0.0311	**significant**
X_2 X_3	1988.71	1	1988.71	10.28	0.0491	**significant**
Curvature	2022.99	1	2022.99	10.46	0.0481	
Residual	580.26	3	193.42			
Lack of Fit	580.26	1	580.26			
Pure Error	0.0000	2	0.0000			
Cor Total	23660.06	10				

4.4.7.2 Main effect and interaction effect between factors (Equation 4.10)

From the equation of the model with ANOVA (Table 4.16), it was noted that the TBHQ (X_1), BHA (X_2), and BHT (X_3) could affirm the significant effect on the response (TOTOX value). This result means that oxidation degradation increases when all these factors change from low to high. These main effect factors, TBHQ (X_1), BHA (X_2), and BHT (X_3), having Prob < 0.05, are considered potentially significant. The $X_1 X_2$, $X_1 X_3$, and $X_2 X_3$ interactions are potentially significant ($p<0.05$). The equation model with ANOVA presented the interaction of TBHQ (X_1), BHA (X_2), and BHT (X_2), showed efficacy against oxidation.

In figure 4.5, half-normal probability plot effect show the absolute value of standardized effect from the largest effect to smallest effect. The TBHQ (X_1), BHA (X_2), BHT (X_3), TBHQ-BHA (X_1X_2), TBHQ-BHT (X_1X_3), and BHA-BHT (X_2X_3) are considered significant ($p<0.05$) given how these effects differ from the near straight line.

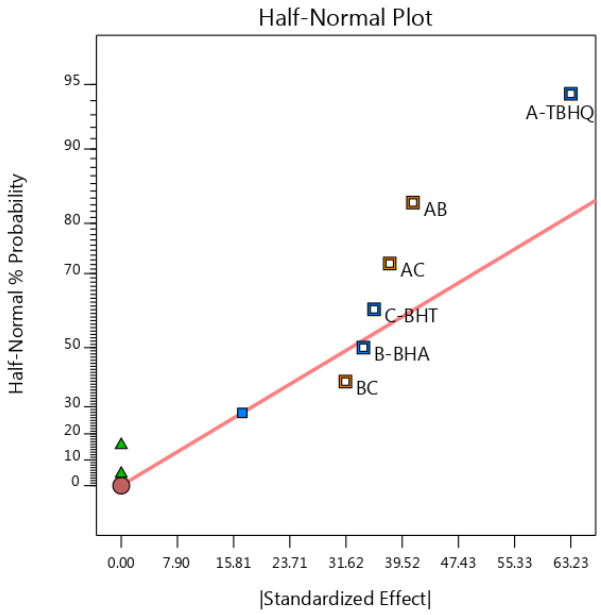


Figure 4.5 Half-Normal plot of the main and interactive effect of synthetic antioxidants factors.

4.4.8 Response surface methodology of synthetic antioxidants

Using a central composite experimental design (2^2), the concentration of synthetic antioxidants was optimized by screening through full factorial analysis. Based on the full factorial (2^3) analysis, all synthetic antioxidants had a significant main and interactive effect ($p<0.05$). It was observed that a higher concentration of TBHQ and BHT and a lower concentration of BHA resulted in a lower TOTOTX value. Conversely, a higher concentration of BHA led to an increase in TOTOX value. Therefore, the combination of TBHQ-BHT was deemed suitable for further analysis. Our chosen antioxidants, TBHQ and BHT, were evaluated to determine the ideal concentration to effectively retard the oxidation of sardine oil by using the TOTOX value as a response. The 2^2 central composite design models studied in synthetic antioxidants studied with different concentrations. The two independent variables: TBHQ (X_1) and BHT (X_2), were optimized in the design model with central and axial points (Table 4.4). Table 4.17 presents the results of 13 experimental trials utilizing the central composite design method. The objective was to establish the correlation between the response variable (TOTOX value) and the factors (TBHQ and BHT).

Table 4.17 Experimental design matrix of the central composite design experiments. The levels of each antioxidant were given in coded values

	X_1	X_2	Response	
Run	**TBHQ**	**BHT**	**TOTOX value**	
	mM	**mM**		
			Experimental	**Predicted**
1	-1.414	0	27.9	27.0
2	0	0	13.2	13.2
3	-1	-1	21.4	22.1
4	0	0	13.2	13.2
5	0	-1.414	19.4	19.2
6	0	0	13.2	13.2
7	0	0	13.2	13.2
8	1	-1	22.1	21.8
9	1	1	23.1	22.8
10	0	0	13.2	13.2
11	-1	1	26.1	26.7
12	1.414	0	23.5	24.0
13	0	1.414	23.4	23.2

Control value:- TOTOX – 336.62

Based on the data presented in Table 4.17, trial 2 had the lowest TOTOX value of 13.29. This was achieved by maintaining a concentration of 0.18mM for TBHQ and 0.18mM of BHT for 50 days while measuring peroxide and anisidine values. A quadratic experimental design matrix was applied to test all runs to obtain the regression equation for the degradation of the TOTOX value from sardine oil. A lack of fit analysis was conducted to determine the adequacy of different models. The second-order polynomial expression in terms of coded values from the ANOVA is expressed:

$$Y_{TOTOX} = 13.29 - 1.05X_1 + 1.40X_2 - 0.9267X_1X_2 + 6.13X_1^2 - 3.99X_2^2 \quad \text{(4.11)}$$

After analysing the experimental results, it was concluded that the quadratic model accurately represented the data with an impressive R^2 value of 0.9942. It is important to note that the lack of fit *p*-value must be non-significant, which was the case in this scenario, as the model had an insignificant lack of fit *p*-value (>0.05). As shown in Table 4.18, the quadratic model was deemed suitable for analysing the experimental data and was therefore chosen for further analysis. Based on the equation, it was found the coefficients of linear term X_1 (TBHQ), interaction term X_1X_2, and quadratic term X_2^2 negative sign indicate lowest response with the significant p-value (0.0010, 0.0120, and 0.0001 respectively). The X_2 (BHT) and X_1^2 positive sign represented the highest response with significant p-value (0.0002, and 0.0001).

4.4.8.1 Studies of analysis of variance for equation 4.11

Based on the analysis of the ANOVA quadratic model, it appears that the model is both significant and suitable for the oxidation stability process. This conclusion is supported by the impressive F-value of 238.40 and the *p*-value of <0.0001. The coefficient of variation (CV%) value of 2.82% confirms that the model has acceptable variation and reproducibility. Therefore, it can be confidently used to predict the reduction inTOTOX value within the specific range of variables.

Table 4.18 The ANOVA of synthetic antioxidants from the central composite design.

Source	Sum of Squares	df	Mean Square	F-value	*p*-value	Remarks
Model	361.27	5	72.25	238.40	< 0.0001	significant
X_1-TBHQ	8.80	1	8.80	29.02	0.0010	
X_2-BHT	15.70	1	15.70	51.82	0.0002	
X_1 X_2	3.43	1	3.43	11.33	0.0120	
X_1^2	261.26	1	261.26	862.02	< 0.0001	
X_2^2	110.78	1	110.78	365.51	< 0.0001	

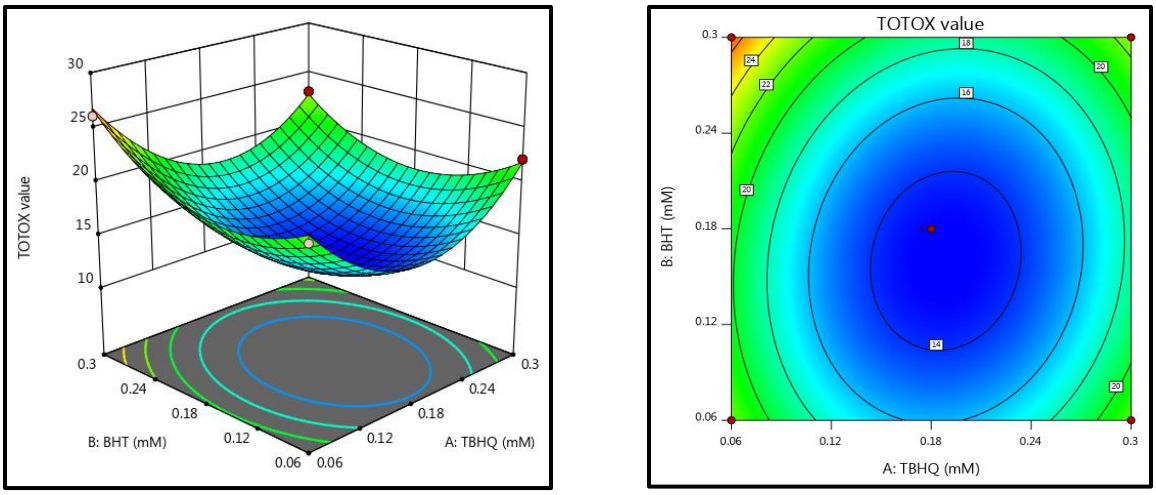
Residual	2.12	7	0.3031			
Lack of Fit	2.12	3	0.7072			
Pure Error	0.0000	4	0.0000			
Cor Total	363.39	12				
Adjusted R^2					0.9900	
Predicted R^2					0.9585	
Regression Coefficient R^2					0.9942	
Coefficient of variation (%)					2.82	
Adeq. precision					36.7359	

The obtained regression coefficient (R^2) value of 0.9942 indicates the significance of the model. However, it is essential to consider that a high R^2 value does not necessarily guarantee a good regression model. It is essential to evaluate the validity of the inference based on the adjusted R^2 value, which was 0.9900 in this study. This suggests that the regression model was significant with the experimental data. The difference between the adjusted and predicted values was less than 20%, indicating that the adjusted R^2 was in reasonable agreement with the predicted R^2 (0.9585). A greater adequate precision (>4.0) is desirable for an appropriate regression model representing the signal-to-noise ratio. In this study, the precision ratio of the quadratic model was found to be 36.7359, which is acceptable. Therefore, it can be concluded that this model is suitable for navigating the design.

4.4.8.2 Main and interactive effect of synthetic antioxidants

Based on Figure 4.6, it appears that the colour blue on the contour and 3D-surface graph indicates a lower response value of TOTOX. This suggests that the concentration of TBHQ and BHT significantly impacts oxidation stability. It is recommended that a combination of TBHQ and BHT, with a synergistic effect, be utilized to achieve a minimum TOTOX value over 50 days at 25°C, which can effectively prevent oxidation. However, the analysis

highlights that the concentration range of independent variables in the model significantly reduces the TOTOX value.



(a) (b)

Figure 4.6 The (a) 3-D surface and (b) Contour plots of TOTOX value of synthetic antioxidants

4.4.8.3 Explored combination of synthetic antioxidants in RSM

Using Design Expert software's optimization tools, a range of TBHQ-BHT combinations can be explored based on desirability (Table 5.1). Analysis of the peroxide values of multiple combinations has demonstrated their effectiveness in preventing oxidation through increased antioxidant activity. The peroxide value was used to analyse primary oxidation products. These findings make way for further studies in kinetics and thermodynamics, developing even better formulations.

4.4.9 Discussion

Synthetic antioxidants are popular in industries because of their effectiveness against oxidation in edible oil. In this analysis, TBHQ, BHA, and BHT are all efficient antioxidants to maintain oxidation stability for a long duration with limited concentration. The

combination of these synthetic antioxidants plays a vital role in inhibiting oxidation by minimizing the concentration. A combination of TBHQ, BHA, and BHT showed high efficacy in maintaining oxidation stability in sardine oil. The RSM analysis TBHQ and BHT combination provide an effective significant response to retard rancidity. Kurechi and Kato (1980) analysed a combination of BHA (0.5mM and BHT (0.5mM) in lard, methyl oleate, soyabean oil at 98°C and clean air passed at a rate of 2.33ml/sec. The combination of BHA/BHT presents an interactive effect in methyl oleate and lard oil to suppress oxidation. However, in soybean oil, the BHA/BHT combination was ineffective. Aardt et al. (2004) investigated the oxidative stability of trilinolein using thermogravimetric analysis and found that the oxidation stability of trilinolein with a mixture of 0.45mM BHA and 0.45mM BHT was higher than that with 0.9mM TBHQ. Jiang et al. (2020) reported that under the temperature of 120°C and the airflow of 20 L/h, the induction period of peanut oil with 0.9mM BHA and 0.9mM BHT extended the induction period of peanut oil was much more than that with 0.9mM of BHA and BHT alone. Ajie et al. (2020) observed that biodiesel with 1.125mM BHA and 1.125mM BHT was more effective than alone with 2.25mM BHA or BHT using the Rancimat method. Jun et al. (2022) investigated lard oil, where each antioxidant contains 0.45mM concentration, and the combination of BHA/BHT, BHA/TBHQ, and BHA/PG is added to 32 grams of lard. It was tested through the Rancimat method with 110°C heating temperature and airflow rate at 20L/h. A combination of BHA/PG is the most prominent antioxidant in lard oil, with a higher induction period of 20.22 hours. BHA/TBHQ showed an effective response; the induction period was 14.11 hrs. BHA/BHT combination induction period was 10.32 hours in lard oil. The synergistic antioxidation of BHA and PG in lard was also observed by Gearhart et al. (1995) using the active oxygen method. The synergistic antioxidation of BHA and TBHQ in biodiesel derived from soybean oils and poultry fats was observed by de Guzman et al. (2009) using the Rancimat method. However, the synergistic antioxidation of BHA and TBHQ in biodiesel derived from safflower oils was not observed by Nogales-Delgado et al. (2019) using the Rancimat method. This analysis provides the synergistic effect of synthetic antioxidants in edible oil. The significant role of synthetic antioxidants

combination inhibits oxidation at lower concentrations in 50 days at 25°C. This combination minimizes the concentration of individual synthetic antioxidants benefiting food industries and customer consumption.

4.5 Summary and conclusion

Full factorial design and Response surface methodology are the statistical tools for screening and optimizing the significant value of antioxidants. These effective values express the antioxidant activity through synergism to maintain the oxidation stability in sardine oil for a long duration. These combinations have different antioxidant mechanisms for inhibiting the various routes of oxidation.

4.5.1 Natural antioxidants

The study of the full factorial design of natural antioxidants, catechin and resveratrol showed the main and interactive effect with a significant *p*-value ($p<0.05$). The significant interactive effect was optimized through response surface methodology. The central composite design in RSM optimizes three models with various combinations to evaluate the lower response of the TOTOX value. Model-III optimizes the optimal concentration of catechin and resveratrol to show a lower TOTOX value. The 3D-surface and contour plot blue colour express the lower response of TOTOX value. The catechin 0.5mM and resveratrol 0.625mM is the optimal value of Model-III with 218.93 TOTOX value. The other models express the higher response of TOTOX value indicated in the 3D surface and contour plots in red. These models also presented the higher concentration that behaves as a pro-oxidant.

The conclusion of natural antioxidant combination for retard the oxidation of sardine oil:

- The selected different class of natural antioxidant from the preliminary study is used for screening through full factorial design.
- Catechin and resveratrol presented the main and interactive effect. Sinapic and vanillic acid did not show the main and interactive effect in FFD.

- Catechin and Resveratrol were selected for optimization through a central composite design with three models of various concentrations.
- Model-III showed catechin 0.5mM and resveratrol 0.625mM presented the lowest response of TOTOX value that was analysed with significant ANOVA and by 3D-surface or contour plots.

4.5.2 Synthetic antioxidants

The study of full factorial design of synthetic antioxidants, TBHQ, BHA, and BHT showed the main and interactive effect with a significant *p*-value ($p<0.05$). All three antioxidants showed a significant interactive effect on each other. TBHQ and BHT combination was optimized for further analysis through response surface methodology. The central composite design in RSM optimized the optimal concentration of synthetic antioxidants for a lower response of TOTOX value. The TBHQ 0.18mM and BHT 0.18mM express the optimal value with a lower response 13.2 TOTOX value. The blue colour of the 3D surface and contour plots indicates the lower response of the TOTOX value. The synthetic antioxidants presented the lowest TOTOX value compared to natural antioxidants, proving the synthetic antioxidants combination is sufficient to suppress the oxidation of sardine oil.

The conclusion of synthetic antioxidant combination for retard the oxidation of sardine oil:

- The preliminary study proved that TBHQ, BHA, and BHT are effective antioxidants for synthetic antioxidants analysis. The combination minimizes the limited concentration of antioxidants and increases the antioxidant activity for a long duration.
- TBHQ, BHA, and BHT presented the main and interactive effects with each other in a full factorial design. All combinations expressed a significant *p*-value ($p<0.05$)
- TBHQ and BHT combination is selected to optimize the optimal concentration through Central composite design.
- TBHQ 0.18mM and BHT 0.18mM combination provides the lowest response of TOTOX value with significant ANOVA and by 3D-surface or contour plots.

CHAPTER – 5

KINETIC AND THERMODYNAMIC ANALYSES OF ENHANCED OXIDATIVE STABILITY AT OPTIMIZED VALUES OF SYNTHETIC ANTIOXIDANTS

CHAPTER 5

In this chapter, the stability of sardine oil is examined at varying temperatures, and kinetic and thermodynamic parameters are determined. This information is valuable for evaluating the efficacy of antioxidants in preventing the propagation of oxidative processes in sardine oil. Understanding oxidation of the oils in different circumstances makes it possible to develop better-quality formulations. The initial study showed that synthetic antioxidants are more effective in preventing oxidation. Using the response surface methodology (RSM) data generated earlier using Design Expert 11.1.2 software, desirability was predicted for different combinations of synthetic antioxidants. These combinations were analysed at different temperatures using kinetic and thermodynamic studies. A linear regression analysis of the natural logarithm of peroxide value versus time was conducted to determine the order of reactions for the formation of hydrogen peroxides. The Arrhenius equation was used to describe the oxidation process as a function of temperature. Additionally, a thermodynamic study is necessary to understand the characteristics of a system, such as whether it is endothermic or exothermic, exergonic or endergonic, and spontaneous or nonspontaneous.

5.1 Materials and Methods

5.1.1 Oxidation stability experiments

A combination of antioxidants was dissolved in ethanol and placed in amber glass vials. Once the solvent was evaporated using nitrogen gas, refined fish oil was added to each vial to achieve a final concentration of antioxidants. This concentration was based on research conducted by Chandrasekar et al. (2016), Maqsood and Benjakul (2010), and Hopia et al. (1996) to prevent any potential prooxidant effect. The samples were mixed for 15 minutes, stored at various temperatures (25, 35, and 45ºC), and exposed to atmospheric air for 40 days. The oxidation experiment was conducted in triplicate, with samples taken every 5th day to determine the peroxide value.

5.1.2 Peroxide value

Peroxide value was analysed using AOCS Cd 8b-90, AOCS (2009) standard method. Peroxide value measures the primary oxidation product, e.g., hydroperoxide concentration based on milliequivalent peroxide per 1000 grams of oil.

$$Peroxide\ value\ (meq\ of\ peroxide/1000\ of\ oil)\ = ((S-B)\times M\times 1000)/m$$

5.1.3 Kinetic and Thermodynamics experiments

Predicted five combinations of synthetic antioxidant (TBHQ and BHT) from RSM is analysed in sardine oil for understanding kinetic-thermodynamic approach was applied, at different temperatures (25, 35, 45 °C) for 40 days under darkness based on the experiment of peroxide value. This experiment is allowed to calculate:

i. Rate constant (k) can assumed as a constant allowing the usual integration of the empirical kinetic model's equations -:

$$zero-order\ kinetic\ model\ (n=0): C_0 - C = k\times t \quad \text{(5.1)}$$

$$first-order\ kinetic\ model\ (n=1): ln\left(\frac{C_0}{C}\right) = k\times t \quad \text{(5.2)}$$

$$second-order\ kinetic\ model\ (n=2): \frac{1}{C} - \frac{C}{C_0} = k\times t \quad \text{(5.3)}$$

ii. The activation energy E_a (KJmol^{-1}) and pre-exponential factor A (h^{-1}) calculated from the Arrhenius equation after linearization (i.e., natural logarithm of the reaction rate constant k, in days^{-1}, versus the inverse of the absolute temperature, in K^{-1}),

$$log_{10}(k, days^{-1}) = intercept_I + slope_I \times \left(\frac{1}{T}\right) \quad \text{(5.4)}$$

$$= log_{10}\,(A, h^{-1}) - \frac{E_a}{R}\times\left(\frac{1}{T}\right)$$

being equal to the product of the slope value by the universal gas constant (R = 8.314 J $mol^{-1}K^{-1}$) and the natural exponential (Euler number basis) of the intercept value, respectively;

$$A\ (h^{-1}) = \ 10^{intercept_I} \_\_\_\_\_ (5.5)$$

$$E_a\ (J\ mol^{-1}) = \ -R\ \times slope_I \_\_\_\_\_ (5.6)$$

The use of this approach assumes the lipid oxidation of oils in an excess of oxygen.

iii. The enthalpies (ΔH^{++}) and entropies (ΔS^{++}) of activation, calculated from the slope and intercept values of the linear regression line established between $log_{10}\left(\frac{1}{T}\right)$ versus the inverse of absolute temperature 1/T, in (K^{-1}), respectively;

$$log_{10}\left(\frac{k}{T}\right) = \ intercept_{II}\ +\ slope_{II}\ \times \left(\frac{1}{T}\right) \_\_\_\_\_ (5.7)$$

$$= \ log_{10}\left(\frac{k_B}{h}\right) + \left(\frac{\Delta S^{++}}{ln\ ln\ (10)\ \times R}\right) - \left(\frac{\Delta H^{++}}{ln\ ln\ (10)\ \times\ R}\right) \times \left(\frac{1}{T}\right)$$

Being k_B the Boltzman constant $k_B = (1.380658 \times 10^{-23} JK^{-1})$ and h the Planck's constant ($h = 1.8405765 \times 10^{-37} Jh = 4.413852585 \times 10^{-36} Jdays$), and so,

$$\Delta H^{++} = \ -slope_{II}\ \times ln\ ln\ (10)\ \times R \_\_\_\_\_ (5.8)$$

$$\Delta S^{++} = ln\ ln\ (10)\ \times R\ \times \left[intercept_{II} - \ log_{10}\left(\frac{k_B}{h}\right)\right] \_\_\_\_\_ 5.9$$

iv. Finally, the Gibbs free energy (ΔG^{++}, in $Jmol^{-1}$) of activation at a given temperature T can be calculated from:

$$\Delta G^{++} = \ \Delta H^{++} - T\ \times\ \Delta S^{++} \_\_\_\_\_ 5.10$$

where T is the absolute temperature (in Kelvin).

5.2 Result and discussion

5.2.1 Combination of synthetic antioxidants

The Table 5.1, is the combination of synthetic antioxidants that is suggested by the pervious central composite design of synthetic antioxidants in RSM studied of Design Expert 11.1.2 (Stat-Ease, Minneapolis, MN, USA) software on the desirability basis.

Table 5.1. Combination of synthetic antioxidant for kinetic-thermodynamics parameters analysis.

Antioxidant	SY1	SY2	SY3	SY4	SY5
TBHQ (mM)	0.187	0.258	0.115	0.250	0.126
BHT (mM)	0.159	0.261	0.069	0.078	0.251

5.2.2 Peroxide value analysis

The AOCS Cd 8b-90, AOCS (2009) standard method was utilized to analyse the Peroxide value (PV). PV is a measure of the primary oxidation product. The obtained data for hydrogen peroxide formation in oils expressed as PV versus time in days was plotted at various temperatures for 40 days under darkness, as shown in Figure 5.1. The sardine oil sample, with synthetic antioxidants, was analysed for peroxide value every 5^{th} day. The results indicated that at 25°C, lipid peroxidation begins at low values, while at higher temperatures, 35 and 45°C, peroxidation rates increase. These findings could be useful in studying the effects of temperature on lipid peroxidation, which can significantly impact food quality and shelf life.

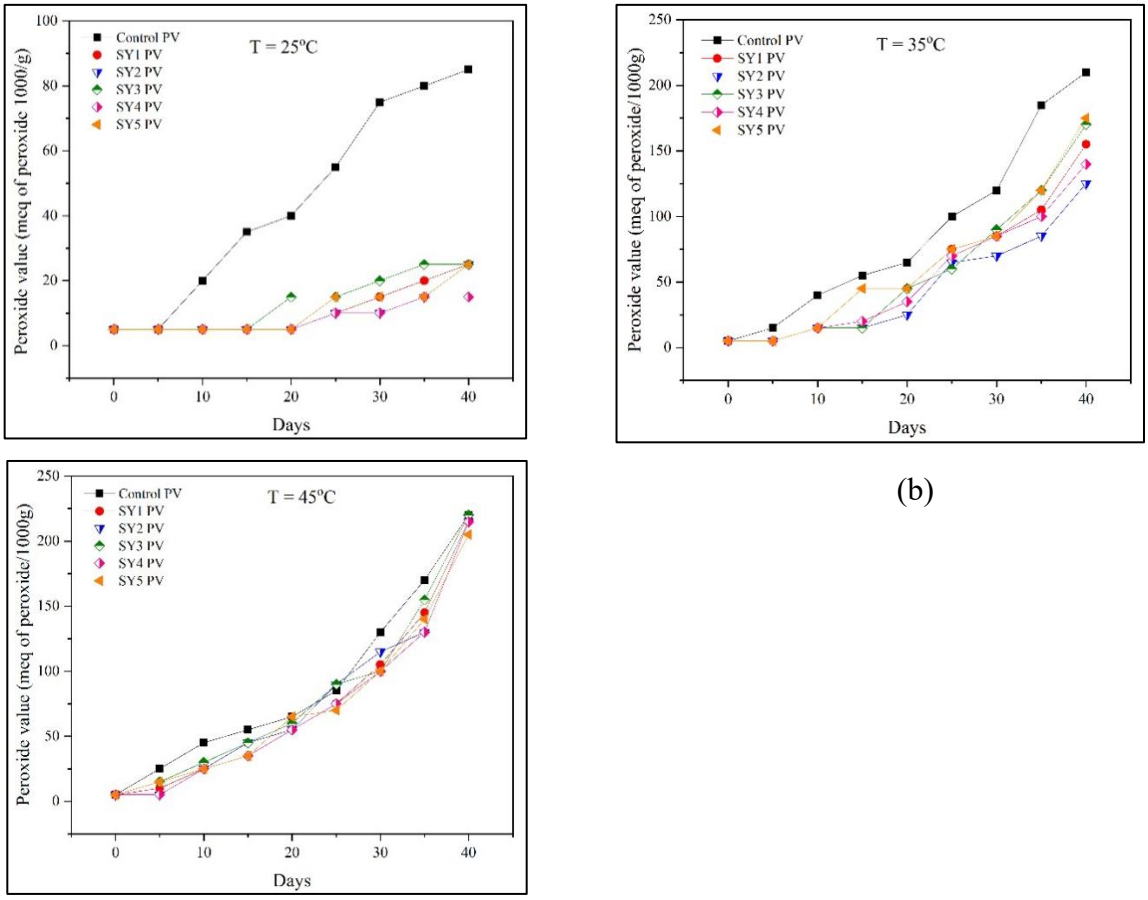


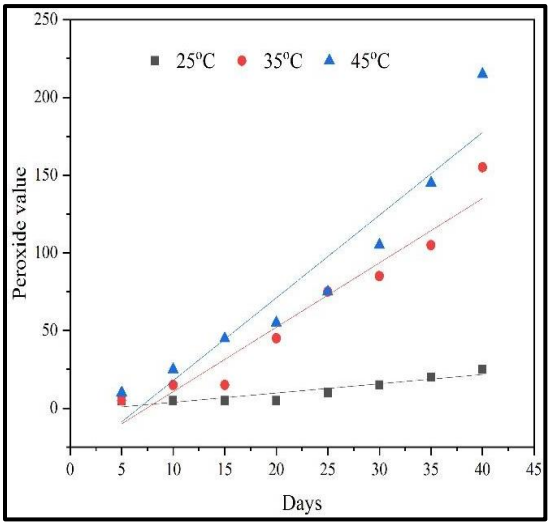
Figure 5.1 Peroxide values of samples incubated at (a) 25°C, (b)35°C and (c) 45°C, in the presence of synergistic mixture of TBHQ and BHT

5.2.3 Kinetic assessment

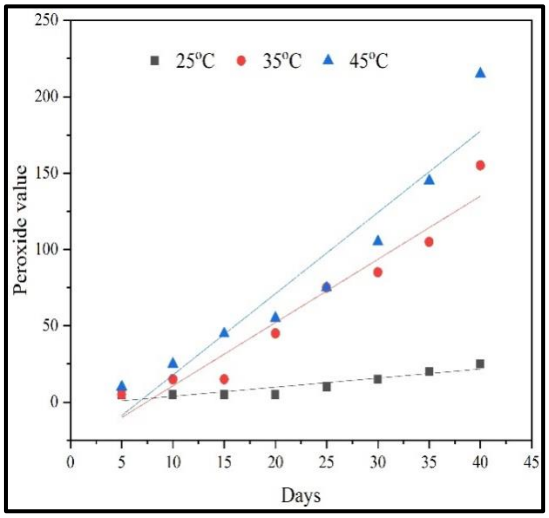
In order to determine the reaction order for hydrogen peroxide formation, the first step involved analysing the natural logarithm of peroxide values over time using linear regression. To categorize reactions that cause food quality loss, most are classified as zero, first, or second order. It is important to keep in mind that kinetic equations vary based on the specific food and temperature being studied.

To evaluate the oxidative stability of oils in the study, the researchers calculated kinetic parameters related to reaction kinetics. Their research showed that peroxide formation in the samples generally followed zero-order reaction kinetics. These findings align with Basturk et al. (2006) conclusion that oxygen consumption is a zero-order reaction in the process of binding oxygen to free radicals, leading to the creation of peroxide radicals and hydroperoxide.

Figure 5.2 displays kinetic models that demonstrate a zero-order reaction kinetic plot, indicating that the reaction rate isn't impacted by the reactant's concentration. This is due to the constant rates of these reactions, regardless of how much the reactant concentration increases or decreases. Such a reaction is referred to as a pseudo-zero-order reaction. However, it is important to note that a zero-order process cannot continue once a reactant has been exhausted. At this point, the reaction will revert to a different rate law instead of falling directly to zero. Table 5.2 provides details on zero-order reaction based on peroxide value.



(a)



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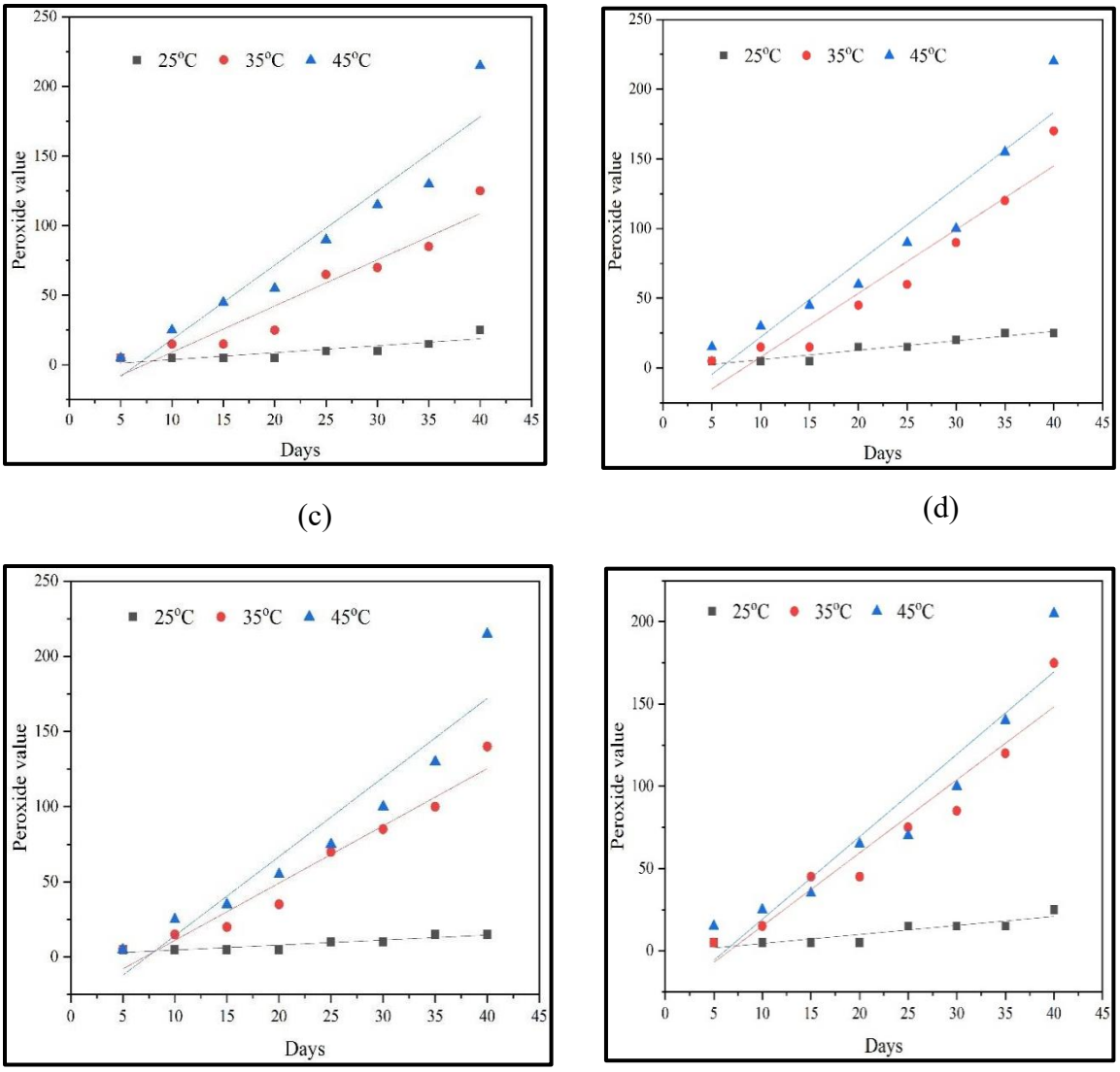


Figure 5.2 Zero order reaction graph of (a) control, and synthetic antioxidants combination (b) SY1, (c) SY2, (d) SY3 (e) SY4 (f) SY5 at various temperature 25°C, 35°C and 45°C.

Table 5.2 Regression equations PV versus time and coefficient of linear regression (R^2) considering zero order reaction rate kinetics.

Oil sample	Temperature (°C)	Intercept	Slope	R^2
Control	25	-3.92	2.36	0.97
	35	-25.53	5.52	0.94
	45	-20.35	5.32	0.91
SY1	25	-2.14	0.59	0.85
	35	-30.71	4.14	0.94
	45	-35.35	5.32	0.90
SY2	25	-1.20	0.5	0.75
	35	-24.10	3.32	0.92
	45	-35	5.33	0.96
SY3	25	-0.89	0.67	0.92
	35	-37.85	4.57	0.92
	45	-31.42	5.36	0.90
SY4	25	1.25	0.33	0.84
	35	-26.96	3.80	0.95
	45	-38.39	5.26	0.89
SY5	25	-1.07	0.54	0.81
	35	-29.28	4.40	0.92
	45	-30.89	5.01	0.90

Table 5.3 First order rate constants, frequency factors, activation energies, enthalpies, entropies of activation and free Gibbs energies of sardine oil with control and different combination of synthetic antioxidants.

Kinetic and Thermodynamic parameter	**Kinetic and Thermodynamic parameter of Synthetic Antioxidants**						***p*-value (ANOVA)**
	Control	SY1	SY2	SY3	SY4	SY5	
$k_{25°C}$	0.06933	0.05283	0.0465	0.05589	0.03799	0.05037	***0.00014***
$k_{35°C}$	0.06881	0.094	0.08758	0.09701	0.09011	0.08977	***0.00014***
$k_{45°C}$	0.05885	0.07885	0.09019	0.07098	0.09055	0.07177	***0.00014***
***p*-value (ANOVA)**	***0.99***	***0.99***	***0.99***	***0.99***	***0.99***	***0.99***	
R^2 of *k* at 25°C	0.81046	0.8787	0.85281	0.87868	0.85637	0.83147	
R^2 of *k* at 35°C	0.94151	0.92924	0.93911	0.95188	0.94157	0.89049	
R^2 of *k* at 45°C	0.98333	0.95495	0.87181	0.97604	0.8972	0.98375	
A (h^{-1})	0.00023	20.97	63.95×10^2	0.403	118.91×10^2	0.822	
Ea ($KJmol^{-1}$)	-2.77	7.085	11.546	4.342	15.157	6.301	
R^2 of log*k* vs. 1/T	**0.76135**	**0.48716**	**0.80539**	**0.20866**	**0.77748**	**0.3959**	
ΔH^{++} ($KJmol^{-1}$)	8.941	13.748	24.021	7.430	32.337	11.943	
ΔS^{++} ($J\ mol^{-1}\ K^{-1}$)	-338.62	-264.05	-230.91	-284.59	-204.41	-270.42	
R^2 of log(*k*/T) vs. 1/T	**0.85928**	**0.40136**	**0.77025**	**0.12653**	**0.74894**	**0.30626**	
$\Delta G_{25°C}^{++}$ $KJmol^{-1}$	109.9040124	92.47603031	92.86791779	92.28179365	93.28257092	92.57165708	***0.47***
$\Delta G_{35°C}^{++}$ $KJmol^{-1}$	113.2903028	95.11657213	95.17702641	95.1277326	95.32667927	95.27593399	***0.47***
$\Delta G_{45°C}^{++}$ $KJmol^{-1}$	116.6765933	97.75711394	97.48613503	97.97367156	97.37078761	97.9802109	***0.47***
***p*-value (ANOVA)**	***<0.0001***	***<0.0001***	***<0.0001***	***<0.0001***	***<0.0001***	***<0.0001***	

Table 5.4 Second order rate constants, frequency factors, activation energies, enthalpies, entropies of activation and free Gibbs energies of sardine oil with control and different combination of synthetic antioxidants.

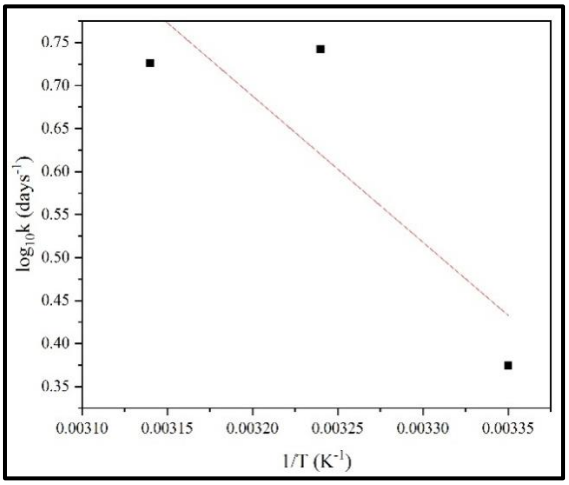
Kinetic and Thermodynamic parameter	**Kinetic and Thermodynamic parameter of Synthetic Antioxidants**						***p*-value (one-way ANOVA)**
	Control	SY1	SY2	SY3	SY4	SY5	
$k_{25°C}$	-0.00371	-0.00564	-0.00521	-0.00564	-0.00476	-0.00552	***0.0015***
$k_{35°C}$	-0.00135	-0.00432	-0.00429	-0.00434	-0.00420	-0.00403	***0.0015***
$k_{45°C}$	-0.00086	-0.00209	-0.00375	-0.00146	-0.00378	-0.00156	***0.0015***
p-value (one-way ANOVA)	***0.48779***	***0.48779***	***0.48779***	***0.48779***	***0.48779***	***0.48779***	
R^2 of *k* at 25°C	0.50494	0.85780	0.86363	0.80718	0.84034	0.80633	
R^2 of *k* at 35°C	0.65724	0.64939	0.67007	065629	0.62892	0.55839	
R^2 of *k* at 45°C	0.84375	0.65292	0.48523	0.75244	0.49948	0.81806	

Based on the analysis of three reaction orders, zero-, first-, and second-order kinetic models were considered to evaluate the degradation kinetics of sardine oil oxidation. The peroxide value versus time and a brief survey pointed out the zero-order kinetic model as the most suitable. The zero-order reaction kinetic plot shows that the concentration of the reactant has no impact on the reaction rate. This is because the reaction rates remain constant, regardless of the increase or decrease of the reactant concentration. These findings are consistent with Basturk et al.'s (2006) conclusion that oxygen consumption is a zero-order reaction in the process of binding oxygen to free radicals, leading to the formation of peroxide radicals and hydroperoxide. Therefore, the zero-order reaction will be further analysed.

The oxidation process can be described as a function of temperature using the Arrhenius equation. By applying Eq. 5.4-5.6, the temperature dependence of rate constant (k) and activation energy (E_a) for antioxidants were determined based on the slope of the lines (E_a/R) and pre-exponential factor (A) as intercept obtained. Figure 5.3 illustrates that the log$k$ versus 1/T obtained using the peroxide value test follows a linear relationship over the temperature range studied, with a regression coefficient (R^2).

Evaluating the effect of temperature on the oxidative degradation process of fish oil, the activation energy (E_a) was determined for the hydroperoxide formation of each treatment. E_a is a measure of the energy required to overcome the reaction barrier and is indicative of the ability of synthetic antioxidant combinations to stabilize fish oil. The control fish oil sample had an E_a of approximately 14.14 KJ/mol for hydroperoxide formation, while SY1 had 38.09 KJ/mol, SY2 had 41.05 KJ/mol, SY3 had 36.01 KJ/mol, SY4 had 47.98 KJ/mol, and SY5 had 38.57 KJ/mol. These results indicate that the synthetic antioxidant combination process rendered further oxidative stability of fish oil.

The pre-exponential factor reflects the number of collisions with sufficient kinetic energy required for successful reaction to happen. The SY1, SY3, and SY5 had 7.12×10^{13} h^{-1}, 1.1×10^{13} h^{-1}, and 1.04×10^{14} h^{-1}. A higher value of the pre-exponential factor signifies a lower probability of successful collisions that cause a chemical change. In the present study, SY4 had 4.08×10^{17} h^{-1}, and SY2 had an 8.95×10^{14} h^{-1} pre-exponential factor, which has a lower probability of successful collisions compared to the control (Control sample had a pre-exponential factor of 5.64×10^{4} h^{-1}), as expressed in Table 5.3.



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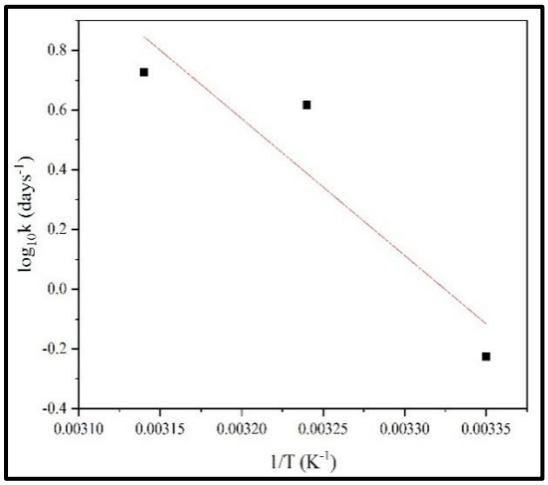

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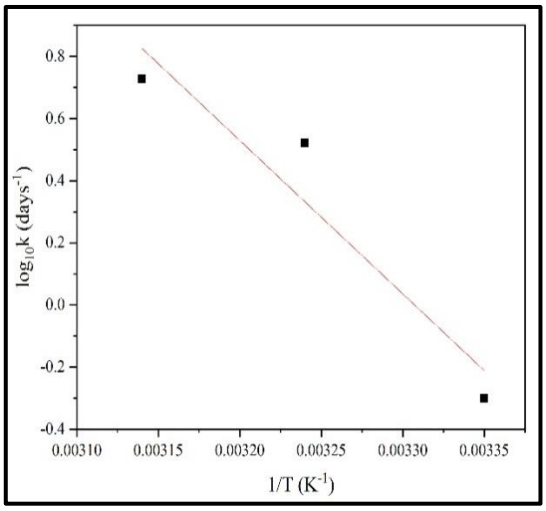

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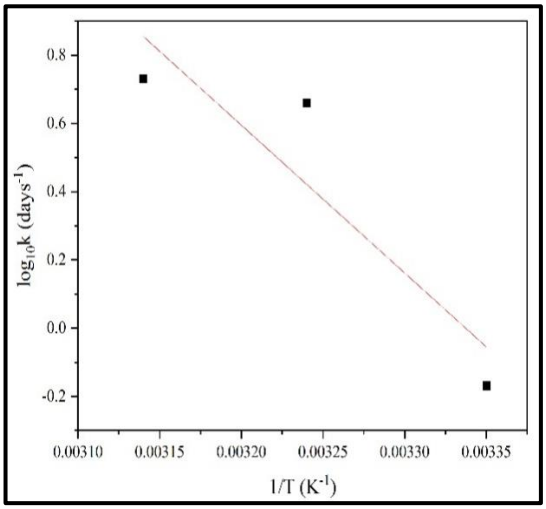

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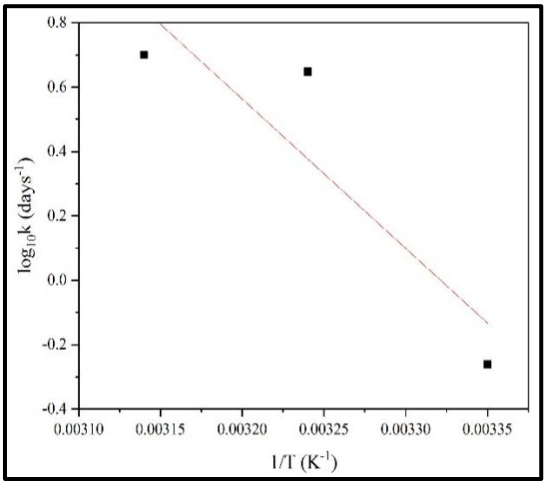

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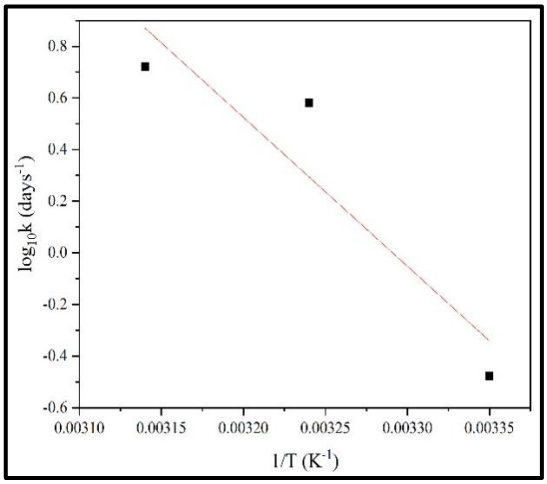


(f)

	Control	SY1	SY2	SY3	SY4	SY5
Intercept	6.13	15.23	16.33	14.45	18.99	15.41
Slope	-1701.32	-4582.18	-4938.25	-4331.69	-5771.04	-4639.90
R^2	0.73	0.85	0.90	0.82	0.85	0.81
Adj R^2	0.47	0.70	0.81	0.65	0.71	0.62

Figure 5.3 Effect of temperature on the rate constant of peroxide formation in sardine oil with (a) Control, and synthetic antioxidant combination (b) SY1, (c) SY2, (d) SY3, (e) SY4, (f) SY5.

Table 5.5 Zero order kinetic parameters for the oxidation process occurred in sardine oil at different temperatures.

Oil sample	$k_{pv\ 25°C}$ ($days^{-1}$)	$k_{pv\ 35°C}$ ($days^{-1}$)	$k_{pv\ 45°C}$ ($days^{-1}$)	E_a (KJ/mol)	A (h^{-1})
Control	2.369	5.523	5.321	14.14	5.64×10^{4}
SY1	0.595	4.142	5.321	38.09	7.12×10^{13}
SY2	0.500	3.321	5.333	41.05	8.95×10^{14}
SY3	0.678	4.571	5.369	36.01	1.1×10^{13}
SY4	0.333	3.809	5.261	47.98	4.08×10^{17}
SY5	0.547	4.440	5.011	38.57	1.04×10^{14}

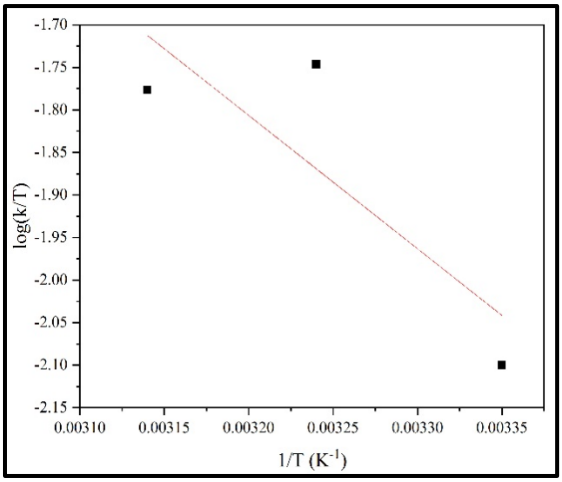
5.2.4 Thermodynamic assessment

To develop high-quality formulations, it is essential to understand the oxidation reaction in the related product and predict lipid oxidation under different conditions. A detailed system characteristic, including the oxidation process, is necessary to determine whether it's endothermic/exothermic, exergonic/endergonic, and spontaneous/non-spontaneous. The Eyring equation derived from Activated Complex Theory was used to calculate Enthalpy (ΔH^{++}) and Entropy (ΔS^{++}) changes. In contrast, the fundamental thermodynamics equation (Eq. 5.7-5.10) was utilized to determine Gibbs free energy (ΔG^{++}) of activation for all temperatures.

The ΔH^{++} represents the difference in energy between the ground state and the transition state in a chemical reaction. A higher activation enthalpy indicates that more energy is required for the products to form in an activated state. The entropy (ΔS^{++}) change shows the increase in randomness after the adsorption is completed, resulting from the change in energy distribution. Exothermic reactions release energy as heat, leading to a rise in temperature, while endothermic reactions absorb energy from the surroundings in the form of heat. Endergonic reactions absorb energy from the surroundings, whereas exergonic

reactions release energy to the surroundings. Spontaneous reactions proceed without energy input, and the reaction occurs slowly with negative ΔG^{++}. Non-spontaneous reactions require energy input, and the reaction occurs with positive ΔG^{++}. The outcome of the thermodynamic study is presented in Figure 5.4.

According to Table 5.4 in this study, the trials involving combinations of synthetic antioxidants, namely SY1, SY3, SY5, and the control sample, exhibited positive enthalpy ($\Delta H^{++} > 0$), negative entropy ($\Delta S^{++} < 0$), and positive Gibb's energy (ΔG^{++}), signifying that the reaction was endothermic, endergonic, and non-spontaneous at all temperatures. These combinations of synthetic antioxidants had a synergistic effect, effectively suppressing hydroperoxide formation at all temperatures. On the other hand, trials SY2 and SY4 exhibited positive enthalpy ($\Delta H^{++} > 0$), positive entropy ($\Delta S^{++} < 0$), and positive Gibb's energy (ΔG^{++}), indicating that the reaction was endothermic, endergonic, and non-spontaneous at low temperatures. Nevertheless, the synergistic effect of SY2 and SY4 against hydroperoxide formation was still effective at low temperatures.



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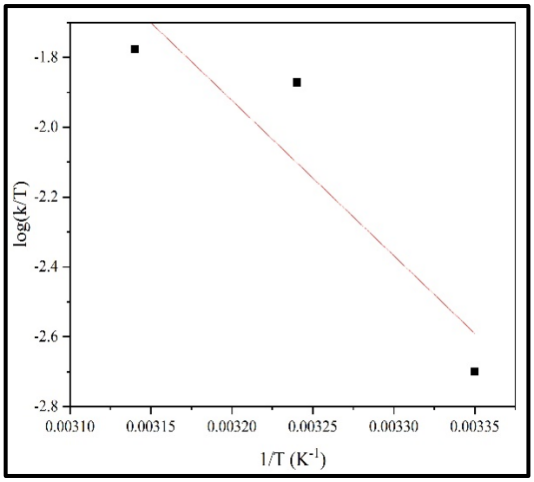

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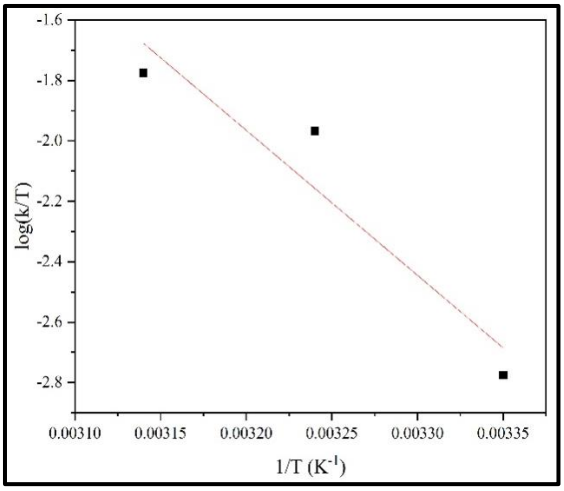

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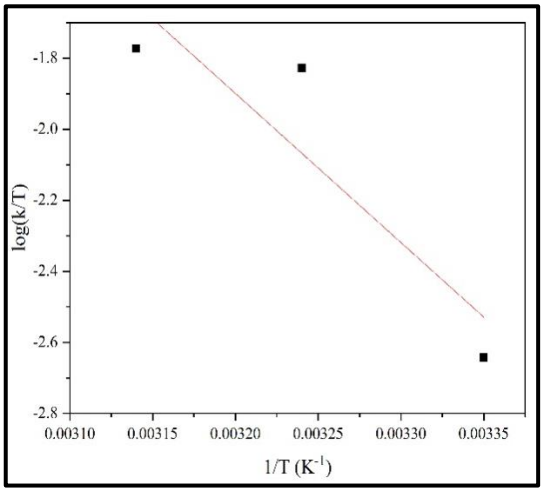

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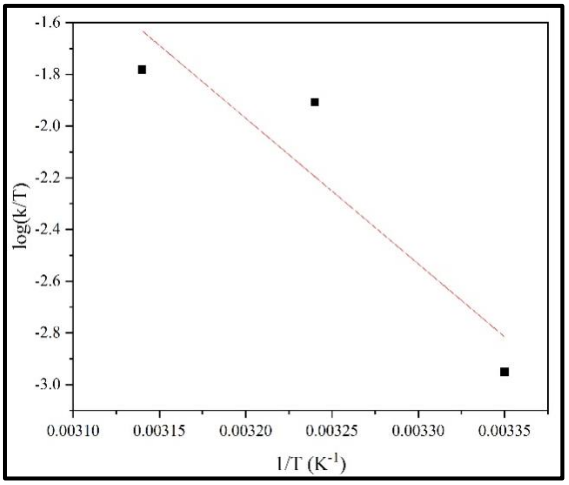

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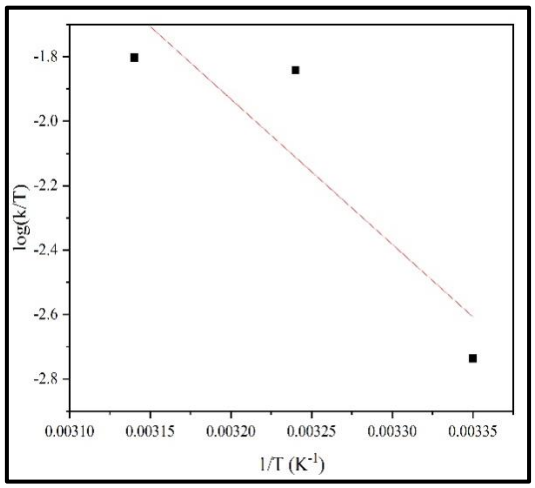


(f)

	Control	SY1	SY2	SY3	SY4	SY5
Intercept	3.20	12.31	13.40	11.53	16.06	12.48
Slope	-1567.11	-4447.97	-4804.05	-4197.49	-5636.84	-4505.70
R^2	0.70	0.84	0.90	0.81	0.85	0.80
Adj R^2	0.41	0.69	0.80	0.63	0.70	0.60

Figure 5.4 Thermodynamic effect for the oxidation process occurred in sardine oil at different temperatures with (a) control, and synthetic antioxidant combination (b) SY1, (c) SY2, (d) SY3, (e) SY4, (f) SY5.

Table 5.6 Thermodynamic parameters for the oxidation process occurred sardine oil at different temperatures.

Oil sample Temp. (°C)	**ΔH^{++} (KJmol^{-1})**	**ΔS^{++} (J mol^{-1} K^{-1})**	**ΔG^{++} (KJmol^{-1})**	
Control				
25	30.005	-177.81	83.022	non-spontaneous at all temperature
35			84.800	
45			86.578	
SY1				
25	85.16	-3.54	86.221	non-spontaneous at all temperature
35			86.257	
45			86.292	
SY2				
25	91.98	17.48	86.771	non-spontaneous at low temperature
35			86.596	
45			86.421	
SY3				
25	80.37	-18.43	85.866	non-spontaneous at all temperature
35			86.050	
45			86.235	
SY4				
25	107.92	68.41	87.530	non-spontaneous at low temperature
35			86.846	
45			86.162	
SY5				
25	86.27	-0.160	86.319	non-spontaneous at all temperature
35			86.320	
45			86.322	

5.3 Discussion

When it comes to suppressing oxidation, even a limited concentration of synthetic antioxidants can be effective. In fact, using a combination of synthetic antioxidants can have a synergistic effect and maintain the oxidation stability of sardine oil even at lower concentrations. To better understand lipid oxidation in relevant products and develop higher-quality formulations, it is important to consider kinetic and thermodynamic parameters. To test the effectiveness of synthetic antioxidants, we analysed their combination at temperatures of 25, 35, and 45°C over a period of 40 days under darkness.

A thorough analysis of various reaction orders has concluded that sardine oil oxidation degradation kinetics are best evaluated using the zero-order kinetic model. A peroxide value confirmed this determination versus time analysis and a brief survey. The zero-order reaction kinetic plot shows a constant reaction rate independent of the reactant concentration, indicating that the reaction rate is not affected by the concentration of the reactant.

The evaluated effect of temperature on the oxidative degradation process of fish oil showed that the synthetic antioxidant combination process rendered further oxidative stability. The results showed that the control fish oil sample had an activation energy (E_a) of approximately 14.14 KJ/mol for hydroperoxide formation, while SY1 had 38.09 KJ/mol, SY2 had 41.05 KJ/mol, SY3 had 36.01 KJ/mol, SY4 had 47.98 KJ/mol, and SY5 had 38.57 KJ/mol.

The pre-exponential factor, which reflects the number of collisions with sufficient kinetic energy required for the successful reaction, was also measured. SY1, SY3, and SY5 had 7.12×1013 h^{-1}, 1.1×1013 h^{-1}, and 1.04×1014 h^{-1} respectively. A higher value of the pre-exponential factor signifies a lower probability of successful collisions that cause a chemical change. In contrast, SY4 had 4.08×1017 h^{-1}, and SY2 had an 8.95×1014 h^{-1} pre-exponential factor, which has a lower probability of successful collisions compared to the control (Control sample had a pre-exponential factor of 5.64×104 h^{-1}).

In previous studies, Tan et al. (2001) discovered that kinetics and activation energy for oxidation of various vegetable oils varied between 79 and 104 KJ mol^{-1}, leading to differences in their fatty acid profiles. Souza et al. (2004) found that the activation energy for sunflower oil oxidation increased with the amount of synthetic antioxidants added. Moreover, Aktar and Adal (2019) presented the pre-exponential value of 5.8×1012 h^{-1} for avocado oil oxidation under isothermal conditions. Tan et al. (2001) also noted that even slight changes in activation energy values could significantly modify pre-exponential factors. Yeşilsu and Özyurt (2019) examined the oxidative stability of microencapsulated fish oil in the presence of rosemary, thyme, and laurel extracts with BHT as a control. The results showed that the rosemary extracts effectively suppressed oxidation, with an activation energy of 31.62 KJ/mol, and BHT, with a 30.46 KJ/mol sample activation energy. These findings were supported by Moomand and Lim's (2014) research, which found that the encapsulation process significantly enhanced the oxidative stability of fish oil. Specifically, the activation energy of hydroperoxide formation reaction in the encapsulated fish oil with electrospun zein fibers (Z3 and Z6) was 11.76 KJ/mol and 12.56 KJ/mol, compared to 10.95 KJ/mol in unencapsulated fish oil. Finally, Mihaylova et al. (2020) reported that ascorbyl palmitate suppresses echium oil oxidation, with an activation energy of 54.74 KJ/mol and 2.36×10^{9} h^{-1}.

According to the thermodynamic study of sardine oil, the SY1, SY3, and SY5 samples indicate that the reaction nature is endothermic, endergonic, and non-spontaneous at all temperatures, with positive $\Delta H^{++,}$ negative ΔS^{++}, and positive ΔG^{++}. Meanwhile, the SY2 and SY4 samples represent the same reaction nature at low temperatures, with positive ΔH^{++}, positive ΔS^{++}, and positive ΔG^{++}. Kurtulbaş et al. (2018) reported that cottonseed oil treated with various antioxidants was found non-spontaneous. Mihaylova et al. (2020) mentioned the echium oil analysed with several antioxidants was non-spontaneous, exothermic, and endergonic. Elhussein et al. (2018) reported the sesame oil was oxidized under the Rancimat test condition, indicating a non-spontaneous reaction. Gülmez, and Şahin (2019), expressed that hazelnut oil treated with various antioxidants under the Rancimat condition indicates a non-spontaneous reaction. Santos et al. (2012) showed that

the TBHQ is heat resistance commercial synthetic antioxidants as compared to BHT. TBHQ generally being sufficient to increase the durability of all fat species. These findings are consistent with a report which states that TBHQ is effective in increasing the durability of all fat species. Furthermore, *Oleum hyperici* (St. John's Wort oil) was analysed for its oxidative stability under the Rancimat condition and presented a non-spontaneous reaction, as reported by Şahin et al. (2020).

5.4 Summary and conclusion

Synthetic antioxidants have been found to effectively delay the oxidation of sardine oil at varying temperatures over an extended period of time. To create superior-quality formulations and understand lipid oxidation in relevant products, it is necessary to determine kinetic and thermodynamic parameters for fish oil oxidation under different conditions. The zero-order reaction is used for the kinetic analysis of sardine oil with and without antioxidants. By evaluating the activation energy (E_a), it becomes possible to determine the impact of temperature on the oxidative degradation process of fish oil. Synthetic antioxidant trials SY4 (with an activation energy of 47.98 KJ/mol) and SY2 (with an activation energy of 41.05 KJ/mol) have been shown to have sufficient energy to inhibit hydroperoxide formation. The pre-exponential factor illustrates the fraction of collisions with enough kinetic energy to trigger a reaction. A higher pre-exponential factor indicates a greater likelihood of successful collisions that cause a chemical change. The pre-exponential factor reflects the number of collisions with sufficient kinetic energy required for successful reaction to happen. A higher value of the pre-exponential factor signifies a lower probability of successful collisions that cause a chemical change. In the present study, SY4 had 4.08×10^{17} h^{-1}, and SY2 had a 8.95×10^{14} h^{-1} pre-exponential factor, which has a lower probability of successful collisions compared to the control (Control sample had a pre-exponential factor of 5.64×10^{4} h^{-1}).

Thermodynamic evaluation help to develop high-quality formulations for which it is essential to understand the oxidation reaction in the related product and predict lipid oxidation under different conditions. Developing the trials involving combinations of

synthetic antioxidants, namely SY1, SY3, SY5, and the control sample, exhibited positive enthalpy change ($\Delta H^{++} > 0$), negative entropy change ($\Delta S^{++} < 0$), and positive Gibb's energy change (ΔG^{++}), signifying that the reaction was endothermic, endergonic, and non-spontaneous at all temperatures. These combinations of synthetic antioxidants had a synergistic effect, effectively suppressing hydroperoxide formation at all temperatures. On the other hand, trials SY2 and SY4 exhibited positive change in enthalpy ($\Delta H^{++} > 0$), positive change in entropy ($\Delta S^{++} < 0$), and positive Gibb's energy (ΔG^{++}), indicating that the reaction was endothermic, endergonic, and non-spontaneous at low temperatures. Nevertheless, the synergistic effect of SY2 and SY4 against hydroperoxide formation was still effective at low temperatures.

CHAPTER – 6

SUMMARY AND CONCLUSIONS

CHAPTER 6

6.1 Summary

- Natural antioxidants such as catechin, caffeic acid, sinapic acid, and Resveratrol have proved to be the better performers among the ten chosen natural antioxidants, in one-factor-at-a-time study.
- TBHQ, BHA, and BHT were the most effective antioxidants among all the thirteen antioxidants studied, over an extended period of 50 days storage.
- The statistical study on natural antioxidants revealed an interactive effect of catechin-resveratrol, while TBHQ, BHA, and BHT displayed an interactive effect among the synthetic antioxidants.
- When compared to the natural and synthetic antioxidants, it is noteworthy to mention that synergistic mixtures of catechin and resveratrol are far inferior compared to the synergistic mixture of TBHQ and BHT. The sardine oil samples showed a TOTOX value of 218.93 for optimal natural antioxidant mixture, whereas a mere 13.29 was recorded with the synergistic mixture of synthetic antioxidants.
- Kinetic analyses revealed that the oil decomposition follows a zero-order reaction irrespective of the presence /absence of antioxidants, their concentrations and storage temperatures. The thermodynamic analysis revealed that the formation of peroxides in the sardine oil is an endothermic, endergonic and a non-spontaneous reaction at ambient temperature.
- The synergistic mixture of synthetic antioxidants having only 0.36mM concentration demonstrated very high effectiveness in slowing down oxidation compared to the individual antioxidants used at the normally accepted concentration of 0.9mM.

6.2 Significant findings

- A one-factor-at-a-time analysis found that catechin exhibited a more significant response against oxidation compared to BHA until the 40^{th} day.

- Through the statistical study conducted on natural antioxidants, it was discovered that catechin-resveratrol has an interactive effect. On the other hand, TBHQ, BHA, and BHT displayed an interactive effect among the synthetic antioxidants.
- The use of a synergistic mixture of synthetic antioxidants with a concentration of only 0.36mM/69.6ppm (TBHQ 0.18mM/29.9ppm + BHT 0.18mM/39.6ppm) has shown remarkable effectiveness in slowing down oxidation, outperforming the individual antioxidants used at the normally accepted concentration of 0.9mM.

6.3 Conclusions

Synergistic mixtures of natural and synthetic antioxidants were designed by the effective use of one-factor-at-a-time experiments, Full Factorial Design and RSM sequentially. Further, it was demonstrated that natural antioxidants can exhibit pro-oxidant effect when they are applied at higher dosage. Kinetic and thermodynamic studies revealed that initiation of oxidation reaction can be suppressed effectively by the synergistic mixtures of TBHQ and BHT.

6.4 The scope for future work

- Various natural combinations that possess metal-chelating activity have been studied for their oxidation stability. These combinations effectively suppress the initial oxidation stage, which in turn decreases hydroperoxide formation.
- Analysing natural antioxidant combinations alongside synthetic antioxidants can lead to a reduction in the standard concentration of synthetic antioxidants found in food and pharmaceutical industries.
- Reduce the standard concentration of synthetic antioxidants with the minimum amount of synthetic antioxidant combinations that can combat oxidation effectively.
- The combination of esterified antioxidants may have the potential to enhance the suppression of oxidation through increased antioxidant activity.

APPENDICES

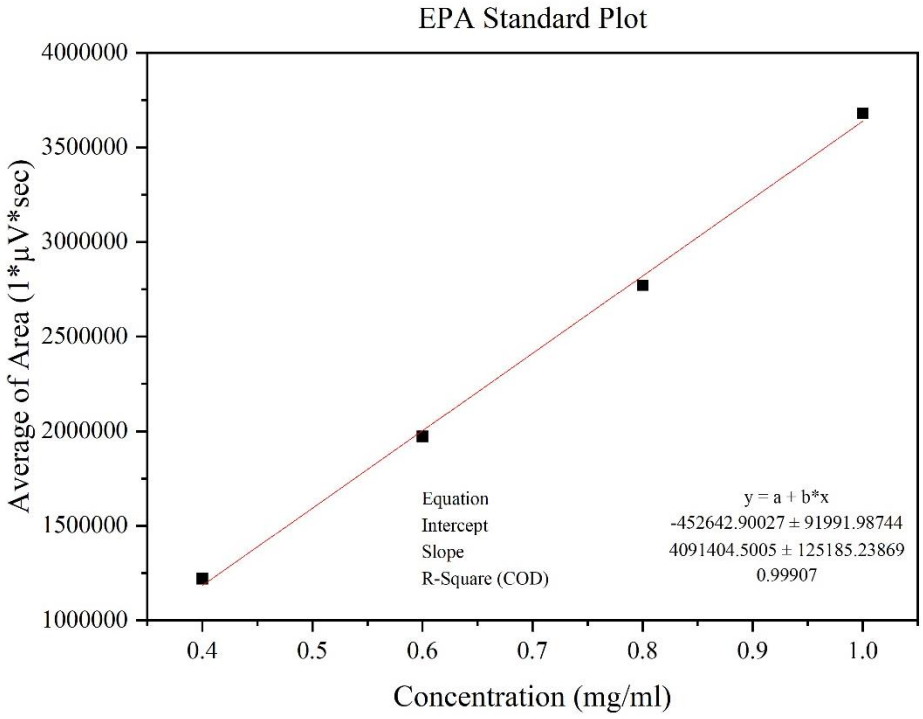
Appendix I

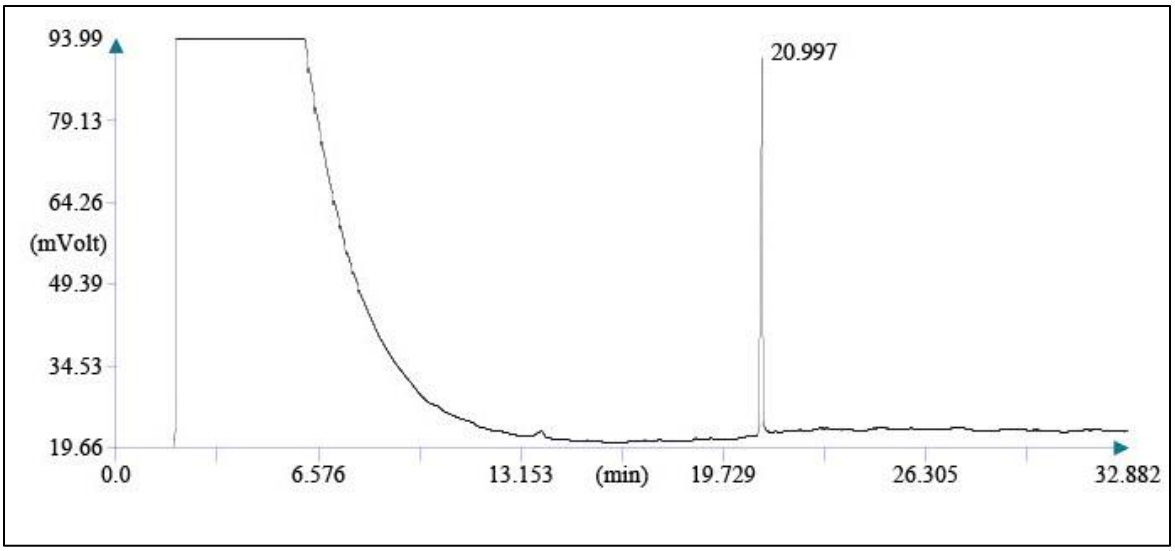
Sardine Fish

Scientific Name	*Sardinella longiceps*
Phylum	Chordata
Class	Actinopterygii
Order	Clupeiformes
Family	Clupeidae
Environment	Deep water (Benthopelagic)
Depth range (meter)	20-200

Appendix II

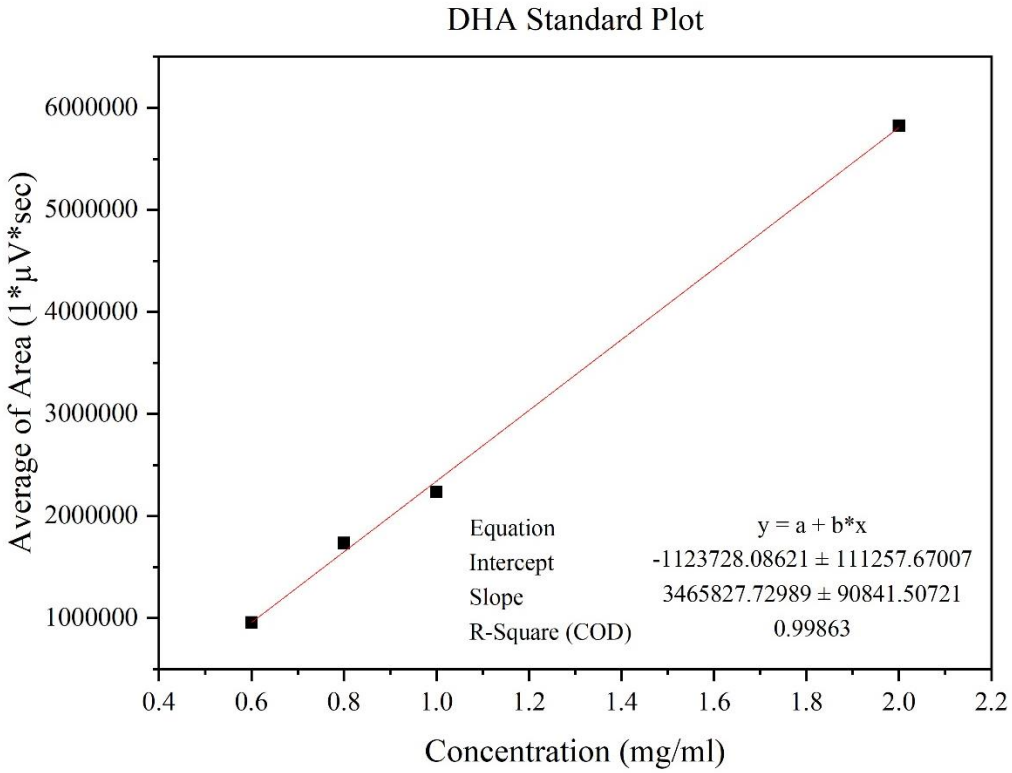
EPA methyl ester standard plot and peak

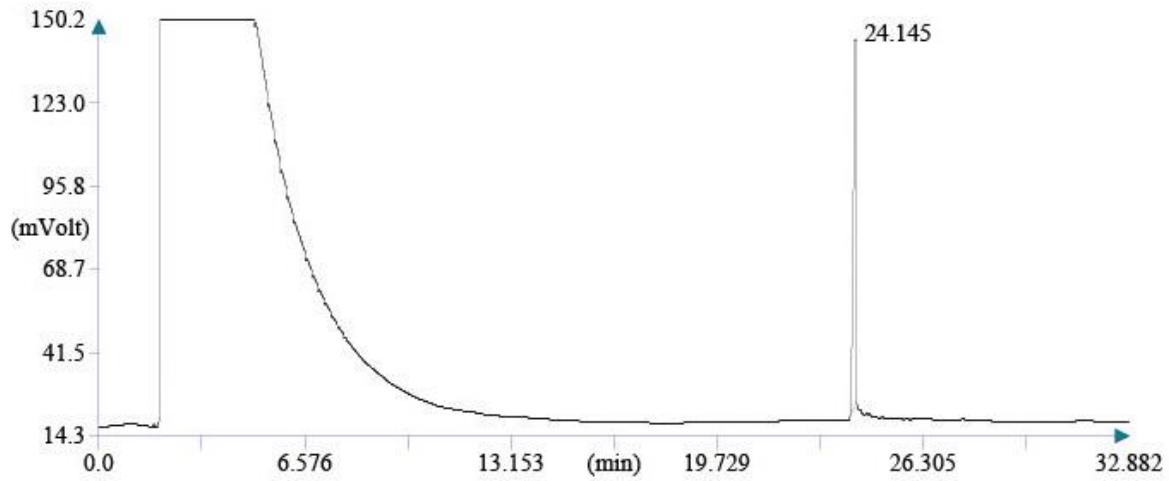




Appendix III

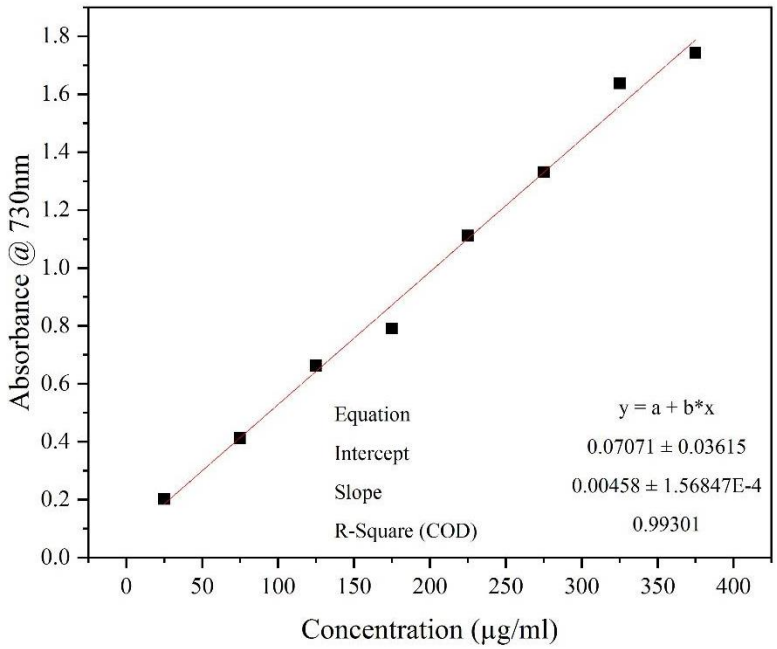
DHA methyl ester standard plot and peak



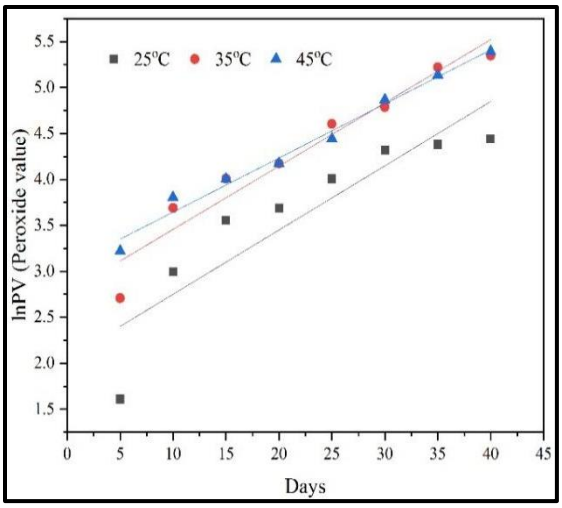


Appendix IV

Phospholipid standard plot



Appendix V - First order kinetic plot



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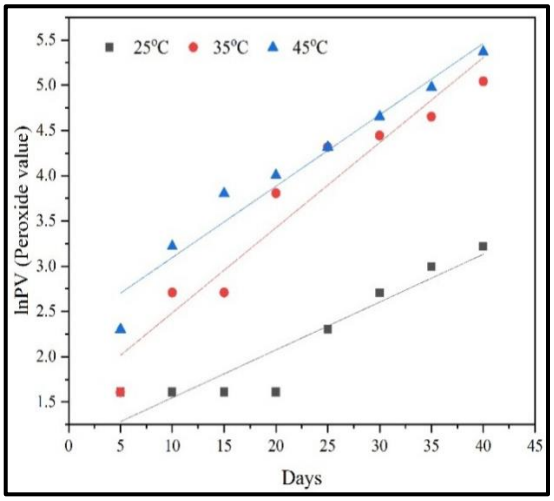

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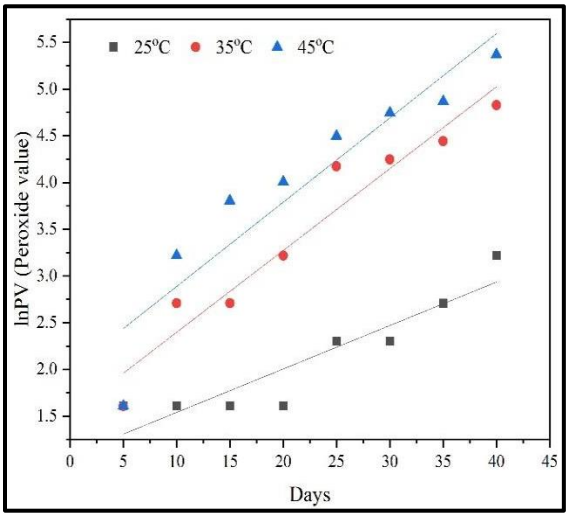

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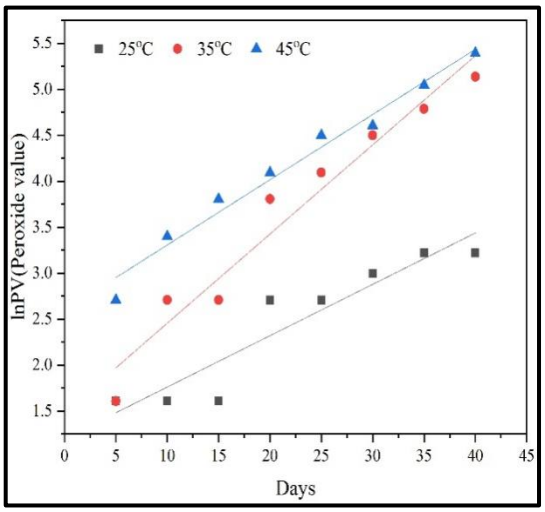

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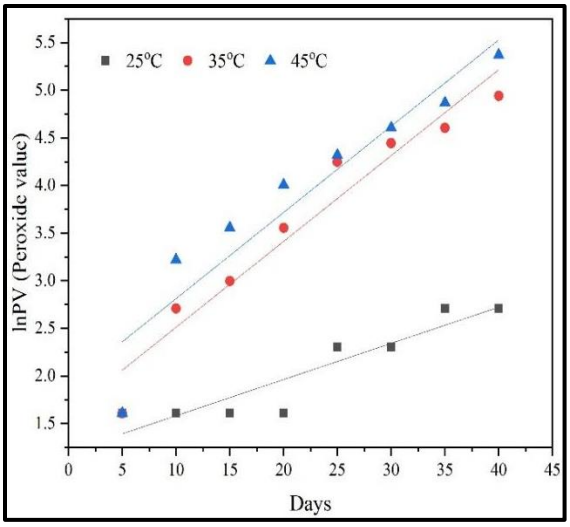

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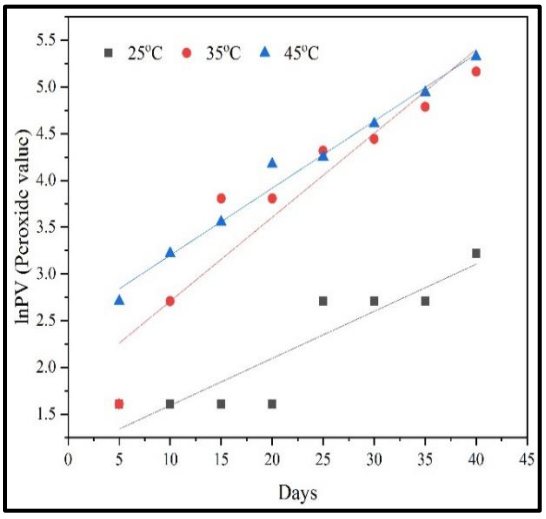


(f)

First order reaction graph of (a) Control, and synthetic antioxidants combination (b) SY1, (c) SY2, (d) SY3 (e) SY4 (f) SY5 at various temperature 25°C, 35°C and 45°C.

Appendix - VI

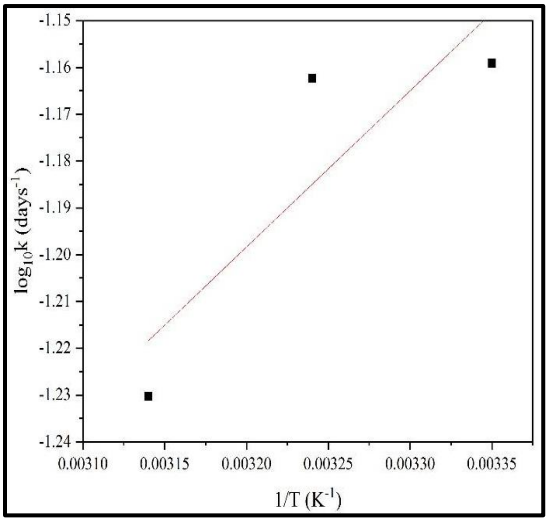
Table -: Regression equations lnPV versus time and coefficient of linear regression (R^2) considering first order reaction rate kinetics.

Oil sample	Temperature (°C)	Intercept	Slope	R^2
Control	25	2.05	0.06	0.81
	35	2.76	0.06	0.94
	45	3.05	0.05	0.98
SY1	25	1.01	0.05	0.87
	35	1.54	0.09	0.92
	45	2.30	0.07	0.95
SY2	25	1.07	0.04	0.85
	35	1.52	0.08	0.93
	45	1.98	0.09	0.87

SY3	25	1.20	0.05	0.87
	35	1.48	0.09	0.95
	45	2.59	0.07	0.97
SY4	25	1.20	0.03	0.85
	35	1.61	0.09	0.94
	45	1.90	0.09	0.89
SY5	25	1.08	0.05	0.83
	35	1.81	0.08	0.89
	45	2.48	0.07	0.98

Appendix – VII

Arrhenius equation plots of first order kinetic



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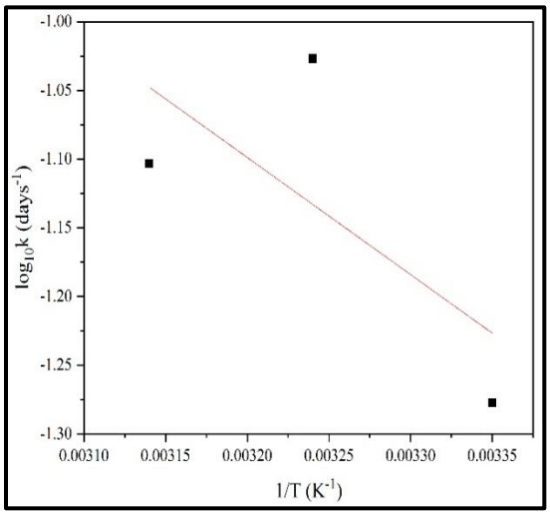

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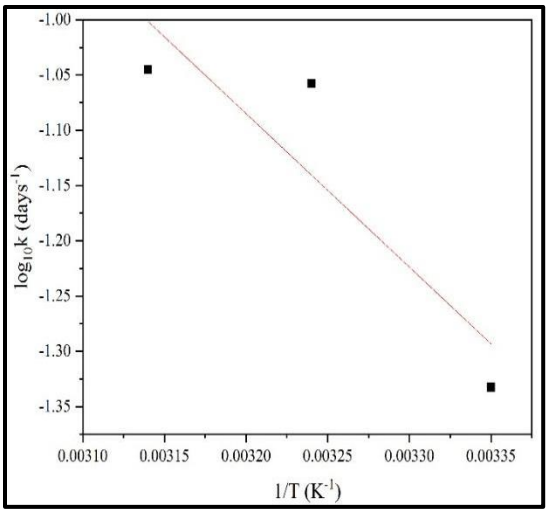

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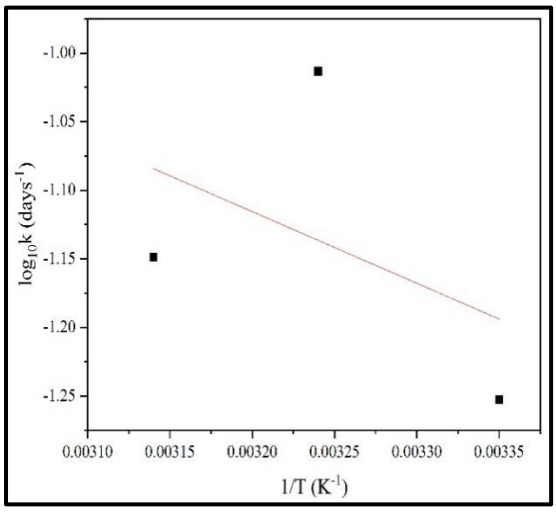

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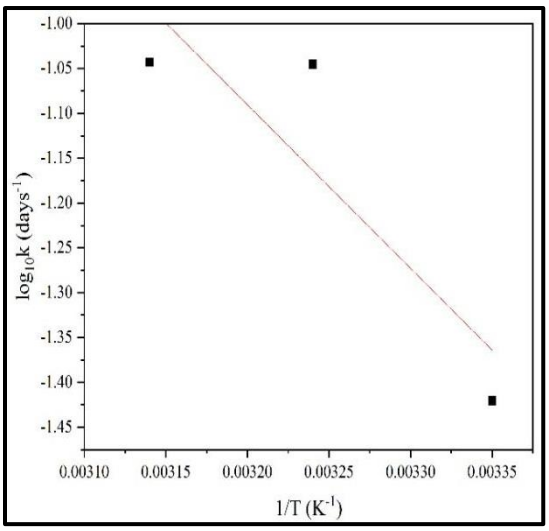

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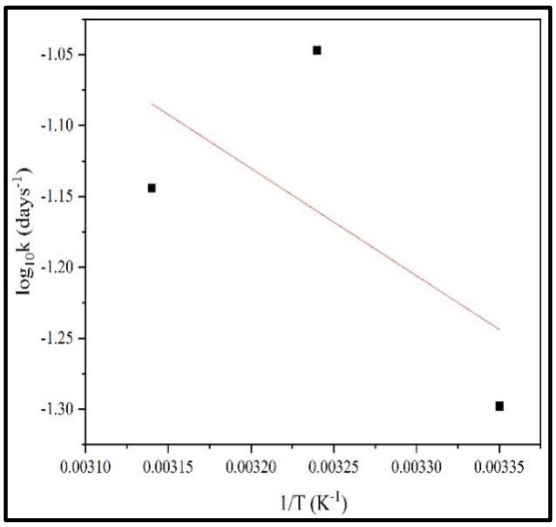


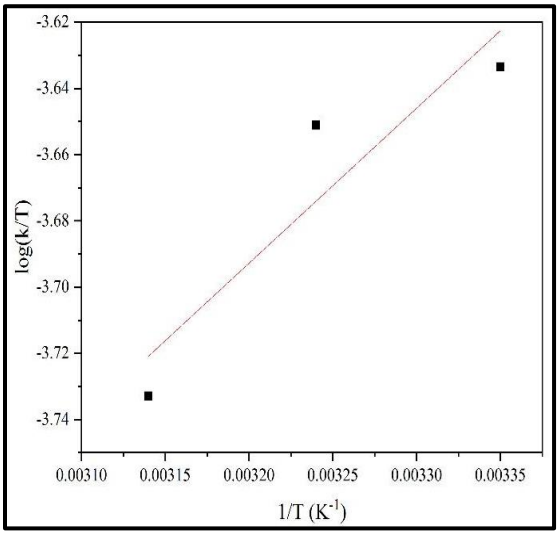
(f)

	Control	SY1	SY2	SY3	SY4	SY5
Intercept	-2.26	1.62	3.35	0.55	4.74	1.29
Slope	333.78	-852.23	-1388.79	-522.25	-1823.09	-757.98
R^2	0.76	-0.48	0.80	0.20	0.77	0.39
Adj R^2	0.52	-0.02	0.61	-0.58	0.55	-0.20

Figure -: Effect of temperature on the rate constant of peroxide formation in sardine oil with (a) Control, and synthetic antioxidant combination (b) SY1, (c) SY2, (d) SY3, (e) SY4, (f) SY5.

Appendix VIII

Thermodynamic plots of first order kinetic



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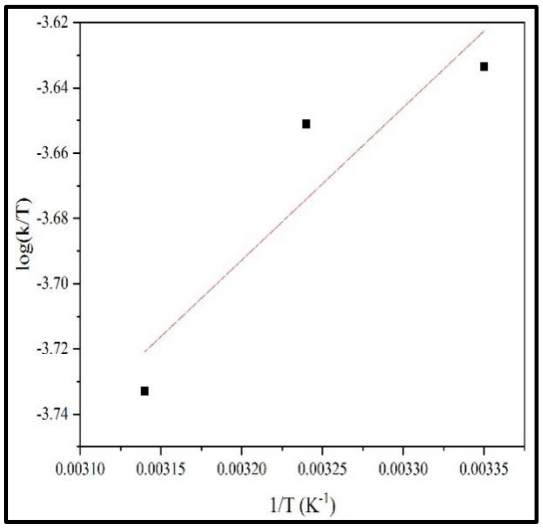

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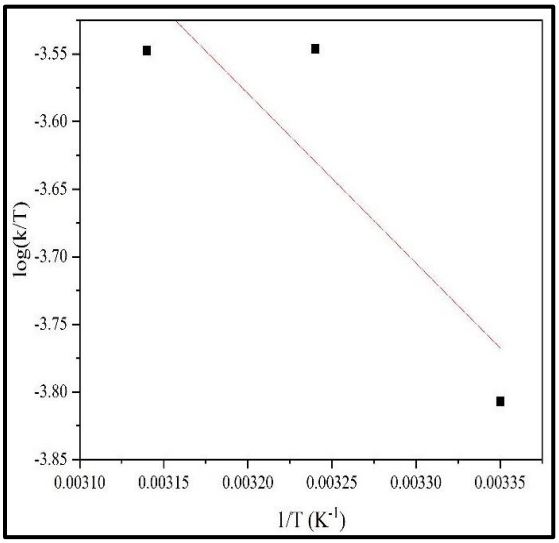

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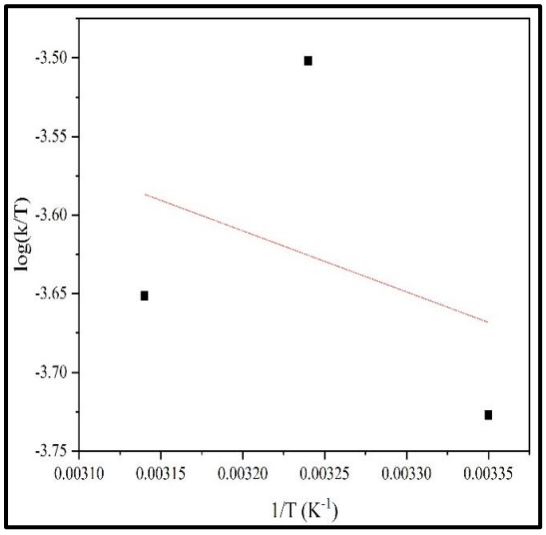

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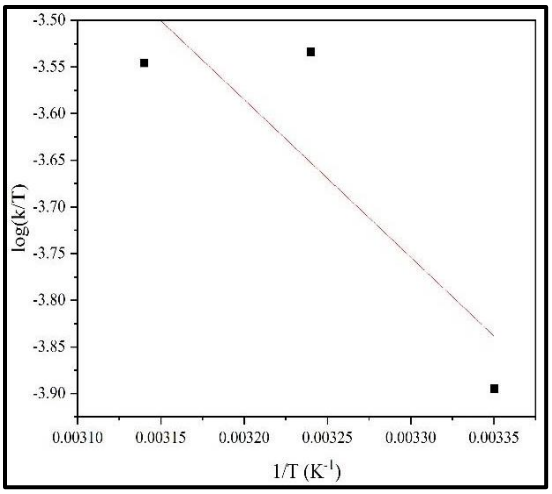

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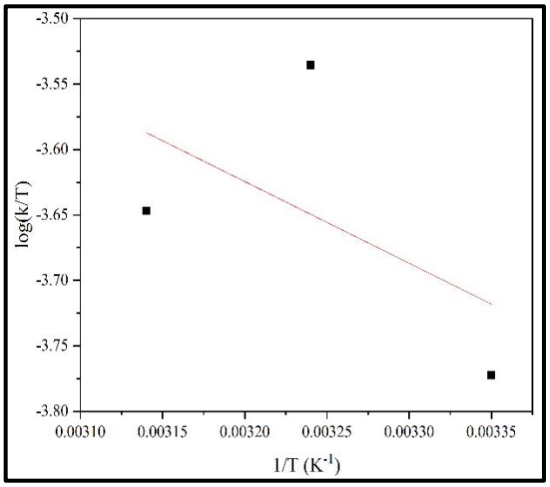
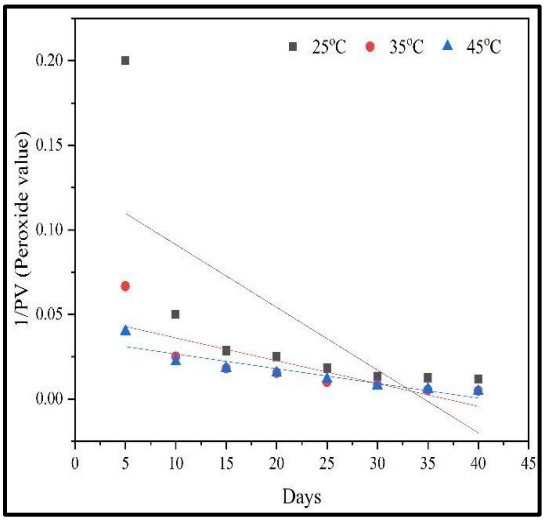


(f)

	Control	SY1	SY2	SY3	SY4	SY5
Intercept	-5.19	-1.29	0.43	-2.36	1.81	-1.62
Slope	467.99	-718.03	-1254.59	-388.05	-1688.89	-623.78
R^2	0.85	0.40	0.77	0.12	0.74	-0.30
Adj R^2	0.71	-0.19	0.54	-0.74	0.49	-0.38

Figure -: Thermodynamic effect for the oxidation process occurred in sardine oil at different temperatures with (a) Control, and synthetic antioxidant combination (b) SY1, (c) SY2, (d) SY3, (e) SY4, (f) SY5.

Appendix IX – Second order kinetic



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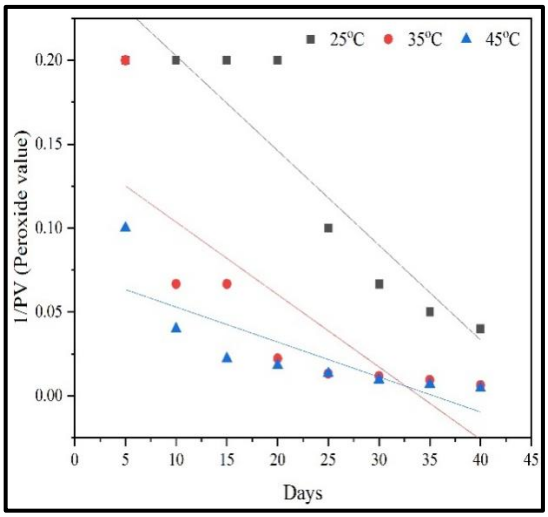

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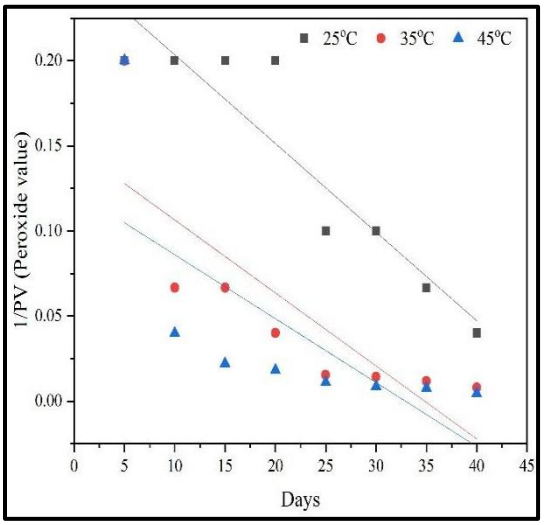

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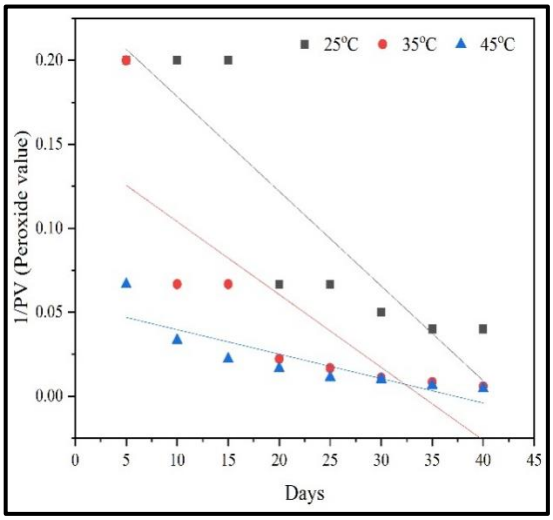

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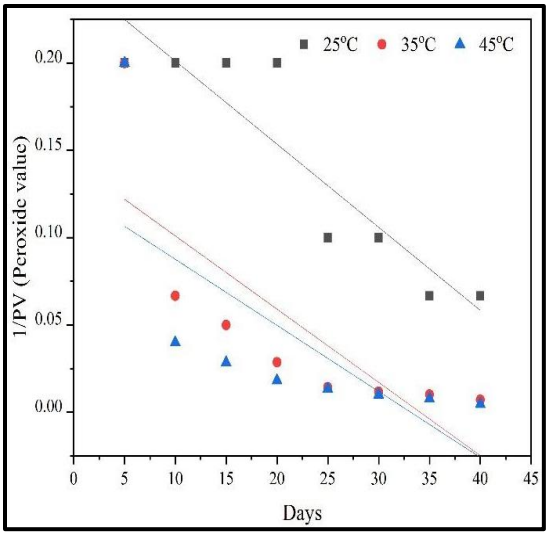

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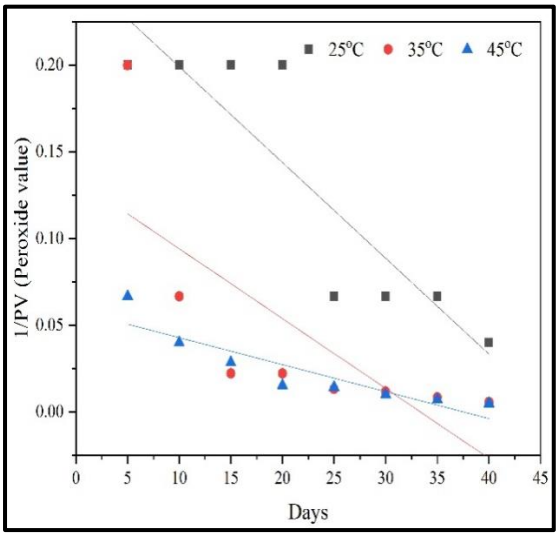

(f)

Figure -: Second order reaction graph of (a) Control, and synthetic antioxidants combination (b) SY1, (c) SY2, (d) SY3 (e) SY4 (f) SY5 at various temperature 25°C, 35°C and 45°C.

Appendix – X

Table -: Regression equations 1/PV versus time and coefficient of linear regression (R^2) considering second order reaction rate kinetics.

Oil sample	Temperature (°C)	Intercept	Slope	R^2
Control	25	0.12	-0.003	0.50
	35	0.04	-0.001	0.65
	45	0.03	-0.0001	0.84
SY1	25	0.25	-0.005	0.85
	35	0.14	-0.004	0.64
	45	0.07	-0.002	0.65
SY2	25	0.25	-0.005	0.86
	35	0.14	-0.004	0.67

	45	0.12	-0.003	0.48
SY3	25	0.23	-0.005	0.77
	35	0.14	0.010	0.59
	45	0.05	0.0007	0.71
SY4	25	0.24	-0.004	0.84
	35	0.14	-0.004	0.62
	45	0.12	-0.003	0.49
SY5	25	0.25	-0.005	0.80
	35	0.13	-0.004	0.55
	45	0.05	-0.001	0.81

REFERENCES

References

Aardt, M. V., Duncan, S. E., Long, T. E., O'Keefe, S. F., Marcy, J. E. and Sims, S. R. (2004). "Effect of antioxidants on oxidative stability of edible fats and oils: thermogravimetric analysis." *J. Agric. Food Chem.*, 52(3), 587-591.

Abdelazim, A. A., Mahmoud, A. and Ramadan-Hassanien, M. F. (2013). "Oxidative stability of vegetable oils as affected by sesame extracts during accelerated oxidative storage." *J. Food Sci. Technol.*, 50(5), 868–878.

Ajie, D. P., Sutanto, H., Lucia, A., Siregar, A. R. and Nasikin, M. (2020). "Increasing the stability oxidation of palm oil biodiesel by the addition of binary antioxidant." *AIP Conf. Proc.* on *Tropical Renewable Energy Conference*, Bali, Indonesia, 030006.

Aktar, T. and Adal, E. (2019). "Determining the arrhenius kinetics of avocado oil: Oxidative stability under rancimat test conditions." *Foods*, 8(7), 236.

Almajano, M. P., Delgado, M. E. and Gordon, M. H. (2007). "Albumin causes a synergistic increase in the antioxidant activity of green tea catechins in oil-in-water emulsions." *Food Chem.*, 102(4), 1375-1382.

Ambigaipalam, P., Camargo, A. C. de, Shahidi F., (2016). "Phenolic compounds of pomegranate byproducts (outer skin, mesocarp, divider membrane) and their antioxidant activities." *J. Agric. Food Chem*. 31, 64, 6584-6604.

Ana, C. A., Veloso, C. A. A., Rodrigues, N., Ouarouer, Y., Zaghdoudi, K., Pereira, A. J. and Peres, M. A. (2020). "A kinetic-thermodynamic study of the effect of the cultivar/total phenols on the oxidative stability of olive oils." *J. Am. Oil Chem. Soc.* 97(6), 625-636.

AOCS. (2009). "Official methods and recommended practices of the AOCS." American Oil Chemist's Society.

Arunachalam, S. S., Chandrasekar, V., Belur, P. D. (2024). Synthesis and

characterization of 3, 4-dihydroxyphenyl acetic acid esters and study of their efficacy in bulk fish oil. *Food Chem*., 441, 138380.

Bao, D. Q., Mori, T. A., Burke, V., Puddey, I. B. and Beilin, L. J. (1998). "Effects of dietary fish and weight reduction on ambulatory blood pressure in overweight hypertensives." *Hypertension*, 32(4), 710-717.

Bartee, S. D., Kim, H. J. and Min, D. B. (2007). "Effects of antioxidants on the oxidative stability of oils containing arachidonic, docosapentaenoic and docosahexaenoic acids." *J. Am. Oil Chem. Soc.*, 84(4), 363–368.

Belur, P. D., Iyyasami, R., Sampath, C. and Chandrasekar, V. (2017). "Refining technologies for edible oils." in: *edible oils - extraction, processing and applications,* C. Smain eds., Boca Raton, CRC press, Taylor and Francis Group, 99-127.

Berger, A. A., Sherburne, R., Urits, I., Patel, H., and Eskander, J. (2020). "Icosapent ethyl – a successful treatment for symptomatic COVID-19 infection." *Cureus*, 12(9), e10211.

Beyzi, E.; Gunes, A.; Buyukkilic Beyzi, S.; Konca, Y. (2019). Changes in fatty acid and mineral composition of rapeseed (Brassica napus ssp. oleifera L.) oil with seed sizes. *Ind. Crop. Prod.*, 129, 10–14.

Bhale, S. D., Xu, Z., Prinyawiwatkul, W., King, J. M. and Godber, J. S. (2007). "Oregano and rosemary extracts inhibit oxidation of long-chain n-3 fatty acids in menhaden oil." *J. Food Sci.*, 72(9), C504–C508.

Burns-Whitmore, B., Froyen, E., Heskey, C., Parker, T., San Pablo, G. (2019). "Alpha-Linolenic and Linoleic Fatty Acids in the Vegan Diet: Do They Require Dietary Reference Intake/Adequate Intake Special Consideration?" *Nutrients*, 11, 2365.

Calder, P. C. (2003). "n-3 polyunsaturated fatty acids and inflammation: from molecular biology to the clinic." *Lipids*, 38(4), 343-352.

Campbell, M. (2018). “Camelina–An alternative oil crop.” In *Biokerosene*; Springer: Berlin/Heidelberg, Germany, pp. 259–275.

Carocho, M., Morales, P. and Ferreira, I. C. F. R. (2018). “Antioxidants: reviewing the chemistry, food applications, legislation and role as preservatives.” *Trends Food Sci. Technol.*, 71, 107–120.

Chakraborty, K. and Joseph, D. (2015). “Production and characterization of refined oils obtained from Indian oil sardine (*Sardinella longiceps*).” *J. Agric. Food Chem.*, 63(3), 998–1009.

Chandrasekar, V. Sampath, C., Belur, P. D., Iyyaswami, R. (2015). Refining of edible oils: a critical appraisal of current and potential technologies. *Int. J. of Food Sci. & Tech*., 50 (1), 13-23.

Chandrasekar, V., Belur, P. D. and Iyyaswami, R. (2016). “Effect of hydroxybenzoic acids antioxidants on the oxidative stability of sardine oil.” *Resour.-Effic. Technol.*, 2, S114–S118.

Chandrasekar, V., Belur, P. D. and Iyyaswami, R. (2017). “Effectiveness of rutin and its lipophilic ester in improving oxidative stability of sardine oil containing trace water.” *Int. J. Food Sci. Technol.*, 53(2), 541–548.

Charanyaa, S., Belur, P. D. and Regupathi, I. (2017). “A New Strategy to Refine Crude Indian Sardine Oil.” *J. Oleo Sci.*, 66(5), 425–434.

Chirinos, R., Zuloeta, G., Pedreschi, R., Mignolet, E., Larondelle, Y., Campos, D. (2013). Sacha inchi (*Plukenetia volubilis*): “A seed source of polyunsaturated fatty acids, tocopherols, phytosterols, phenolic compounds and antioxidant capacity.” *Food Chem*., 141, 1732–1739.

Chirinos, R.; Zuloeta, G.; Pedreschi, R.; Mignolet, E.; Larondelle, Y.; Campos, D. (2013). Sacha inchi (Plukenetia volubilis): A seed source of polyunsaturated fatty acids,

tocopherols, phytosterols, phenolic compounds and antioxidant capacity. *Food Chem*., 141, 1732–1739.

Choe, E. and Min, D. B. (2006). "Mechanisms and factors for edible oil oxidation." *Comprehensive Reviews in Food Science and Food Safety*, 5(4), 169–186.

Choe, E., and Min, B. D. (2009). "Mechanisms of antioxidant in the oxidation of foods." *Compr. Rev. Food Sci. Food Saf.*, 8(4), 345–358.

Copetta, A.; Bazzicalupo, M.; Cassetti, A.; Marchioni, I.; Mascarello, C.; Cornara, L.; Pistelli, L.; Ruffoni, B. (2021). Plant production and leaf anatomy of mertensia maritima (l.) gray: comparison of in vitro culture methods to improve acclimatization. horticulturae, 7, 111.

Cumberford, G.; Hebard, A. (2015). Ahiflower oil: A novel non-GM plant-based omega-3+6 source. *Lipid Technol*., 27, 207–210.

Dhama, K., Sharun, K., Gugjoo, M.B., Tiwari, R., Alagawany, M., Iqbal Yatoo, M., Thakur, P., Iqbal, H.M.N., Chaicumpa, W., Michalak, I. (2021). "A Comprehensive Review on Chemical Profile and Pharmacological Activities of *Ocimum basilicum*." *Food Rev. Int*., 1–29.

Dziedzic, S. Z. and Hudson, B. J. F. (1984). "Phosphatidyl ethanolamine as a synergist for primary antioxidants in edible oils." *J. Am. Oil Chem. Soc.*, 61, 1042–1045.

Eggersdorfer, M. and Wyss, A. (2018). "Carotenoids in human nutrition and health." *Arch. Biochem. Biophys.*, 652, 18–26.

Elhussein, E., Bilgin, M. and Şahin, S. (2018). "Oxidative stability of sesame oil extracted from the seeds with different origins: Kinetic and thermodynamic studies under accelerated conditions." *J. Food Process Eng.*, 41(8), e12878.

Fadiyah, N. N., Megawati, G. and Luftimas, D. E. (2022). "Potential of omega 3

supplementation for coronavirus disease 2019 (COVID-19): A Scoping review." *Int. J. Gen. Med.*, 15, 3915-3922.

Fakourelis, N., Lee, E. C. and Min, D. B. (1987). "Effects of chlorophyll and β-carotene on the oxidation stability of olive oil." *J. Food Sci.*, 52, 234–235.

Fhaner, M., Hwang, H. S., Winkler-Moser, J. K., Bakota, E. L. and Liu, S. X. (2016). "Protection of fish oil from oxidation with sesamol." *Eur. J. Lipid Sci. Technol.*, 118(6), 885–897.

Finley, J. W. and Shahidi, F. (2001). "The chemistry, processing, and health benefits of highly unsaturated fatty acids: An overview." *ACS Symp. Ser.*, 788, 2-11

Frankel, E. N., Huang, S. W., Aeschbach, R. and Prior, E. (1996). "Antioxidant activity of a rosemary extract and its constituents, carnosic acid, carnosol, and rosmarinic acid, in bulk oil and oil-in-water emulsion." *J. Agric. Food Chem.*, 44(1), 131–135.

Fsanz. dhasco and Arasco. (2003). Oils as sources of long-chain polyunsaturated fatty acids in infant formula; food standards Australia New Zealand: Canberra, BC, Australia.

Fukuda, Y., Nagata, M., Osawa, T. and Namiki, M. (1986). "Contribution of lignan analogues to antioxidative activity of refined unroasted sesame seed oil." *J. Am. Oil Chem. Soc.*, 63(8), 1027–1031.

Gearhart, W. M. and Stuckey, B. N. (1955). "A comparison of commercially used phenolic antioxidants in edible animal fats." *J. Am. Oil Chem. Soc.*, 32, 386-390.

Ghobadi, S.; Hassanzadeh-Rostami, Z.; Mohammadian, F.; Zare, M.; Faghih, S. (2019). Effects of Canola Oil Consumption on Lipid Profile: A Systematic Review and Meta-Analysis of Randomized Controlled Clinical Trials. *J. Am. Coll. Nutr*., 38, 185–196.

Gopalam, R., Tumaney, A.W. (2021). Functional characterization of acyltransferases from Salvia hispanica that can selectively catalyze the formation of trilinolenin. *Phytochemistry*, 186, 112712.

Gordon, M. H. (1990). "The mechanism of antioxidant action in vitro." In: *Food* Antioxidants, B. J. F. Hudson eds. 1-18. London and Newyork: Elsevier Science Publishing Inc.

Gouveia De Souza, A., Oliveira Santos, J. C., Conceição, M. M., Dantas Silva, M. C. and Prasad, S. (2004). "A thermoanalytic and kinetic study of sunflower oil." *Brazilian J. Chem. Eng.*, 21(2), 265-273.

Goyal, A., Sharma, V., Upadhyay, N., Gill, S., Sihag, M., (2015). "Flax and flaxseed oil: an ancient medicine & modern functional food." *J. Food Sci. Technol*. 51 (9), 1633-1653.

Gray, R.J. (2017). Application for the authorization of DHA and EPA-rich algal oil from *Schizochytrium sp*.; Martek Biosciences Corporation: Columbia, ML, USA.

Guil-Guerrero, J.L., Gómez-Mercado, F., Ramos-Bueno, R.P., González-Fernández, M.J., Urrestarazu, M., Jiménez-Becker, S., de Bélair, G. (2018). "Fatty acid profiles and sn-2 fatty acid distribution of γ-linolenic acid-rich Borago species." *J. Food Compos. Anal*., 66, 74–80.

Guil-Guerrero, J.L.; Gómez-Mercado, F.; Ramos-Bueno, R.P.; Rincón-Cervera, M.Á.; Venegas-Venegas, E. (2014). Restricted-range boraginaceae species constitute potential sources of valuable fatty acids. *J. Am. Oil Chem. Soc.*, 91, 301–308.

Guillen, M. D. and Cabo, N. (2002). "Fourier transform infrared spectra data versus peroxide and anisidine values to determine oxidative stability of edible oils." *Food Chem.,* 77(4), 503-510.

Gülmez, Ö. and Şahin, S. (2019). "Evaluation of oxidative stability in hazelnut oil

treated with several antioxidants: kinetics and thermodynamics studies." *LWT- Food Sci. Technol.*, 111, 478-483.

Günç Ergönül, P.; Aksoylu Özbek, Z. (2020). Chapter 21—Cold pressed camelina (Camelina sativa L.) seed oil. In Cold Pressed Oils; Ramadan, M.F., Ed.; Academic Press: Cambridge, MA, USA, pp. 255–266.

Guzman, R. D., Tang, H., Salley, S. and Ng, K. Y. S. (2009). "Synergistic effects of antioxidants on the oxidative stability of soybean oil- and poultry fat-based biodiesel." *J. Am. Oil Chem. Soc.*, 86(5), 459–467.

Haila, K. and Heinonen, M. (1994). "Action of β-carotene on purified rapeseed oil during light storage." *LWT-Food Sci. Technol.*, 27(6), 573-577.

Haila, K. M., Lievonen, S. M. and Heinonen, M. I. (1996). "Effects of lutein, lycopene, annatto, and γ-tocopherol on autoxidation of triglycerides." *J. Agric. Food Chem.*, 44(8), 2096–2100.

Hamilton, R. J., Kalu, C., McNeill, G. P., Padley, F. B. and Pierce, J. H. (1998). "Effects of tocopherols, ascorbyl palmitate, and lecithin on autoxidation of fish oil." *J. Agric. Food Chem.*, 75, 813–822.

Hamilton, R.J. and Hamilton, S. (1994) Lipid Analysis: A Practical Approach. Oxford University Press, New York.

Han, L., Usher, S., Sandgrind, S., Hassall, K., Sayanova, O., Michaelson, L.V., Haslam, R.P., Napier, J.A. (2020). "High level accumulation of EPA and DHA in field-grown transgenic Camelina—A multi-territory evaluation of TAG accumulation and heterogeneity." *Plant Biotechnol*. J. 18, 2280–2291.

Hang, Y., Yang, L., Zu, Y., Chen, X., Wang, F. and Liu, F. (2010). "Oxidative stability of sunflower oil supplemented with carnosic acid compared with synthetic antioxidants during accelerated storage." *Food Chem.*, 118(3), 656–662.

Henry, L. K., Catignani, C. L. and Schwartz, S. J. (1998a). “The influence of carotenoids and tocopherols on the stability of safflower seed oil during heat-catalyzed oxidation.” *J. Am. Oil Chem. Soc.*, 75(10), 1399–1402.

Henry, L. K., Catignani, G. L. and Schwartz, S. J. (1998b) “Oxidative degradation kinetics of lycopene , lutein , and 9-cis and all-trans β-carotene.” *J. Am. Oil Chem. Soc.*, 75(7), 823–829.

Hopia, A., Huang, S., Schwarz, K., German, J. and Frankel, E. (1996). “Effect of different lipid systems on antioxidant activity of rosemary constituents carnosol and carnosic acid with and without α-tocopherol.” *J. Agric. Food Chem*., 44(8), 2030–2036.

Huang, J., Sun, Q., Song, G., Qi, S., Chen, J., Zhang, P., Geng, T., Lin, Q. and Duan, Y. (2020). “Antioxidant and anti-isomerization effects of sesamol and resveratrol on high oleic acid peanut oil.” *LWT- Food Sci. Technol.*, 123, 109077.

Huang, T.Y., Lu, W.C., Chu, I.M. (2012). A fermentation strategy for producing docosahexaenoic acid in *Aurantiochytrium limacinum* SR21 and increasing C22:6 proportions in total fatty acid. *Bioresour. Technol*., 123, 8–14.

Huang, T.Y.; Lu, W.C.; Chu, I.M. (2012). A fermentation strategy for producing docosahexaenoic acid in *Aurantiochytrium limacinum* SR21 and increasing C22:6 proportions in total fatty acid. *Bioresour. Technol.*, 123, 8–14.

Hussain, S. A., Hameed, A., Ajmal, I., Nosheen, S., Suleria, H. A. R. and Song, Y. (2018). “Effects of sesame seed extract as a natural antioxidant on the oxidative stability of sunflower oil.” *J. Food Sci. Technol.*, 55, 4099–4110.

Jiang, S., Xie, Y., Li, M., Guo, Y., Cheng, Y., Qian, H. and Yao, W. (2020). “Evaluation on the oxidative stability of edible oil by electron spin resonance spectroscopy.” *Food Chem.*, 309,125714.

Jung, L., Lee, E. and Choe, E. (2014). “Effects of catechin and α-tocopherol addition on the autoxidative stability of diacylglycerol oil derived from an olive oil and perilla oil mixture.” *Food Sci. Biotechnol.*, 23, 1793–1798.

Jung, M. Y. and Min, D. B. (1992). “Effects of oxidized α-, γ- and δ-tocopherols on the oxidative stability of purified soybean oil.” *Food Chem.*, 45(3), 183–187.

Karabulut, I. (2010). “Effects of α-tocopherol, β-carotene and ascorbyl palmitate on oxidative stability of butter oil triacylglycerols.” *Food Chem.*, 123(3), 622–627.

Kennedy. T, A. and Liebler, D. C. (1992). “Peroxyl radical scavenging by β-carotene in lipid bilayers. Effect of oxygen partial pressure.” *J. Biol. Chem.*, 267(7), 4658–4663.

Kim, J. Y., Kim, M. J., Yi, B., Oh, S. and Lee, J. (2015a). “Effects of relative humidity on the antioxidant properties of α-tocopherol in stripped corn oil.” *Food Chemi.*, 167, 191-196.

Kim, J. Y., Kim, M. J., Yi, B., Oh, S. and Lee, J. (2015b). “Antioxidant properties of ascorbic acid in bulk oils at different relative humidity.” *Food Chem.*, 176, 302–307.

Kiy, T.; Luy, M.; Zeumer, O. (2010). Production of omega-3 fatty acids in microflora of thraustochytriales using modified media. google patents. available online: https://data.epo.org/gpi/EP2084290B1

Knez Hrnˇciˇc, M., Ivanovski, M., Cör, D., Knez, Ž. (2019). “Chia Seeds (*Salvia Hispanica* L.): An Overview—Phytochemical Profile, Isolation Methods, and Application.” *Molecules*, 25, 11.

Kochevar, I. E. and Redmond, R. W. (2000). “Photosensitized production of singlet oxygen.” *Methods Enzymol.*, 319, 20–28.

Koketsu, M. and Satoh, Y. I. (1997). “Antioxidative activity of green tea polyphenols in edible oils.” *J. Food Lipids*, 4(1), 1–9.

Kozłowska, M. and Gruczyńska, E. (2018). “Comparison of the oxidative stability of soybean and sunflower oils enriched with herbal plant extracts.” *Chem. Pap.*, 72, 2607–2615.

Kulås, E. and Ackman, R. G. (2001). “Properties of α-, γ-, and δ-tocopherol in purified fish oil triacylglycerols.” *J. Am. Oil Chem. Soc.*, 78, 361–367.

Kurechi, T. and Kato, T. (1980). “Studies on the antioxidants: XI. oxidation products of concomitantly used butylated hydroxyanisole and butylated hydroxytoluene.” *J. Am. Oil Chem. Soc.*, 57(7), 220-223.

Kurtulbaş, E., Bilgin, M. and Şahin, S. (2018). “Assessment of lipid oxidation in cottonseed oil treated with phytonutrients: Kinetic and thermodynamic studies.” *Ind. Crops Prod.*, 124, 593-599.

Laguerre, M., Bayrasy, C., Panya, A., Weiss, J., McClements, D. J., Lecomte, J., Decker, E. A. and Villeneuve, P. (2015). “What Makes Good Antioxidants in Lipid-Based Systems? The Next Theories Beyond the Polar Paradox.” *Crit. Rev. Food Sci. Nutr.*, 55(2), 183–201.

Laidlaw, M., Cockerline, C. A., Rowe, W. J., (2014). “A randomized clinical trial to determine the efficacy of manufacturers’ recommended doses of omega-3 fatty acids from different sources in facilitating cardiovascular disease risk reduction.” *Lipids Health Dis*. 13, 99.

Lampi, A. M., Kataja, L., Kamal-Eldin, A. and Vieno, P. (1999). “Antioxidant activities of α- and γ-tocopherols in the oxidation of rapeseed oil triacylglycerols.” *Crit. Rev. Food Sci. Nutr.*, 76, 749–755.

Lee, C., Gim, S. Y., Kim, M. J. and Lee, J. (2016). “Effects of quercetin or rutin on the oxidative stability of stripped or non-stripped soybean oils containing α-tocopherol.” *Eur. J. Lipid Sci. Technol.*, 119(7), 1600329.

Lee, E. C. and Min, D. B. (1988). “Quenching mechanism of β-carotene on the chlorophyll sensitized photooxidation of soybean oil.” *J. Food Sci.*, 53(6), 1894-1895.

Li, J., Chen, J., Bi, Y. and Yang, H. (2022). “Insight into synergistic antioxidation mechanisms of butyl hydroxyanisole with common synthetic antioxidants.” *Grain Oil Sci. Technol.*, 5(3), 114-130.

Li, S.-S., Yuan, R.-Y., Chen, L.-G., Wang, L.-S., Hao, X.-H., Wang, L.-J.; Zheng, X.-C.; Du, H. (2015). “Systematic qualitative and quantitative assessment of fatty acids in the seeds of 60 tree peony (*Paeonia section Moutan* DC.) cultivars by GC–MS.” *Food Chem*., 173, 133–140.

Lim, D.K.Y.; Garg, S.; Timmins, M.; Zhang, E.S.B.; Thomas-Hall, S.R.; Schuhmann, H.; Li, Y.; Schenk, P.M. (2012). Isolation and Evaluation of Oil-Producing Microalgae from Subtropical Coastal and Brackish Waters. *PLoS ONE*, 7, e40751.

Liu, L., Jin, C. and Zhang, Y. (2014). “Lipophilic phenolic compounds (Lipo-PCs): Emerging antioxidants applied in lipid systems.” *RSC Adv.*, 4, 2879–2891.

Lopez, L. B., Kritz-Silverstein, D. and Barrett-Connor, E. (2011). “HIgh dietary and plasma levels of the omega-3 fatty acid docosahexaenoic acid are associated with decreased dementia risk: The rancho bernardo study.” *J. Nutr. Heal. Aging*, 15(1), 25-31.

Louca, P., Murray, B., Klaser, K., Graham, M. S., Mazidi, M., Leeming, E. R., Thompson, E., Bowyer, R., Drew, D. A., Nguyen, L. H., Merino, J., Gomez, M., Mompeo, O., Costeira, R., Sudre, C. H., Gibson, R., Steves, C. J., Wolf, J., Franks, P. W., Ourselin, S., Chan, A. T., Berry, S. E., Valdes, A. M., Calder, P. C., Spector, T. D. and Menni, C. (2021). “Modest effects of dietary supplements during the COVID-19 pandemic: Insights from 445 850 users of the COVID-19 Symptom Study app.” *BMJ Nutr. Prev. Heal.*, 4(1), 149-157.

Lyashenko, S.; González-Fernández, M.J.; Borisova, S.; Belarbi, E.-H.; Guil-Guerrero, J.L. (2021). Mertensia (Boraginaceae) seeds are new sources of γ-linolenic acid and minor functional compounds. *Food Chem*., 350, 128635.

Ma, X.-N., Chen, T.-P., Yang, B., Liu, J., Chen, F. (2016). Lipid Production from *Nannochloropsis*. *Mar*. *Drugs*, 14, 61.

Maehre, H.K.; Malde, M.K.; Eilertsen, K.-E.; Elvevoll, E.O. (2014). Characterization of protein, lipid and mineral contents in common Norwegian seaweeds and evaluation of their potential as food and feed. *J. Sci. Food Agric.*, 94, 3281–3290.

Maqsood, S. and Benjakul, S. (2010). "Comparative studies of four different phenolic compounds on in vitro antioxidative activity and the preventive effect on lipid oxidation of fish oil emulsion and fish mince." *Food Chem*., 119(1), 123–132.

Marineli, R.D.S., Moraes, É.A., Lenquiste, S.A., Godoy, A.T., Eberlin, M.N., Maróstica, M.R., Jr. (2014). "Chemical characterization and antioxidant potential of chilean chia seeds and oil (*Salvia hispanica* L.)." *LWT—Food Sci. Technol*., 59, 1304–1310.

Marinova, E., Toneva, A. and Yanishlieva, N. (2008). "Synergistic antioxidant effect of α-tocopherol and myricetin on the autoxidation of triacylglycerols of sunflower oil." *Food Chem.*, 106(2), 628–633.

Martínez, M. L., Penci, M. C., Ixtaina, V., Ribotta, P. D. and Maestri, D. (2013). "Effect of natural and synthetic antioxidants on the oxidative stability of walnut oil under different storage conditions." *LWT-Food Sci. Technol.*, 51(1), 44–50.

Martin-Rubio, A. S., Sopelana, P., Ibargoitia, M. L. and Guillén, M. D. (2017). "Prooxidant effect of α-tocopherol on soybean oil. Global monitoring of its oxidation process under accelerated storage conditions by ^{1}H nuclear magnetic resonance." *Food Chem.*, 245, 312–323.

Mas, E., Woodman, R. J., Burke, V., Puddey, I. B., Beilin, L. J., Durand, T. and Mori, T. A. (2010). “The omega-3 fatty acids EPA and DHA decrease plasma F2-isoprostanes: Results from two placebo-controlled interventions.” *Free Radic. Res.*, 44(9), 983-990.

Medina, I., Alcántara, D., González, M. J., Torres, P., Lucas, R., Roque, J., Plou, F. J. and Morales, J. C. (2010). “Antioxidant activity of resveratrol in several fish lipid matrices: Effect of acylation and glucosylation.” *J. Agric. Food Chem.*, 58(17), 9778–9786.

Melo, D.; Machado, T.B.; Oliveira, M.B.P.P. (2019). Chia seeds: An ancient grain trending in modern human diets. *Food Funct.*, 10, 3068–3089.

Mihaylova, D., Gandova, V., Deseva, I., Tschuikowa, S., Schalow, S. and Westphal, G. (2020). "Arrhenius Equation Modeling for the Oxidative Stability Evaluation of Echium Oil Enriched with a Natural Preservative". *Eur. J. Lipid Sci. Technol.* 122(11), 2000118.

Mishra, K., S., Belur, D., P., and Regupathi, I. (2021). “Review on use of antioxidants for enhancing oxidative stability of bulk edible oils.” *Int. J. Food Sci. Technol.* 56(1) 1-12.

Mishra, K., S., Belur, D., P., and Regupathi, I. (2022) "Comparison of efficacy of various natural and synthetic antioxidants in stabilizing the fish oil." *J. Food Process. Preserv.*, 46(11), e16970.

Mitra, M.; Patidar, S.K.; Mishra, S. (2015). Integrated process of two stage cultivation of Nannochloropsis sp. for nutraceutically valuable eicosapentaenoic acid along with biodiesel. *Bioresour. Technol.*, 193, 363–369.

Moen, V., Stoknes, I. and Breivik, H. (2017). “Antioxidant efficacy of a new synergistic, multicomponent formulation for fish oil omega-3 concentrates.” *J. Am. Oil*

Chem. Soc., 94(7), 947–957.

Mohanan, A., Nickerson, M.T. and Ghosh, S. (2018). "Oxidative stability of flaxseed oil: Effect of hydrophilic, hydrophobic and intermediate polarity antioxidants." *Food Chem.*, 266, 524–533.

Mohdaly, A. A. A., Smetanska, I., Ramadan, M. F., Sarhan, M. A. and Mahmoud, A. (2011). "Antioxidant potential of sesame (*Sesamum indicum*) cake extract in stabilization of sunflower and soybean oils." *Ind. Crops Prod.*, 34(1), 952–959.

Moomand, K. and Lim, L. T. (2014). "Oxidative stability of encapsulated fish oil in electrospun zein fibres." *Food Res. Int.*, 62, 523-532.

Mori, T. A. (2017). "Marine omega-3 fatty acids in the prevention of cardiovascular disease." *Fitoterapia*, 123, 51-58.

Mori, T. A., Mas, E., Woodman, R.J., Burke, V., Puddey, I.B., Beilin, L.J., Durand, T. (2010). "The omega-3 fatty acids EPA and DHA decrease plasma F2-isoprostanes: Results from two placebo-controlled interventions." *Free Radic. Res*., 44, 983–990.

Moser, B.R. (2008). "Efficacy of myricetin as an antioxidant in methyl esters of soybean oil." *Eur. J. Lipid Sci. Technol*., 110(12), 1167-1174.

Mozaffarian, D., Geelen, A., Brouwer, I.A., Geleijnse, J.M., Zock, P.L., Katan, M.B. (2005). "Effect of fish oil on heart rate in humans: A meta-analysis of randomized controlled trials." *Circulation.*, 112, 1945–1952

Nain, C. W., Mignolet, E., Herent, M. F., Quetin-Leclercq, J., Debier, C., Page, M. M. and Larondelle, Y. (2022). "The catechins profile of green tea extracts affects the antioxidant activity and degradation of catechins in DHA-rich oil." *Antioxidants*, 11(9), 1844.

Nazir, S. Wani, I.A. (2021). "Physicochemical characterization of basil (*Ocimum*

basilicum L.) seeds." *J. Appl. Res. Med. Aromat. Plants*, 22, 100295.

Nemzer, B.; Al-Taher, F.; Abshiru, N. Phytochemical composition and nutritional value of different plant parts in two cultivated and wild purslane (*Portulaca oleracea* L.) genotypes. Food Chem. 2020, 320, 126621.

Nogales-Delgado, S., Encinar, J. M. and González, J. F. (2019). "Safflower biodiesel: improvement of its oxidative stability by using BHA and TBHQ." *Energies*, 12(10), 1940.

Olsen, E., Vogt, G., Saarem, K., Greibrokk, T. and Nilsson, A. (2005). "Autoxidation of cod liver oil with tocopherol and ascorbyl palmitate." *J. Am. Oil Chem. Soc.*, 82, 97–103.

Oroian, M. and Escriche, I. (2015). "Antioxidants: characterization, natural sources, extraction and analysis." *Food Res. Int.*, 74, 10–36.

Osman, F., Jaswir, I., Khaza, H., Hashim, R. (2007). "Fin fish contained higher AA and DHA levels than menhaden fish oil." *J. Oleo Sc*i., 56, (3), 107-113.

Osman, H., Suriah, A. R., Law, E. C., (2001). "Fatty acid composition and cholesterol content of selected marine fish in Malaysian waters." *Food Chem*. 73 (1). 55-60.

Panigrahy, D., Gilligan, M. M., Huang, S., Gartung, A., Cortés-Puch, I., Sime, P. J., Phipps, R. P., Serhan, C. N. and Hammock, B. D. (2020). "Inflammation resolution: a dual-pronged approach to averting cytokine storms in COVID-19?" *Cancer Metastasis Rev.*, 39(2), 337-340.

Park, H.Y., Kim, D.H., Saini, R.K., Gopal, J., Keum, Y.S.; Sivanesan, I. (2019). "Micropropagation and quantification of bioactive compounds in *Mertensia maritima* (L.) Gray." *Int. J. Mol*. Sci. 20, 2141.

Patel, A.; Mu, L.; Shi, Y.; Rova, U.; Christakopoulos, P.; Matsakas, L. (2020). Novel

Biorefinery Approach Aimed at Vegetarians Reduces the Dependency on Marine Fish Stocks for Obtaining Squalene and Docosahexaenoic Acid. ACS Sustain. *Chem. Eng*., 8, 8803–8813

Pei, X. C., Yin, F. W., Zhong, X., Liu, H. L., Song, L., Zhao, G. H., Wang, Y. F. and Zhou, D. Y. (2022). "Effects of different antioxidants and their combinations on the oxidative stability of DHA algae oil and walnut oil." *Food Sci. Nutr.*, 10(8), 2804-2812.

Pereira, H., Barreira, L., Figueiredo, F., Custódio, L., Vizetto-Duarte, C., Polo, C., Rešek, E., Engelen, A., Varela, J. (2012). Polyunsaturated fatty acids of marine macroalgae: potential for nutritional and pharmaceutical applications. *Mar. Drugs*, 10, 1920–1935.

Petrie, J.R., Shrestha, P., Belide, S., Kennedy, Y., Lester, G., Liu, Q., Divi, U.K., Mulder, R.J., Mansour, M.P., Nichols, P.D. (2014). "Metabolic Engineering Camelina sativa with Fish Oil-Like Levels of DHA." *PLoS ONE*. 9, e85061.

Piskernik, S., Vidrih, R., Demsar, L., Koron, D., Rogelj, M., Zontar, T.P. (2018). "Fatty acid profiles of seeds from different ribes species." *LWT—Food Sci. Technol*., 98, 424–427.

Prabu, S.L. Umamaheswari, A. Puratchikody, A. (2019). "Phytopharmacological potential of the natural gift *Moringa oleifera* Lam and its therapeutic application: An overview." *Asian Pac. J. Trop. Med*., 12, 485–498.

Prasad, P., Anjali, P., Sreedhar, R.V. (2020). "Plant-based stearidonic acid as sustainable source of omega-3 fatty acid with functional outcomes on human health." *Crit. Rev. Food Sci. Nutr*., 61, 1725–1737.

Qiao, H.; Cong, C.; Sun, C.; Li, B.; Wang, J.; Zhang, L. (2016). Effect of culture conditions on growth, fatty acid composition and DHA/EPA ratio of Phaeodactylum tricornutum. Aquaculture, 452, 311–317.

Ramalho, B.V.C. and Jorge, N. (2008). Antioxidant action of rosemary extract in soybean oil submitted to thermoxidation. *Int. J. Fats Oils*, 59(2), 128–131.

Rao, A. R., Sarada, R. and Ravishankar, G. A. (2007). "Stabilization of astaxanthin in edible oils and its use as an antioxidant." *J. Sci. Food Agric.*, 87(6), 957–965.

Redondo-Cuevas, L., Castellano, G. and Raikos, V. (2017). "Natural antioxidants from herbs and spices improve the oxidative stability and frying performance of vegetable oils." *Int. J. Food Sci. Technol.*, 52(11), 2422–2428.

Reische, D. W., Lillard, D. A. and Eitenmiller, R. R. (2017). "Antioxidants." In: *Food lipids : Chemistry, Nutrition, and Biotechnolog*, C. C. Akoh and D. B. Min eds., Newyork, Marcel Dekker, Inc., 507-534.

Richards, M. P., Chaiyasit, W., McClements, D. J. and Decker, E. A. (2002). "Ability of surfactant micelles to alter the partitioning of phenolic antioxidants in oil-in-water emulsions." *J. Agric. Food Chem.*, 50(5), 1254–1259.

Rincón-Cervera, M.Á., Galleguillos-Fernández, R., González-Barriga, V., Valenzuela, R., Speisky, H., Fuentes, J., Valenzuela, A. (2020). "Fatty Acid Profile and Bioactive Compound Extraction in Purple Viper's Bugloss Seed Oil Extracted with Green Solvents." *J. Am. Oil Chem. Soc*., 97, 319–327.

Roedig-Penman, A. and Gordon, M. H. (2012). "Antioxidant properties of myricetin and quercetin in oil and emulsions." *J. Am. Oil Chem. Soc.*, 75, 169–180.

RV, S., Kumari, P., Rupwate, S.D., Rajasekharan, R., Srinivasan, M. (2015). "Exploring triacylglycerol biosynthetic pathway in developing seeds of chia (*Salvia hispanica* L.): A transcriptomic approach." *PLoS ONE*, 10, e0123580.

Şahin, S., Ciğeroğlu, Z., Kurtulbas E.¸ Pekel, G., A. and Ibibik, K. (2020). "Kinetics and thermodynamics evaluation of oxidative stability in *Oleum hyperici*: A comparative study." *J. Pharm. Biomed. Anal*., 183, 113148.

Saini, R. K., Prasad, P., Sreedhar, R. V., Naidu, K. A., Shang, X., Keum, Y. S. (2021). "Omega−3 polyunsaturated fatty acids (pufas): emerging plant and microbial sources, oxidative stability, bioavailability, and health benefits—A Review." *Molecules*, 10, 1627.

Saini, R.K. Keum, Y.-S. Rengasamy, K.R. (2020). "Profiling of nutritionally important metabolites in green/red and green perilla (*Perilla frutescens Britt*.) cultivars: A comparative study." *Ind. Crop. Prod*., 151, 112441.

San Andrés, M. P., Otero, J. and Vera, S. (2011). "High performance liquid chromatography method for the simultaneous determination of α-, γ- and δ-tocopherol in vegetable oils in presence of hexadecyltrimethylammonium bromide/n-propanol in mobile phase." *Food Chem.*, 126(3), 1470–1474.

Santos, N. A., Cordeiro, A. M. T. M., Damasceno, S. S., Aguiar, R. T., Rosenhaim, R., Carvalho Filho, J. R., Santos, I. M. G., Maia, A. S. and Souza, A. G. (2012). "Commercial antioxidants and thermal stability evaluations." *Fuel*, 97, 638-643.

Schmid, M.; Guihéneuf, F.; Stengel, D.B. (2014). Fatty acid contents and profiles of 16 macroalgae collected from the Irish Coast at two seasons. *J. Appl. Phycol*., 26, 451–463.

Scott, S.D.; Armenta, R.E.; Berryman, K.T.; Norman, A.W. (2011). Use of raw glycerol to produce oil rich in polyunsaturated fatty acids by a thraustochytrid. *Enzym. Microb. Technol.*, 48, 267–272.

Serhan, C. N., Libreros, S. and Nshimiyimana, R. (2022). "E-series resolvin metabolome, biosynthesis and critical role of stereochemistry of specialized pro-resolving mediators (SPMs) in inflammation-resolution: Preparing SPMs for long COVID-19, human clinical trials, and targeted precision nutrition." *Semin. Immunol.*, 59, 101597.

Serhan, N. C., Libreros, S., Nshimiyimana, R. (2022). “E-series resolvin metabolome, biosynthesis and critical role of stereochemistry of specialized pro-resolving mediators (SPMs) in inflammation-resolution: Preparing SPMs for long COVID-19, human clinical trials, and targeted precision nutrition.” *Semin Immunol*. 59, 101597.

Shadyro, O. I., Sosnovskaya, A. A. and Edimecheva, I. P. (2017). “Flaxseed oil stabilization using natural and synthetic antioxidants.” *Eur. J. Lipid Sci. Technol.*, 119(10), 1700079.

Shahidi, F. and Ambigaipalan, P. (2015). “Phenolics and polyphenolics in foods, beverages and spices: Antioxidant activity and health effects - A review.” *J. Funct. Foods*, 18, 820–897.

Shahidi, F. and Miraliakbari, H. (2004). “Omega-3 (n-3) fatty acids in health and disease: Part 1-cardiovascular disease and cancer.” *J. Med. Food*, 7(4), 387-401.

Shahidi, F. and Miraliakbari, H. (2005). “Omega-3 fatty acids in health and disease: part 2--health effects of omega-3 fatty acids in autoimmune diseases, mental health, and gene expression.” *J. Med. Food*, 8(2), 133-148.

Shahidi, F. and Wanasundara, U. (1995). “Effect of natural antioxidants on the stability of canola oil.” *Dev. Food Sci.*, 37, 469–479.

Shahidi, F. and Zhong, Y. (2010). “Lipid Oxidation and Improving the Oxidative Stability.” *Chem. Soc. Rev.*, 39(11), 4067-4079.

Shetty, U.S.; Akhilender, N.K. (2017). Garden cress (*Lepidium sativum* L.) Seed Oil: Alternative Source for ALA. FASEB J., 31, 971.12–971.12.

Skroza, D., Generalić Mekinić, I., Svilović, S., Šimat, V. and Katalinić, V. (2015). “Investigation of the potential synergistic effect of resveratrol with other phenolic compounds: A case of binary phenolic mixtures.” *J. Food Compos. Anal.*, 38, 13-18.

Sreedhar, R.V.; Prasad, P.; Reddy, L.P.A.; Rajasekharan, R.; Srinivasan, M. (2017). Unravelling a stearidonic acid-rich triacylglycerol biosynthetic pathway in the developing seeds of Buglossoides arvensis: A transcriptomic landscape. *Sci. Rep*., 7, 10473.

Su, Y. L., Xu, J. Z., Ng, C. H., Leung, L. K., Huang, Y. and Chen, Z.Y. (2004). "Antioxidant activity of tea theaflavins and methylated catechins in canola oil." *J. Am. Oil Chem. Soc.*, 81, 269–274.

Suja, K. P., Abraham, J. T., Thamizh, S. N., Jayalekshmy, A. and Arumughan, C. (2003). "Antioxidant efficacy of sesame cake extract in vegetable oil protection." *Food Chem.*, 84(3), 393–400.

Suriah, A. R., Huah, S. T., Nassan, O., Daud, M. N., (1995). "Fatty acid composition of some Malaysian freshwater fish." *Food Chem*. 54 (1), 45-49.

Szydłowska-Czerniak, A., Rabiej, D., Kyselka, J., Dragoun, M. and Filip, V. (2018). "Antioxidative effect of phenolic acids octyl esters on rapeseed oil stability." *LWT-Food Sci. Technol.,* 96*,* 193-198.

Tan, C. P., Che Man, Y. B., Selamat, J. and Yusoff, M. S. A. (2001). "Application of Arrhenius kinetics to evaluate oxidative stability in vegetable oils by isothermal differential scanning calorimetry." *J. Am. Oil Chem. Soc.*, 78(11), 1133–1138.

Thies, F., Garry, J. M. C., Yaqoob, P., Rerkasem, K., Williams, J., Shearman, C. P., Gallagher, P. J., Calder, P. C. and Grimble, R. F. (2003). "Association of n-3 polyunsaturated fatty acids with stability of atherosclerotic plaques: A randomised controlled trial." *Lancet*, 361(9356), 477-485.

Thiyam, U., Stöckmann, H. and Schwarz, K. (2006). "Antioxidant activity of rapeseed phenolics and their interactions with tocopherols during lipid oxidation." *J. Am. Oil Chem. Soc.*, 83, 523-528.

Uddin, M.K., Juraimi, A.S., Hossain, M.S., Nahar, M.A., Ali, M.E., Rahman, M.M. (2014). "Purslane weed (*Portulaca oleracea*): A prospective plant source of nutrition, omega-3 fatty acid, and antioxidant attributes." *Sci. World J*., 951019.

Ullah, R.; Nadeem, M., Khalique, A., Imran, M., Mehmood, S., Javid, A., Hussain, J. (2016). "Nutritional and therapeutic perspectives of Chia (*Salvia hispanica* L.): A review." *J. Food Sci. Technol*., 53, 1750–1758.

Umesha, S.S.; Monahar, B.; Naidu, K.A. (2013). Microencapsulation of α-linolenic acid-rich garden cress seed oil: Physical characteristics and oxidative stability. *Eur. J. Lipid Sci. Technol*., 115, 1474–1482.

Usher, S., Han, L., Haslam, R.P., Michaelson, L.V., Sturtevant, D., Aziz, M.; Chapman, K.D., Sayanova, O., Napier, J.A. "Tailoring seed oil composition in the real world: (2017). Optimising omega-3 long chain polyunsaturated fatty acid accumulation in transgenic Camelina sativa." *Sci. Rep*., 7, 6570.

Velasco, J., Dobarganes, M. C. and Márquez-Ruiz, G. (2004). "Antioxidant activity of phenolic compounds in sunflower oil-in-water emulsions containing sodium caseinate and lactose." *Eur. J. Lipid Sci. Technol.*, 106(5), 325–333.

Viskupicova, J., Danihelova, M., Ondrejovic, M., Liptaj, T. and Sturdik, E. (2010). "Lipophilic rutin derivatives for antioxidant protection of oil-based foods." *Food Chem.*, 123(1), 45–50.

Vollmann, J., Eynck, C. (2015). "Camelina as a sustainable oilseed crop: Contributions of plant breeding and genetic engineering." *Biotechnol. J.*, 10, 525–535.

Wanasundara, U. N. and Shahidi, F. (1996). "Stabilization of seal blubber and menhaden oils with green tea catechins." *J. Am. Oil Chem. Soc.*, 73(9), 1183-1190.

Wang, H., Liu, F., Yang, L., Zu, Y., Wang, H., Qu, S. and Zhang, Y. (2011). "Oxidative stability of fish oil supplemented with carnosic acid compared with synthetic

antioxidants during long-term storage." *Food Chem.*, 128(1), 93–99.

Wang, J., Gu, S. S., Pang, N., Wang, F. Q., Pang, F., Cui, H. S., Wu, X. Y. and Wu, F. A. (2014). "Alkyl caffeates improve the antioxidant activity, antitumor property and oxidation stability of edible oil." *PLoS ONE*, 9(4), e95909.

Wang, S., Zhu, F., Kakuda, Y. (2018). "Sacha inchi (*Plukenetia volubilis* L.): Nutritional composition, biological activity, and uses." *Food Chem*., 265, 316–328.

Watanabe, Y. (2015). "Antioxidative properties of acyl ascorbates on lipid oxidation in bulk, disperse and microcapsule systems." *Food Sci. Technol. Res.*, 21(5), 639–647.

Watanabe, Y., Ishido, E., Fang, X., Adachi, S. and Matsuno, R. (2005). "Oxidation kinetics of linoleic acid in the presence of saturated acyl l-ascorbate." *J. Am. Oil Chem. Soc.*, 82, 389–392.

Xie, D., Jackson, E.N., Zhu, Q. (2015). "Sustainable source of omega-3 eicosapentaenoic acid from metabolically engineered Yarrowia lipolytica: From fundamental research to commercial production." *Appl. Microbiol. Biotechnol*., 99, 1599–1610.

Xin, W., Wei, W. and Li, X. Y. (2013). "Short-term effects of fish-oil supplementation on heart rate variability in humans: A meta-analysis of randomized controlled trials." *Am. J. Clin. Nutr.*, 97(5), 926-935.

Yang, Y., Song, X., Sui, X., Qi, B., Wang, Z., Li, Y. and Jiang, L. (2015). "Rosemary extract can be used as a synthetic antioxidant to improve vegetable oil oxidative stability." *Ind. Crops Prod.*, 80, 141–147.

Yeşilsua, F. A. and Özyurt, G. (2019). "Oxidative stability of microencapsulated fish oil with rosemary, thyme and laurel extracts: A kinetic assessment." *J. Food Eng.*, 240, 171-182.

Yin, X., Dong, H., Cheng, H., Ji, C. and Liang, L. (2022). "Sodium caseinate particles

with co-encapsulated resveratrol and epigallocatechin-3-gallate for inhibiting the oxidation of fish oil emulsions." *Food Hydrocoll.*, 124, 107308.

Zamani Ghaleshahi, A., Ezzatpanah, H., Rajabzadeh, G., Ghavami, M. (2020). "Comparison and analysis characteristics of flax, perilla and basil seed oils cultivated in Iran." *J. Food Sci. Technol*., 57, 1258–1268.

Zamani-Ghaleshahi, A., Rajabzadeh, G., Ezzatpanah, H., Ghavami, M. (2020). "Biopolymer coated nanoliposome as enhanced carrier system of perilla oil." *Food Biophy*. 15, 273-287.

Zeb, A. (2012). "Thermal degradation of β-carotene in food oils." In: *Vitamin A and Carotenoids*, V. R. Preedy eds., Cambridge, UK: Royal Society of Chemistry Public., 129-139.

Zettel, V.; Hitzmann, B. (2018). "Applications of chia (*Salvia hispanica* L.) in food products." *Trends Food Sci. Technol*., 80, 43–50.

Zhang, J., Zhang, Z., Xu, W., Fan, H., Wu, X., Dong, J. (2022). "Application of response surface methodology for analysis of reheat steam temperatures in a double reheat coal-fired boiler." *J. therm. Sci*., 31, 2203-2215.

Zhang, Y. Y., Zhang, F., Thakur, K., Ci, A. T., Wang, H., Zhang, J. G. and Wei, Z. J. (2017). "Effect of natural polyphenol on the oxidative stability of pecan oil." *Food Chem. Toxicol.*, 119, 489–495.

Zhang, Y., Yang, L., Zu, Y., Chen, X., Wang, F. and Liu, F. (2010). "Oxidative stability of sunflower oil supplemented with carnosic acid compared with synthetic antioxidants during accelerated storage." *Food Chem.*, 118(3), 656–662.

Zhang, Z.-S., Liu, Y.-L., Che, L.-M. (2018). "Characterization of a New α-Linolenic Acid-Rich Oil: *Eucommia ulmoides* Seed Oil." *J. Food Sci*., 83, 617–623.

Zhu, S., Li, Y., Li, Z., Ma, C., Lou, Z., Yokoyama, W. and Wang, H. (2014a). “Lipase-catalyzed synthesis of acetylated EGCG and antioxidant properties of the acetylated derivatives”. *J. Food Res. Int.*, 56, 279–286.

Zhu, X.-M., Zou, X.-G., Xia, X., Li, J., Cao, J., Li, H. and Deng, Z.-Y. (2014b). “Effect of fatty acid and tocopherol on oxidative stability of vegetable oils with limited air.” *Int. J. Food Prop.*, 18(4), 808–820.

Zuk M, Richter D, Matuła J, Szopa J (2015). “Linseed, the multipurpose plant.” *Ind Crops Prod* 75: 165–177.

Zunin, P., Leardi, R., Bisio, A., Boggia, R. and Romussi, G. (2010). “Oxidative stability of virgin olive oil enriched with carnosic acid.” *Food Res. Int.*, 43(5), 1511–1516.

Zuta, P. C., Simpson, B. K., Zhao, X. and Leclerc, L. (2007). “The effect of α-tocopherol on the oxidation of mackerel oil.” *Food Chem.*, 100(2), 800–807.

LIST OF PUBLICATION BASEDON PRESENT WORK

1. **Sumit Kumar Mishra**, Prasanna D. Belur, Regupathi Iyyaswami (2020). "Review on Use of antioxidants for enhancing oxidative stability of bulk edible oils." *International Journal of Food Science and Technology,* 56(1) 1-12. Q1 Journal with IF: 3.612
2. **Sumit Kumar Mishra**, Prasanna D. Belur, Regupathi Iyyaswami (2022). "Comparison of efficacy of various natural and synthetic antioxidants in stabilising the fish oil." *Journal of Food Processing and Preservation,* 46(11), e16970. Q2 Journal with IF: 2.609
3. **Sumit Kumar Mishra**, Chandrasekar V., Prasanna D. Belur, Regupathi Iyyaswami (2023). "Development of a synergistic mixture of natural antioxidants through statistical approaches for enhancing the oxidative stability of Sardine oil." *Journal of Oleo Science* – Under Review. Q3 Journal with IF: 1.628

CURRICULUM VITAE

SUMIT KUMAR MISHRA

E-mail ID -: sumit.mishra789@gmail.com

ACADEMIC DETAILS

I. **Ph.D. Chemical Engineering -** (2017-2023)

National Institute of Technology, Surathkal, Karnataka, India

- Advisor -: Dr. Prasanna B.D. (Professor) and Dr. I. Regupathi (Professor)
- Thesis -: "Study of oxidative stability of sardine oil in the presence of antioxidants."

II. **Master of Technology in Biotechnology -** (2013-2015)

Birla Institute of Technology, Mesra, Ranchi, India

- Project title -: Optimization of vinblastine production from bacterial endophyte of *Catharanthus roseus.*
- Score -: **7.49 CGPA**

JOURNAL PUBLICATION

1. Sumit Kumar Mishra, Prasanna D. Belur, Regupathi Iyyaswami (2020). "Review on Use of antioxidants for enhancing oxidative stability of bulk edible oils." *International Journal of Food Science and Technology,* 56(1) 1-12. Q1 Journal with IF: 3.612
2. Sumit Kumar Mishra, Prasanna D. Belur, Regupathi Iyyaswami (2022). "Comparison of efficacy of various natural and synthetic antioxidants in stabilising the fish oil." *Journal of Food Processing and Preservation,* 46(11), e16970. Q2 Journal with IF: 2.609
3. Sumit Kumar Mishra, Chandrasekar V., Prasanna D. Belur, Regupathi Iyyaswami (2023). "Development of a synergistic mixture of natural antioxidants through statistical approaches for enhancing the oxidative stability of Sardine oil." *Journal of Oleo Science* – Under Review. Q3 Journal with IF: 1.628
4. Singh, S., Kamal, E., Mishra, S. K., Pal, A., and Sharma, Y. (2015). "Fermentation of

rice bran with *Saccharomyces cerevisiae* generates metabolites with antimicrobial activity". Biotech Today: An International Journal of Biological Sciences, 4(2), 22-27

5. Khan, J. A., Mishra, S. K., Vandana, K., (2012). "Screening of antibacterial properties of *Stevia rebaudiana*". International Journal of Biology, Pharmacy and Allied Sciences, 1(10), 1517-1523.

PROJECT AND TRANINGS

1. **Project, MRD Life Science, Lucknow**
   Screeing of antibacterial properties of *Stevia rebaudiana*.
2. **Traning, MRD Life Sciences, Lucknow**
   Advanced Biotech Tenchniques – Microbiological techniques, Total protein isolation, Estimation and enzyme assay, SDS-PAGE, Western blotting, Restriction digestion, Ligation and Transformation, Cloning and Gene expression, DNA/RNA isolation, Gel electrophorasis, Sourthen/Northern blotting, Fermentation, Downstream processing, Chromatography, Gel Doc system, and Polymerase Chain Reaction.

CONFERENCES PROCEEDINGS

1. Mishra, S. K., Anjum, N., Chandra, R. (2014). "Optimization of vincristine and vinblastine production from bacterial endophyte of *Cathranthus roseus.*" National conference on empowering mankind with microbial technologies (AMI-EMMT 2014), Tamil Nadu Agricultural University, Coimbatore, India.

LABORAATORY SKILLS

- Chromatographic skills such as HPLC-ESI-MS, Gas Chromatography (GC), and (Fast Protein Liquid Chromatography) FPLC.
- Rotatory evaporator, Pressure homogenizer, Rolling ball viscometer, Density meter, Refractrometer, Tensiometer, Coulometer, Ultra sonicator.
- Analytical techniques for performing oxidation studies.

Declaration:

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

SUMIT KUMAR MISHRA