JAVIER HERNÁN FALCONÍ HEREDIA

ASSESSMENT OF NATURAL AND PYROLYTIC ANTIOXIDANTS AS ADDITIVES TO BIODIESEL PRODUCTION

Thesis submitted to the Agricultural Engineering Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae.*

Adviser: Marcio Arêdes Martins

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APPROVED: March 10, 2022.

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Adviser

This thesis is dedicated,

To my beloved fiancée Adriana

To my parents, Isabel, and Galo

To my brothers, Galo, and Daniel

To my nephews, Leonardo, Federico, Maria Isabel, Pedro, and Ignácio

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BIOGRAPHY

JAVIER HERNÁN FALCONÍ HEREDIA, son of Isabel Heredia and Galo Falconí, was born in Quito-Pichincha (Ecuador), on May 29, 1989.

In March 2009 he starts his undergraduate on Biotechnological Engineering at Universidade Estadual Paulista (UNESP), at the Faculty of Sciences and Letters, and graduated in March 2014. In June 2014 worked as assistant professor on the course in Agroindustrial Engineering at Universidad de Las Américas.

In March 2017 he began his Master in the Agricultural Engineering Program at the Universidade Federal de Viçosa, in the area of Energy in Agriculture. In February of 2019 concluded the dissertation entitled “Vinasse as a source of nutrients for the cultivation of microalgae of the phylum Chlorophyta”.

In March of 2019 he initiated his doctorate in the Agricultural Engineering Program at the Universidade Federal de Viçosa, in the area of Energy in Agriculture. The thesis was submitted to the committee in March 2023, to obtain the Doctor Scientiae degree in Agricultural Engineering.

As we journey through the vast expanse of the galaxy, let us never forget the importance of science in our quest for knowledge. It is the relentless pursuit of scientific discovery that reveals the mysteries of the universe and helps us understand the power of the Force. Science is not just a discipline of reason, but also a catalyst for imagination and adventure. It gives us the tools to explore the unknown and reach for the stars, inspiring us to push the limits of our understanding and unleash the full potential of our minds. So, let us embrace science and the wonders it holds, and may the Force be with us always." –
Star Wars

GENERAL ABSTRACT

FALCONÍ-HEREDIA, Javier Hernán, D.Sc., Universidade Federal de Viçosa, March 2023. **Assessment of natural and pyrolytic antioxidants as additives to biodiesel production.** Adviser: Marcio Arêdes Martins.

Brazil is one of the largest producers and consumers of biodiesel in the world, with a production of 6.7 million m^3 in 2021. Its main sources for biodiesel production are soybean oil and animal fat. The low oxidative stability of biodiesel is a problem that has not yet been satisfactorily solved and represents the main barrier to market expansion. Therefore, a promising approach to improve oxidative stability is the addition of antioxidants. This study evaluated the effect and performance of natural extracts of *Scenedesmus obliquus* (S. obliquus), *Haematococcus pluvialis* (*H. pluvialis*), *curcuma* sp. (turmeric), *Bixa Orellana* (annatto) seeds, coffee husk (C. husk), and commercial *Haematococcus pluvialis* (C.*H. pluvialis*) as antioxidants during long-term storage of biodiesel matrices from soybean (*Glycine max*), canola (*Brassica napus*), and macauba (*Acrocomia aculeata*). In addition, the antioxidant properties of bio-oils produced from the slow pyrolysis of biodiesel synthesis of soybean, canola, macauba, and depigmented *S. obliquus* biomass were also investigated. Among the natural extracts, turmeric and *S. obliquus* stood out, which at the concentration of 3000 ppm produced increased stability time (35.0% for soybean, 51.8% for canola, and 338.4% for macauba) and (36.0% for soybean, 46.7% for canola, and 122.0% for macauba) respectively. Furthermore, the addition of *S. obliquus* bio-oil at a concentration of 4000 ppm produced an increase in stability time of 116.8 % (13.2 h) for soybean, 194.5% (17.7 h) for canola, and 563.5% (13.21 h) for macauba, which meets the Brazilian standard requirement (ANP resolution no. 798/219) of a minimum stability time of 12 hours for biodiesel.

Keywords: Biodiesel. Catalysis. Natural antioxidants. Microalgae. Oxidation stability. Pyrolysis antioxidant.

RESUMO GERAL

FALCONÍ-HEREDIA, Javier Hernán, D.Sc., Universidade Federal de Viçosa, março de 2023. **Avaliação de antioxidants naturais e pirolíticos como aditivos para a produção de biodiesel.** Orientador: Marcio Arêdes Martins.

O Brasil é um dos maiores produtores e consumidores de biodiesel do mundo, com uma produção de 6,7 milhões de m^3 em 2021. Suas principais fontes para a produção de biodiesel são o óleo de soja e a gordura animal. A baixa estabilidade oxidativa do biodiesel é um problema que ainda não foi satisfatoriamente resolvido e representa a principal barreira à expansão do mercado. Portanto, uma abordagem promissora para melhorar a estabilidade oxidativa é a adição de antioxidantes. Este estudo avaliou o efeito e o desempenho de extratos naturais de *Scenedesmus obliquus* (*S. obliquus*), *Haematococcus pluvialis* (*H. pluvialis*), *curcuma* sp. (turmeric), sementes de *Bixa Orellana* (annatto), casca de café (C. husk), e *Haematococcus pluvialis* comercial (C.*H. pluvialis*) como antioxidantes durante o armazenamento de longo prazo de matrizes de biodiesel de soja (*Glycine max*), canola (*Brassica napus*), e macauba (*Acrocomia aculeata*). Além disso, as propriedades antioxidantes dos bio-óleos produzidos a partir da pirólise lenta da síntese de biodiesel de soja, canola, macauba e biomassa de *S. obliquus* despigmentada também foram investigadas. Entre os extratos naturais, destacam-se o açafrão-da-terra e o *S. obliquus*, que na concentração de 3000 ppm produziu maior tempo de estabilidade (35,0% para a soja, 51,8% para a canola e 338,4% para a macauba) e (36,0% para a soja, 46,7% para a canola e 122,0% para a macauba), respectivamente. Além disso, a adição de óleo biológico de *S. obliquus* a uma concentração de 4000 ppm produziu um aumento no tempo de estabilidade de 116,8% (13,2 h) para soja, 194,5% (17,7 h) para canola, e 563,5% (13,21 h) para macaúba, o que atende ao requisito da norma brasileira (resolução ANP nº 798/219) de um tempo mínimo de estabilidade de 12 horas para biodiesel.

Palavras-chave: Biodiesel. Catálise. Antioxidantes naturais. Microalgas. Estabilidade de oxidação. Antioxidante pirolítico.

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SUMMARY

1. General introduction

Human population had experienced a dramatic increase over the past decades, reaching 8 billion in 2022 and is projected to increase up to 9.7 billion in 2050, and 10.4 billion in 2100 (UNITED-NATIONS, 2022). The unregulated, disorganized and over exploration of natural resources has led to several complications, energy crisis, deterioration of ecosystems, global warming and diseases associated with climate change (Klein and Anderegg, 2021).

Ensuring harmonious coexistence of humans and the environment, while also achieving sustainable, human development, and economic growth, entail the use of renewable energy sources (Kazemi et al., 2019). Among the various types of biofuels, bioethanol and biodiesel stand out as alternatives to their counterparts of fossil origin used in the transport sector, in particular gasoline and diesel, respectively (Hosseinzadeh-Bandbafha et al., 2018).

Biodiesel can be produced using a wide variety of feedstocks, since the synthesis process involves using materials that contain triglycerides (Sander et al, 2018). The physicochemical properties of biodiesel, such as density, kinematic viscosity, cetane number, are strongly influenced by the individual characteristics of its constituent methyl esters, which in turn depend on the fatty acid content of the feedstock used (Nor et al., 2015). Thus, each feedstock has a unique fatty acid profile, and this is important because it determines the catalytic methodology to be employed, and the efficiency and quality of biodiesel production (Singh et al., 2019).

Brazil, the main sources for biodiesel production are soybean oil and animal fat (ABIOVE, 2021)Meanwhile, in countries such as Canada, Italy, Germany, Finland, and the United Kingdom, the main source for biodiesel production is canola, in Indonesia and Malaysia it is coconut and palm oils, and in the United States it is soybean oil (Ambat et al., 2018). Furthermore, another biodiesel source is Macauba a oleaginous perennial palm native of South America, which over the past year are gaining attention as promising energy crop, due to its low price, been able to grown in different biomes, not compete with food crops, high oil content, and productivity (Ampese et al., 2021; Pires et al., 2023).

The low oxidation stability of biodiesel is a significant drawback that should be considered for long-term storage. Oxidation processes cause changes in the chemical and physical properties of biodiesel, leading to lower fuel quality (Kumar, 2017). The most widely used substances to inhibit oxidative degradation reactions are synthetic antioxidants like butylated hydroxyanisole, tertiary butylhydroquinone and propyl gallate (Chandra and Sharma, 2020; Rial et al., 2020; Sundus et al., 2017; Varatharajan and Pushparani, 2018).

Besides synthetic antioxidants, natural compounds are gaining distinction as potential renewable substitutes (tocopherols, carotenoids, lycopene, zeaxanthin, canthaxanthin, astaxanthin, gallic acid, caffeic acid, vanillin, and feluric acid). (Makwana et al., 2015; Singh et al., 2020). However, the main disadvantage is the high cost for extraction and purification (Dzah et al., 2020; Galanakis et al., 2018)Another promising source of antioxidant compound is the bio-oil from pyrolysis of biomass and wastes (Wu et al., 2018). Pyrolysis is a thermochemical process of biomass breakdown at high temperatures (400-800 °C) in the absence of oxygen and at atmospheric pressure, leading to the production of a solid product (biochar), condensate liquid (aqueous phase and biocrude or bio-oil), and gaseous product (syngas) (Xia et al., 2022). The liquid phase retain large amount of organic acids, aldehydes, ketones, furans, sugar-based components and phenolics, such as phenol, dimethylphenol, guaiacol and catechol reported in the literature for having antioxidant activity (Bautista et al., 2017; García et al., 2017).

This study assessed the antioxidant effect and performance, during long storage time, of natural extract from green microalgae *Scenedesmus obliquus* (*S. obliquus*) and *Haematococcus pluvialis* (*H. pluvialis*), from turmeric (*Curcum*a sp.), annatto seeds (*Bixa Orellana*), Coffee husk (*Coffea arabica*), commercial *Haematococcus pluvialis* (*C.H. pluvialis*) and of organic phase of bio-oil produced form slow pyrolysis of analytic glycerol, crude glycerol from soybean, canola, macauba, and from depigmented biomass of *Scenedesmus obliquus.* Those extracts and bio-oils were tested as alternative additives for soybean canola and macauba biodiesel.

This thesis is organized in three independent chapters: chapter one discussed the influence of feedstock and catalytic process on biodiesel quality, and consider the potential antioxidant activity of microalgae extract as a new source for biodiesel antioxidant; chapter two aims to evaluated the effect of plant and microalgae extracts

as alternative antioxidants to increased stability time on soybean, canola and macauba biodiesel; Lastly, chapter three evaluated the used of pyrolytic organic phase (bio-oil) produced form slow pyrolysis of residues, as new antioxidant source for soybean, canola, and macauba biodiesel.

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2. Chapter 1 - Biodiesel feedstock, catalytic process, and oxidative stability: review

Abstract

The low oxidative stability of biodiesel is an important disadvantage to be considered during long storage periods. Antioxidants usage is the most cost-effective method. The addition of antioxidants is necessary to reach the minimum limit of 12 hours of oxidative stability establish by ANP resolution No 798/219. The antioxidants performance varies depending on the type of biodiesel. Likewise, most published articles tested a restricted number of synthetic antioxidants, however, there is a great diversity of antioxidants that have not yet been studied. The main drawback of commercial synthetic antioxidant is they are produced from non-renewable sources. Natural antioxidant substances derived from plants, microalgae, and fruits constitutes an alternative, given their renewable and less polluting nature. Microalgae stand out as a promising source for antioxidants since the composition of the strains can be regulated to the metabolite of interest by modulating operational variables and nutritional conditions. Moreover, natural antioxidants have not achieved significant commercial success, mainly due to their high costs when compared to synthetic ones. Therefore, in this review are presented and extensible discusses the feedstock, catalytic production processes, and oxidation on biodiesel.

Keywords: Biodiesel. Catalysis. Natural antioxidants. Microalgae.

2.1 Introduction

Global industrialization has been driven primarily by conventional and non-renewable energy sources such as petroleum and natural gas, providing many benefits to various sectors, including transportation (Watts et al., 2018). However, the overexploitation of these resources has led to several complications, such as the deterioration of ecosystems, global warming, and diseases associated with climate change (Kazemi et al., 2019). In addition, when dealing with non-renewable resources, depletion in the next century has been the subject of intense debate (Devi et al., 2019; Heredia Falconí et al., 2021).

In response to these challenges and to ensure the harmonious coexistence of humans and the environment, while also achieving sustainable economic growth and development, the production and use of renewable energy sources, such as biofuels, has shown an expressive growth (Kazemi et al., 2019). Among the various types of biofuels, bioethanol and biodiesel have stood out as alternatives to their counterparts of fossil origin used in the transport sector, in particular gasoline and diesel (Heredia Falconí et al., 2021; Hosseinzadeh-Bandbafha et al., 2018).

The renewable character of biodiesel has motivated research on the use of different sources of raw material of plant and animal origin. The carbon present in vegetable oil or animal fat originates mainly from biogenic carbon dioxide. Thus, the contribution to global warming is much smaller compared to its fossil equivalent (Hosseinzadeh-Bandbafha et al., 2018). In addition, other technical and environmental benefits include greater combustion efficiency, lower sulfur and aromatic compounds, greater biodegradability, higher flash point. Thus, due to the fact that biodiesel and diesel share similar properties, there has been a gradual replacement of diesel in the transport sector (Aghbashlo et al., 2018).

However, despite the advantageous renewable origin and biodegradable nature, the major disadvantage associated with biodiesel is its low oxidative stability. The esters of unsaturated fatty acids, which constitutes the main chemical components of biodiesel, are very reactive towards atmospheric oxygen, and their oxidation reactions lead to production of aldehydes, ketones, polymers, acids, and peroxides which compromise the quality of biodiesel. Consequently, to avoid such problems, it is

necessary to add antioxidant substances to the biofuel (Varatharajan and Pushparani, 2018). Although oxidation cannot be completely prevented by the use of antioxidants, it can be significantly delayed (Chen et al., 2019).

The most widely used substances to inhibit oxidative degradation reactions are synthetic antioxidants, with phenolic antioxidants such as synthetic butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiary butylhydroquinone (TBHQ) and propyl gallate (PG) being the most used. (However, the use of such compounds shows some disadvantages, including high toxicities, high volatility, low biodegradability, low thermal stability (specifically BHT) and are produced from petroleum (Valenga et al., 2019).

The use of natural compounds derived from plants, microalgae, and fruits have been regarded as promising alternatives to synthetic molecules, since substances such as tocopherols, phenolic compounds, flavonoids, terpenes and carotenoids are natural compounds with antioxidant properties (Kimura et al., 2018). However, despite the benefits indicated for the use of antioxidants from renewable sources, which grant greater stability to the oxidation of biodiesel, few studies have been developed to demonstrate the efficiency in inhibiting the oxidation reaction.

Still, few studies are reported on the effectiveness of different mixtures of natural antioxidants added to biodiesel from different oilseed sources and available on the market. Additionally, the extraction and use of antioxidants from the same sources of matter used as a source of oil for biodiesel make it possible to add value to the biofuel production chain.

2.2 Biodiesel

According to resolution No. 789/2019, from the National Petroleum Agency (ANP), biodiesel (B100) is a fuel composed of alkyl esters of long-chain carboxylic acids. It is produced from the transesterification and/or esterification of fatty materials derived from vegetable or animal fats. Biodiesel mainly consists of a mixture of long-chain fatty acid methyl esters (FAME), and it can be commercially produced by reacting triglycerides from renewable sources with short-chain alcohols through transesterification. Biodiesel can be made from a wide variety of feedstocks (refer to **Table 2.1**). Therefore, the availability of feedstocks and economic factors are crucial for biodiesel production. When selecting feedstocks, they should ideally meet two requirements: be low-cost, as they represent 75% of the final cost, and enable large-scale production (Chyuan and Silitonga, 2020; Singh et al., 2020).

The availability of feedstocks for biodiesel production depends on several factors, such as regional climate, geographic location, local soil conditions, and agricultural practices in each country. Brazil is one of the largest producers and consumers of biodiesel in the world with production of 3.8 million m^3 in 2016, 4.30 million m^3 in 2017 and 5.33 million m^3 in 2018, 5,90 million m^3 2019, 6,43 million m^3 2020, 6,75 million m^3 in 2021.

From **figure 2.1,** it can be seen that in Brazil, the main sources for biodiesel production are soybean oil and animal fat (ABIOVE, 2021)Meanwhile, in countries such as Canada, Italy, Germany, Finland, and the United Kingdom, the main source for biodiesel production is canola, in Indonesia and Malaysia it is coconut and palm oils, and in the United States it is soybean oil (Ambat et al., 2018). In addition, Karanja and Jatropha are considered future biodiesel feedstocks in India(Singh et al., 2020).

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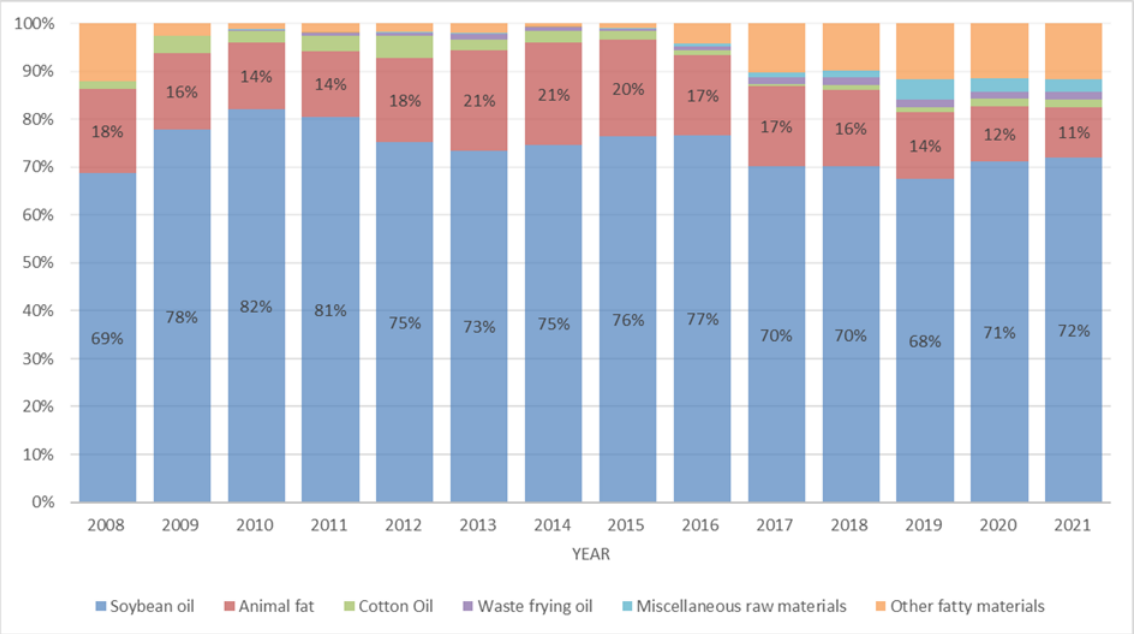

Figure 2. 1. Biodiesel production by feedstock in percent in Brazil. Source (ABIOVE, 2021)

2.3 Feedstock

Biodiesel can be produced using a wide variety of feedstocks (**Table 2.1**), since the synthesis process involves using materials that contain triglycerides (Sander et al., 2018a). The physicochemical properties of biodiesel, such as density, kinematic viscosity, cetane number, are strongly influenced by the individual characteristics of its constituent methyl esters, which in turn depend on the fatty acid content of the feedstock used (Nor et al., 2015). Thus, each feedstock has a unique fatty acid profile, and this is important because it determines the catalytic methodology to be employed, and the efficiency and quality of biodiesel production (D. Singh et al., 2019).

The fatty acid profile in vegetable oils and animal fat shows some striking differences, such as carbon chain length and degrees of unsaturation, as shown in **Table 2.1** (Bankovi et al., 2014; Singh and Singh, 2010). Vegetable oils have greater number of unsaturation in the chain, so they are in a liquid state at room temperature (25 ℃). Animal fat, on the other hand, because it contains a lower percentage of unsaturation, can be present in a solid state at 25 ℃. (Adewale et al., 2015). In addition, the degree of unsaturation exerts a great influence on the characteristics of biodiesel, since an increase in the number of unsaturation results in a decrease in the cetane number and lower oxidative stability, but improves performance at low temperatures (Kumar, 2017).

The feedstocks used for biodiesel production are categorized into three generations. Biodiesel produced from edible oils is termed first generation, the use of non-edible oils as second generation, the use of waste oils as third generation (waste frying oils and microalgae) (Yuan et al., 2020). Although this technology is still in its pioneering stage, it has an unparallel potential for shifting energy generation from fossil resources to renewable sources (Aro, 2016).

Table 2.1.Profile of fatty acids in different raw materials used for biodiesel production

Feedstock	% Mass													
	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:1	20:5	22:00	22:5	22:6
1ª Generation														
Palm	-	0.5-2	39-48	0.3	3-6	35-42	9-12	0.2	-	-	-	-	-	-
Soybean	-	-	6-10	-	2-5	20-30	50-60	5-13	0.23	-	-	0.46	-	-
Sunflower	-	0.08	6.1	0,1	3.26	17.7	72.8		-	-	-		-	-
Canola		0.05	6.23	0.34	2.49	61.46	22.12	5.11	1.43	-	-	0.37	-	-
Corn	-	0.02	12.23	0.13	2.62	31.40	51.21	0.85	0.32	-	-	0.13	-	-
Peanut	-		11		2	50.1	34	1.5	1	-	-	2	-	-
Colza	-		3.5	0.1	0.9	64.1	22.3	8.23	-	-	-	-	-	-
Coconut	46.50	19.50	9.80	-	3	6.90	2.20	-						
Olive	-		13.27	0.86	3.69	68	12.48	0.76	0.48	-	-	0.24	-	-
2 Generation														
rubber seed	-		10.6		8.7	24.6	39.6	16.3	-	-	-	-	-	-
Jatropha	-		14.2		6.9	43.1	34.3	0.15	-	-	-	-	-	-
Linseed			4.4	0.3	3.8	20.7	15.9	54.6	-	-	-	-	-	-
Neen	-	0.26	14.9	0,1	20.6	43.9	17.9	0.4	-	-	-	-	-	-
Cottonseed	-	0.69	21.47	0.56	2.61	18.21	55.45	0.15	0.06	-	-	0.14	-	-
Pongamia pinnata	-	11.65	5.8		5.8	51.5	12.65	-	-	10.95	-	-	-	-
3ª generation														
Chicken fat	-	0.5	24	5.8	5.8	37.8	23.8	1.9	-	-	-	-	-	-
Brown fat	-	1.7	23.8	3.1	12.5	42.4	12.1	0.8	-	-	-	-	-	-
Yellow fat		1.02	14.83	1.50	8.41	47.88	19.11	4.68	2.56	-	-	-	-	-
Fish oil	0.14	5.77	16.94	5.42	4.31	19.20	16.05	2.82	-	-	15,55		2.45	11.36
beef tallow	-	23.3	19.3	-	47.4	2.9	0.9	2.9	0.44	1.88	-	-	-	-
lard		1.41	25.69	2.82	14.50	40.88	12.93	-	0.50	1.34	-	-	-	-
Microalgae (C. Vulgaris)	0.91	6.1	22.6	10.4	21.4	6.9	6.6	14.3	2.3	-	6	-	-	-

Adapted from Singh et al.(2019), Nor et al. (2015), e Bankovi et al. (2014) Sander et al. (2018a), Ambat et al. (2018), Atabani et al., (2013), and Yaşar (2020) **.**

2.3.1 First generation

The first generation currently accounts for more than 95% of the world biodiesel production. The raw materials used are edible vegetable oils such as sunflower, canola, rice bran, soybean, coconut, corn, palm, olive, among others (Silitonga et al., 2016; Yuan et al., 2020). However, there has been much concern and debate over the use of these oils for biodiesel production as they directly compete with food crops, leading to market disruptions and increasing food prices (Ambat et al., 2018). **Table 2.1** and **2.2** presents the fatty acid profile and parameters used in the transesterification process using edible oils.

Coconut oil, *cocos nuceifera*, has a high yield in biodiesel production, is a triglyceride and is characterized by having a low percentage of monounsaturated fatty acids (<6%), polyunsaturated fatty acids (2%) and a high percentage of saturated fatty acids (86%). Thus, it consists mainly of lauric (45%), palmitic (8%) and myristic (17%) acids (Singh et al., 2020). Some of the physical properties such as density, viscosity and calorific value are 914kg m^{-3} (at 15 °C), 27 mm^2 S^{-1} (at 40 °C) and 37,806 MJ kg^{-1}, respectively (D. Singh et al., 2019).

Table 2.2. Parameters used in transesterification of edible vegetable oils and methanol

Feedstock	Catalytic Process	Catalyst (weight/fat weight)	Molar ratio	T (°C)	Time (h)	Yield (%)	Reference
Palm	Basic Homogeneous	KOH 1%	6:1	60	1	88%	Ali and Isis, (2013)
Soybean	Basic Homogeneous	NaOH 0,5%	6:1	60	1	99.1	Churasia et al. (2016)
Sunflower	Basic heterogeneous	CaO 1%	13:1	60	1,6	91.0	Poves et al. (2007)
Canola	Basic heterogeneous	$CaO–La_2O_3$	15:1	65	2,5	96.3	(Maleki et al., 2017)

2.3.2 Second generation

In recent years, several research have been conducted to determine the feasibility of producing biodiesel from non-edible oilseed plants such as *Oleaginous, Leguminosae, Brassicaceae and Euphorbiaceae* (Atabani et al., 2013; Badday et al., 2013). These seeds are characterized by having a high oil content but are not suitable

for human consumption or animal feed due to their high content of free fatty acids and toxic substances.

Some non-edible crops used in biodiesel production are: chestnut (*Jatropha curcas L.*), karanja (*Pongamia pinnata*), rubber seed (*Hevea brasiliensis*), castor bean (*Ricinus communis*), yellow oleander (*Thevettia peruviana*), tobacco (*Nicotiana tabacum L*), Chinese tallow (*Sapium sebiferum*), rice bran (*Oryza sativa L.*), sea mango (*Cerbera odollam Gaertn*), cotton (*Gossypium hirsutum L.),* mahua (*Madhuca indica (J. Koenig*)), *and* Ethiopian mustard (*Brassica carinata A. Braun*) (Ambat et al., 2018).

The use of these oils as feedstock for biodiesel production **(Table 2.3)** has several notable advantages such as, oil plants have the ability to produce large amounts of inedible oil and are widely distributed around the world; they can grow on unsuitable land because they are adapted to arid and semi-arid conditions; require less water and fertilizer for growth, and have no impact on the food chain, they are easily available raw materials in some parts of the world and can reduce of deforestation because they are more environmentally and economically favorable (Aro, 2016; Rezania et al., 2019)

Table 2.3. Parameters used in the transesterification of non-edible vegetable oils and methanol

Feedstock	Catalytic Process	Catalyst (weight/fat weight)	Molar ratio	T (°C)	Time (h)	Yield (%)	Reference
Seed Rubber	Basic Heterogeneous Acid Pre-treatment	CaO 9%	9:1	65	0.6	97.5	Roschat et al. (2017)
Jatropha	Basic Heterogeneous Acid Pre-treatment	Sr^{2+}CaO/MgO 5%	9:1	70	2	99.6	Sudsakorn et al.(2017)
Linseed	Basic Heterogeneous	CaO 0,98%	9,41:1	30	1	98.7	(Gargari and Sadrameli, 2019)
Neen	Nano heterogeneous	CZO 10%	10:1	55	1	97.1	(Gurunathan and Ravi, 2015)
Pongamia pinnata	Basic Heterogeneous Acid Pre-treatment	KOH 1%	6:1	65	3	92.0	Karmee and Chadha. (2005)

2.3.3 Third generation

Third-generation biodiesel comprises microalgae oil, as well as residual and recycled oils generated from food processing in snack bars, commercial and domestic kitchens and industries that process fried food products (Rezania et al., 2019).

Residual oils and fats are classified according to the concentration of free fatty acids: brown fat (<15 FFA) and yellow fat (>15%) (Talebian-kiakalaieh et al., 2013).

Residual oils are considered a more economical alternative due to their availability and generation volume. **Table 2.4** shows the concentration of lipids in the main effluents). Thus, the price ranges from 0.04-0.09 and 0.04-0.014 US$ Kg^{-1} for yellow and brown fat, respectively (César et al., 2017; Sharma et al., 2008).

Table 2.4. Concentration of lipids in some waste effluents

Effluent type Lipid	**Concentration (mg L^{-1})**
Domestic	40-100
Slaughterhouses and poultry	>500
Dairy products	4.680
Restaurants	98
Oil extraction industry	16.000
Ice cream factory	845

Adapted from (Aguiar et al., 2005).

The main source of the generation of large amounts of this oil and fats are the large restaurants and industrial processing plants. In Brazil, the annual production of residual oils and fats is 1.2 million tons (Fonseca et al., 2019). In some cases, these oils can be reused and mixed with animal feed and fertilizers. However, almost all of it is discharged directly into the sewage system.

These residual oils and fats are generated during the frying process, which involves immersing food in oils or fats at high temperatures (150 to 200 °C) in the presence of oxygen (Safari et al., 2018). The frying process leads to the formation of a wide variety of compounds that are directly related to the specific type of oil used. Thus, during the frying process, a series of reactions occur, such as oxidation, hydrolysis, polymerization, isomerization and, evidently, decomposition of the oil into various volatile compounds (Fonseca et al., 2019).

The oxidation reaction that takes place during the frying process leads to the formation of various compounds in the oil, including free fatty acids, conjugated dienes and trienes, peroxides, alcohols, ketones, and aldehydes. Peroxide is one of the main reactive by-products of oxidation, reaching a peak level and gradually decreasing. The primary oxidation reaction rate is influenced by temperature, exposure time, light, oxygen and type of oil (Koh and Surh, 2015). Furthermore, polymerization of the oil occurs when it is repeatedly heated to high temperatures. Together with the polymers, non-volatile polar compounds and triacylglycerol dimers are formed.

The amount of these compounds formed varies according to the type of oil. The increase in these compounds accelerates oil degradation, increases viscosity, reduces heat transfer, produces foam during the frying process, and develops an undesirable color for food (Fonseca et al., 2019). Hydrolysis and oxidation are primarily responsible for the formation of polar compounds such as free fatty acids, monoacylglycerols, diacylglycerols, and polymers among others. Thus, the polar compounds in frying oils are one of the main parameters to evaluate the quality (Zhu et al., 2018).

The production of biodiesel from these raw materials needs additional steps due to the presence of a high amount of free fatty acids (César et al., 2017). In most cases, simple heating and subsequent filtration are sufficient to remove impurities. However, there are cases in which the quality of the oil is compromised due to the decomposition of the raw material, requiring the use of pre-treatments (steam injection, neutralization, evaporation, and vacuum filtration), which prevent the formation of undesirable products and allow an increase in biodiesel yield (Fonseca et al., 2019). **Table 2.5** shows the parameters and catalyst used for third generation biodiesel production.

The main challenge in using these residual matrices is related to harvest logistics and infrastructure. Thus, the problem is due to inherent characteristics of the sewage system itself, as it is widely spread throughout the city, has no quality control system, and does not have a collection system (more than 80% of the waste oils produced in family homes are discarded directly into the sewage system) (Nor et al., 2015).

Table 2.5 Parameters used in the transesterification of residual oils using methanol

Feedstock	Catalytic Process	Catalyst (weight/fat weight)	Molar ratio	T (°C)	Time (h)	Yield (%)	Reference
Chicken fat	Homogeneous acid	H_2SO_4 25%	30:1	50	24	90,1	Adewale et al.(2015)
Fish oil	Basic homogeneous Acid pre-treatment	KOH 1,25%	20:1	150	0,4	96,57	Kumar et al.(2019)
Beef tallow	Heterogeneous Acid Pre-treatment	CaO 5,42% Casca de ovo	39:16	63	3,38	95,94	(Okwundu et al., 2019)
Lard	Basic homogeneous	CH_3OK 1,25%	1:6	65	0,6	96,2	(Ezekannagha et al., 2017)
Waste cooking oil	Acid pre-treatment basic homogeneous	KOH 1%	1:3	60	0,8	94	(Sadaf et al., 2018)

2.3.4 Animal fat

This raw material is generally cheaper than refined oils because, instead of being a primary product, it represents a by-product of the animal agroindustry and because the demand for this product is lower than most common vegetable oils. Thus, during the deboning process in slaughterhouses, waste is generated, such as the trimmings resulting from this operation, which are generally used in the production of meat derivatives. Bones and non-edible fatty parts are sent to rendering plants to be transformed into tallow or animal fat (Sander et al., 2018b). Some raw materials based on animal fat are already used on an industrial scale (chicken fat, tallow, lard) for the production of biodiesel (Singh et al., 2020).

These raw materials offer economic and environmental advantages, since their use can prevent the improper disposal of these residues, minimize negative impacts and maintain food safety in relation to edible vegetable oils. Furthermore, this raw material contains a high content of saturated fatty acids (**Table 2.1**). Therefore, production requires more complex processing techniques. In addition, biodiesel derived from animal fats, due to its high content of saturated fatty acid esters, generally has a cetane number and oxidative stability higher than those observed in vegetable oil biodiesel (**Table 2.5**) (Adewale et al., 2015).

2.3.5 Lard

Basically, it's pork fat in its processed form. Pork fat can be generated by two types of processes: wet and dry. In wet processing, pork fat is boiled or steamed at high temperature, the lard, being insoluble in water, is removed from the surface of the mix (Sander et al., 2018a). In the dry process, lard is exposed to a high temperature in an oven without the presence of water. Thus, these two types of processes generate different products. Moist lard has a more neutral flavor, lighter color, and a high smoke point. Dry lard is brown in color and has a lower smoke point (Sander et al., 2018a).

2.3.6 Tallow

Tallow is characterized by having a content of 50% saturated fatty acids. Furthermore, tallow has a high content of stearic and palmitic acid, which leads to a

high melting point and viscosity, thus being solid at room temperature (Adewale et al., 2014). The animal fats most used as a substrate for biodiesel production are derived from beef or lamb in the form of edible or inedible tallow. The edible form is relatively expensive and has a low FFA content, which makes it a viable substrate for an alkaline transesterification reaction (Ambat et al., 2018).

2.3.7 Fish oil

The fish processing industry generates a significant amount of oil-rich waste products suitable for biodiesel production. The effluent from the fish processing industry contains heads, guts, bones, trimmings, tails, fins, and skin mixed with the wastewater, thus its indiscriminate disposal poses a threat to the environment (R. C. Rodrigues et al., 2020). Thus, between 6-11% of this wastewater represents oil, which can be recovered by simple separation processes. Furthermore, the recovery of this oil can be done specifically by homogenizing the effluent, heating it to a temperature of between 95 to 100 °C for about 15 to20 minutes, filtering and then centrifuging it to separate the water from the oil (Adewale et al., 2015; Papargyriou et al., 2019).

2.3.8 Microalgae

Algae, including both micro and macro, are widely documented in the literature as a very promising source for biodiesel production. They have high photosynthetic efficiency in biomass production, a high growth ratio and productivity, high amount of oil generated compared to edible and non-edible oils, and do not require agricultural land or potable water for growth (Baskar and Aiswarya, 2016). However, the main disadvantage of commercializing algae is the very high cost of production required for oil removal and the use of large-scale bioreactors.

It should be noted that each microalgae species presents a different composition of lipids, carbohydrates and proteins, pigments and other cellular components (**Table 2.6**). Among these components, lipid content is an essential parameter to determine the suitability of the microalgae for commercial biodiesel production. It is reported in the literature that this content ranges from 30 to 80% (Sun et al., 2019).

Table 2.6. Lipid content of microalgae species

Microalgae species	Lipid concentration (%)
Anabaena cylindrica	4--7
Chlamydomonas einhardtii	6
Chlorella vulgaris	49--52
Chlorella pyrenoidosa	38
Chlorella sorokiniana	22--24
Dunaliella bioculata	8
Dunaliella salina	6--25
Nannochloropsis sp.	30
Nannochloropsis. Granulata	28,5
Nannochloropsis oculata	45
Neochloris oleoabundans	35--54
Porphyridium cruentum	9--14
Prymnesium parvum	22--38
Scenedesmus dimorphus	10
Scenedesmus obliquus	30--50
Scenedesmus quadricauda	1--9
Tetraselmis sp.	20--50

Adapted from (Han et al., 2019), (Demirbas et al., 2011) e (B. R. Kumar et al., 2019).

As can be seen in **Table 2.1** the fatty acid profile in microalgae differs from most vegetable oils in that they are quite rich in polyunsaturated fatty acids with four or more double bonds (Deshmukh et al., 2019). Thus, microalgae are the only renewable source of biodiesel capable of meeting the global demand for transportation fuels (Han et al., 2019). **Table 2.7** presents the parameters used for biodiesel production from some microalgae.

Table 2.7. Parameters used in transesterification of microalgae using methanol

Feedstock	Catalytic Process	Catalyst (weight/fat weight)	Molar ratio	T (°C)	Time (h)	Yield (%)	Reference
S*pirulina maxima*	H_2SO_4 acid pretreatment, Basic Homogeneous	KOH 0.75%	9:1	65	0.6	86.16	Rahman et al.(2017)
***Nannochloropsis* sp.**	Acid pretreatment Heterogeneous basic	$Ca(OCH_3)_2$ 3%	30:1	86	3	99.00	(Hwa et al., 2016)
Scenedesmus obliquus	Heterogeneous acid	Oxide mixture Cr/Al 14%	20:1	80	4	98.28	(Guldhe et al., 2017)
Chlorella	Acid homogeneous	H_2SO_4 10%	20:1	90	2	90.00	(Y. Zhang et al., 2015)

2.4 Catalytic biodiesel production processes

Transesterification is the reversible chemical reaction between a triglyceride and a chain alcohol in 1:3 molar ratios, respectively. The products of this reaction are alkyl esters and glycerol, as well as unreacted triglycerides and diglycerides. In addition, various alcohols such as methanol, ethanol, propanol, isopropanol, butanol, or amyl alcohol can be used in biodiesel production (Aransiola et al., 2013). Methanol and ethanol are often employed due to their physicochemical properties (smaller carbon chain and higher polarity), fast reaction with triacylglycerides and easy dissolution of the catalyst (Mohadesi et al., 2019).

Homogeneous catalysis is a process in which the catalyst and the reactants are in the same reaction phase, which may be acidic or basic, depending on the catalyst used. Homogeneous catalysis in an alkaline medium is the most widely used commercial technological route in the world for the production of biodiesel, with NaOH, CH_3ONa and KOH catalysts are often used (Thangaraj et al., 2019). This catalysis stands out for taking place under mild operating conditions: 1atm, temperature 40-60°C, and reaction time ranging from 30-90 minutes, achieving high conversions to fatty acid methyl esters (FAMES) >98 (Baskar and Aiswarya, 2016).

The great disadvantage of this process is related to the presence of water and a high content of FFA in the raw material > 1%, as higher content of FFA contributes to the saponification reaction that reduces the yield of the process (Thangaraj et al., 2019). Additionally, the use of homogeneous catalysts implies the use of acid neutralizing agents in the purification step, producing salts that are dissolved in glycerol (Fonseca et al., 2019). Glycerol, a by-product of the reaction, is produced in large quantities and does not have the purity required for commercialization, becoming an important liquid contaminant from the process (Arumugam and Sankaranarayanan, 2020).

Homogeneous acid -catalyzed esterification usually employs acids such as H_2SO_4, HCl, HNO_3, H_3PO_4 (Fonseca et al., 2019). In addition, in this process it is possible to obtain high FAMES conversions >98%, but specific conditions are necessary, such as alcohol-to-oil molar ratio of 30:1, reaction time greater than 3

hours, temperature greater than 100 °C and a percentage of catalyst ranging from 0.5 to 1% m m^{-1} in relation to the oil (Baskar and Aiswarya, 2016).

This synthesis method is used when raw materials of low quality, free fatty acid content > 5% and high humidity are used, such as oils with a lower degree of refinement or residual oils (frying) (Fonseca et al., 2019). In the acid catalysis process, catalyst residues must be completely eliminated from the product as they represent a serious problem for equipment and may cause engine corrosion (Arumugam and Sankaranarayanan, 2020). The biggest disadvantage of homogeneous catalysts, both alkaline and acidic, is that they cannot be reused or regenerated. The process requires several separation steps, resulting in increased production costs (Mohamed et al., 2019).

On the other hand, the limitations of the homogeneous process can be solved using heterogeneous catalysis, also called contact catalysis. Heterogeneous catalysis occurs when the catalyst is in a phase different from the reaction medium, which is generally a non-soluble solid in a fluid phase (gas or liquid).

In heterogeneous catalysis, the chemical reaction takes place at the interface between the two phases that constitute the reaction system. Therefore, the catalytic performance is related to the basic or acidic properties of the active sites of the solid material (Dal et al., 2019; Mansir et al., 2017). Heterogeneous catalysts can be used pure or supported on a catalytic matrix. In a pure (or bulk) catalyst, all of its mass is made up of active centers. Supported catalysts, on the other hand, consist of an active phase and an inert phase to the catalytic process, which can be a chemically and thermally stable material, such as alumina (Dhawane et al., 2016; Guldhe et al., 2017).

Heterogeneous catalysis has several advantages: use of simpler reactors, avoidance of saponification reaction, greater mechanical resistance of catalysts, and their ability to be separated, recovered, and reused from the reaction mixture, by a simple process such as filtration or centrifugation. This minimizes purification steps and consequently reduces the cost of production (Yuan et al., 2020).

However, heterogeneous catalysis requires longer reaction times (> 10 hours) and high temperatures (> 200 ºC) to achieve conversions greater than 95% FAME (Mardhiah et al., 2017). Thus, the main disadvantage of heterogeneous catalysis is the low reaction rate, which directly influences the reaction yield, due to the formation of a three-phase system in the process (alcohol-oil-catalyst). The low reaction rate is mainly due to the difficulty of reagents' diffusion through the pores of the catalyst (Arumugam

and Sankaranarayanan, 2020). However, this limitation can be overcome by using a co-solvent, such as hexane, tetrahydrofuran and ethanol, which increases the miscibility of the oil and the solvent (Baskar and Aiswarya, 2016).

Thus, a wide variety of compounds have been evaluated as potential heterogeneous catalysts for biodiesel production, such as ion exchange resins, heteropoly acids, acidic and basic zeolites, hydrotalcites, and metallic oxides (Mansir et al., 2017). Among the heterogeneous catalysts used in the synthesis of biodiesel, those of a basic nature have the highest catalytic performance compared to acid catalysts (Nath et al., 2020). Therefore, alkali metal oxides, especially CaO, have attracted much attention due to their relatively high basicity, low solubility in methanol and the possibility of being synthesized from low-cost raw materials such as limestone and calcium hydroxide (Kesserwan et al., 2020).

2.5 Oxidative stability

Biodiesel has significant technical and environmental advantages over its fossil counterpart, diesel. For example, it is renewable, non-toxic, emits smaller amounts of polluting gases during combustion and has greater combustion efficiency, lower sulfur and aromatic compounds, greater biodegradability, and higher flash point (Aghbashlo et al., 2018). However, the low oxidation stability of biodiesel is an important disadvantage to be considered during storage periods. The oxidation process causes changes in the chemical and physical properties of biodiesel, resulting in the loss of fuel quality due to the formation of oxidation products such as aldehydes, alcohols, carboxylic acids, insoluble gums, and sediments The products can cause fouling problems and reduce life of the engine (Kumar, 2017).

The vulnerability of biodiesel to oxidation is mainly related to the presence of polyunsaturated fatty acid chains in the ester molecules. These chains easily react with oxygen when exposed to air, especially in the presence of heat. Generally, the rate of oxidation of fatty acid alkyl esters depends on the number of double bonds and their position in the chain. The greater the number of bis-allyl-methylene groups in biodiesel, the greater the probability of oxidation (Kumar, 2017). Saturated fatty acids, in turn, have good oxidation stability, but negatively impact biodiesel properties associated

with flow, such as viscosity and cloud point, especially at low temperatures (Bing et al., 2020).

Another factor that influences oxidative stability is the storage temperature. It is widely described in the literature that the oxidation rate increases linearly with increasing temperature (Bär et al., 2018). The degradation rate of biodiesel decreases when stored at low temperatures, as reported in the study developed by Leung et al.(2006). However, the commercial use of low temperatures is an economically impractical technique, and storage at room temperature being generally preferred. Thus, under these storage conditions, the influence of temperature is low, and oxidative degradation is minimal. On the other hand, in the engine, specifically in the combustion chamber, biodiesel is subjected to high temperatures, leading to fuel oxidation and the generation of debris (Varatharajan and Pushparani, 2018).

Furthermore, biodiesel that have a high content of diglycerides or glycerol are likely to absorb more water and, consequently, will be subject to hydrolytic degradation. The water content of biodiesel reduces its calorific value, increases the corrosion rate and can serve as a breeding ground for microbes. (Varatharajan and Pushparani, 2018).

The main processes that cause deterioration in the quality of biodiesel are auto-oxidation, photo-oxidation, thermal or enzymatic oxidation, but auto-oxidation is the most common process and can be defined as the spontaneous reaction between free radicals with atmospheric oxygen (R. Kumar et al., 2016).

Biodiesel auto-oxidation is divided into three stages: initiation, propagation, and termination steps, as epicted in **figure 2.2**. In the initiation stage, the is the initial formation of free radicals, generated by initiators such as heat, metals, enzymes and light, which removed hydrogen from the unsaturated fatty acid (Cardoso et al., 2020).

In the second stage, called propagation, the previously formed radicals react with oxygen, forming peroxide radicals, which can break and form aldehydes and ketones (R. Kumar et al., 2016). Furthermore, this peroxide radical can react with biodiesel, forming a hydroperoxide and another free radical, which will react with another fatty acid molecule in biodiesel, thus constituting a chain reaction. In the third stage, termination reactions between two radicals takes place, forming a non-radical product (Silva et al., 2017). Furthermore, in this stage, the formation of secondary oxidation products obtained by splitting and rearrangement of peroxides (epoxides, volatile and non-volatile compounds) occurs. This stage is characterized by strong

odors, changes in color and viscosity (Cardoso et al., 2020; Varatharajan and Pushparani, 2018)

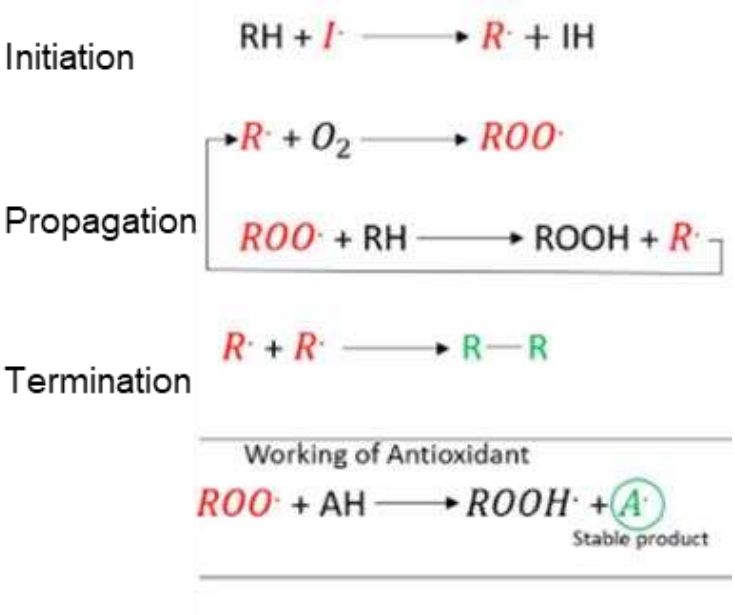

Figure 2.2. Mechanism of triglyceride oxidation and action of primary antioxidants. In which I• is the initiator radical, RH is the unsaturated fatty acid, ROO• and R• are the free radicals formed from fatty acids in biodiesel. Adapted by Singh et al. (2019).

Consequently, due to the great problem of oxidative degradation, a wide variety of oxidative degradation inhibition techniques have been developed, such as vacuum technology, inert gas packaging, reduction of oxygen partial pressure and use of antioxidants (R. Kumar et al., 2016). In addition, there are advanced inhibition techniques based on structural modifications, such as changing the location of the installations, removing hydroxyl groups and converting cis unsaturation into trans (Sundus et al., 2017).

2.5.1 Antioxidants

The use of antioxidants is the most cost-effective method to reach the minimum limit of oxidative stability recommended for commercialization of biodiesel (Agência Nacional do Petróleo, 2019). Although it cannot completely prevent oxidation, it can significantly delay it (Chen et al., 2019).

Antioxidants are classified into primary and secondary based on their mechanism of action (Shearer and Blain, 1966). Primary antioxidants (**Figure 2.2**) are based on interrupting the chain of reactions involved in lipid oxidation by donating

electrons or hydrogens to free radicals (peroxides and hydroperoxides), converting these into more thermodynamically stable products (Chandra and Sharma, 2020). On the other hand, secondary antioxidants retard the auto-oxidation reaction by different mechanisms, which include complexation with metals, oxygen scavenging, decomposition of hydroperoxides to form non-radical species, absorption of ultraviolet radiation or singlet oxygen deactivation (Westhuizen and Focke, 2018).

Antioxidants must be added immediately after biodiesel production, as the oxidative degradation begins, the addition is no longer effective. In addition, an ideal antioxidants must be non-toxic, low volatile, effective at low concentrations, photostable, thermal stable, high soluble in biodiesel, present long shelf life, and be accessible (Sundus et al., 2017).

2.5.2 Concentration of Antioxidants

The effectiveness of antioxidants is directly related to the ideal concentration range, which varieties for each type and substrate used. Therefore, the effect on stability is only significant when the antioxidant reaches the critical concentration. At the same time, there is a saturation concentration, in which successive antioxidant additions do not produce any appreciable improvement in oxidative stability (Lawan et al., 2019; Varatharajan and Pushparani, 2018; Wypych, 2020).

The critical concentration increases with temperature, therefore, a greater amount of antioxidant is required to obtain effective stability at elevated temperatures. Lapuerta et al. (2012) studied the effect of temperature on the stability time in biodiesel derived from animal fat, soybean oil and residual oil with BHT, they observed that a temperature greater than 130 °C required a concentration of 25000 ppm to reach an stability time of. 8 hours.

On the other hand, Moser (2008) reported that the ideal concentration of 700 ppm of α-tocopherol is necessary for soybean biodiesel, successive antioxidant additions do not produce any appreciable improvement in oxidative stability. Consequently, higher concentration of antioxidant additives should be avoided, as don’t have a significant effect on increasing the delay period in combustion, increasing only the overall production cost (Varatharajan and Pushparani, 2018).

2.5.3 **Types of antioxidants**

Antioxidants have varying efficiency in different types of biodiesel, and therefore, it is necessary to screen antioxidants for each specific biodiesel. The main 3 antioxidant are phenolics, amines and thiophenols. Most of the natural antioxidants contain polyphenols, which act similarly to phenolics. However, thiophenols are not commonly used to increase the stability time due to toxicity effects and tend to generate sulfur emissions (VARATHARAJAN; PUSHPARANI, 2018).

There are several main antioxidants used in the industry, including hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), propylgallate (PG), oxytol gallate (OG), dodecyl gallate (DG), pyrogallol (PY) and ethoxyquin (EQ) (Chandra and Sharma, 2020; Rial et al., 2020; Sundus et al., 2017).

BHT is a synthetic phenolic antioxidant widely used in the food and biodiesel industry, characterized by being extremely soluble in animal fat and insoluble in water (Yehye et al., 2015). It is an efficient antioxidant in liquid hydrocarbons, particularly gasoline and low concentration biodiesel blends. However, it is inefficient in protecting pure biodiesel or blends with high content of biodiesel (Yehye et al., 2015). The main disadvantage of BHT is its rapid depletion and low thermal stability. The presence of two butyl groups gives a higher steric hindrance, thus affecting the hydrogen donating ability. Nonetheless, BHT is relatively low-cost compared to the other phenolic antioxidants (Kumar, 2017).

BHA is a white solid with a waxy appearance, produced from the mixture of the isomers 3-*tert*-butyl-4-hydroxyanisole (90%) and 2-*tert*-butyl-4-hydroxyanisole (10%). Similar to BHT, it is soluble in animal fat and insoluble in water (Yehye et al., 2015). In addition, BHA inhibits the growth of gram-positive and gram-negative bacteria and yeasts, thus preventing microbial contamination (Chandra and Sharma, 2020).

BHA is effective in improving the stability of short-chain fatty acids, such as those found in palm and coconut oils, and can be used alone or in combination with another antioxidant, such as BHT and TBHQ, enhancing the antioxidant effect (Yehye et al., 2015). However, one issue with BHA is that it reacts with alkali metals, such as sodium or potassium, present in lipids, and generates a pink color (Varatharajan and Pushparani, 2018).

TBHQ is a very effective antioxidant in stabilizing oils and fats, particularly in polyunsaturated vegetable oils. The hydroxyls positioned in the para position are responsible for stabilizing the radical originating from oxidation reactions. It is stable

when subjected to high temperatures, and it is considered the best antioxidant for frying oils and fried products (Anderson et al., 2016; Zhang et al., 2004). It is slightly soluble in water, and its efficiency in vegetable oils can be increased when combined with citric acid (Mbah et al., 2019; Varatharajan and Pushparani, 2018).

2.5.4 Natural antioxidants

Besides synthetic antioxidants, natural antioxidants are also gaining prominence due to their renewable and less polluting nature. However, their high cost is a significant disadvantage. Vegetable feedstocks with a high concentration of phenols can act as antioxidants in oils and fats. While vegetable oils have natural antioxidants, such as tocopherols, tocotrienols, polyphenols, chlorophylls, ascorbates, lignin, and carotenoids, among others, which prevent the oxidation of fatty acids (Dzah et al., 2020; Galanakis et al., 2018). However, natural antioxidants experiences a drastic reduction during transesterification and biodiesel refining due to inherent characteristics of the process (Lawan et al., 2019).

Some phenolic compounds of plant origin that exhibit antioxidant properties, such as tocopherols, carotenoids (e.g. lycopene, zeaxanthin, canthaxanthin, astaxanthin), gallic acid, caffeic acid, vanillin, ferulic acid, protocatechuic acid, *p*-coumaric acid, eugenol, sesamol, and vanillic acid, are produced commercially on a large scale (Makwana et al., 2015; Singh et al., 2020). However, few studies have been conducted to test these natural antioxidants, except for tocopherols, which are only efficient if the concentration is similar to that found in vegetable oils. At higher concentrations, tocopherols act as a pro-oxidant (Oswell et al., 2018; Varatharajan and Pushparani, 2018).

The second more used compound, after phenolics, to improve the stability is secondary aromatic amines. Aromatic amines antioxidant activity is relate to the formation of nitroxides and benzoquinone imine compounds, which are responsible for the antioxidant properties of secondary aromatic amines (Rashed et al., 2016).

However, aromatic amines tend to generated insoluble products that can cause deposits on pistons and discolor biodiesel during the oxidation process in long-term storage. Therefore, they are not commonly used to ensure oxidation stability in biodiesel. On the other hand, secondary amines have a number of advantages over

phenolic antioxidants, such as greater hydrogen-donating ability, because the N-H bond is not as strong as the O-H bond in phenols (Denisov and Afanas'ev, 2005). For instance, some phenolic antioxidants can capture only two peroxide radicals per molecule, while aromatic amines, depending on the type, can capture 50-500 peroxide radicals per molecule (Varatharajan and Pushparani, 2018).

Moreover, natural antioxidants have not achieved significant commercial success mainly due to high costs compared to synthetic ones (Kumar, 2017; Lau et al., 2022). Another promising source of compound with the ability to improve oxidative stability is using thermochemical processes (Wu et al., 2018). Among them, the pyrolysis of lignocellulosic biomass stands out, which has the ability to produce a liquor (bio-oil) with a large amount of phenolic compounds, such as organic acids, aldehydes, ketones, furans, sugar-based components, and phenolic compounds like phenol, dimethylphenol, guaiacol, and catechol, which are reported in the literature for having antioxidant activity (Bautista et al., 2017).

2.5.5 Secondary antioxidants

Secondary antioxidants act by trapping metal ions or decompose hydroperoxides. Typically, secondary antioxidants are added to biodiesel to improve the shelf life of primary antioxidants, as primary antioxidants can be expensive (WESTHUIZEN; FOCKE, 2018). Chelating agents are one type of secondary antioxidant that form complexes with metal ions, preventing them from participating in the catalytic reaction that cause oxidation. Ethylenediaminetetraacetic acid (EDTA) and its sodium (Na_2EDTA) and calcium ($CaNa_2EDTA$) salts are widely used chelating agents in the food industry, along with citric acid, diethylenetriaminepentaacetic acid (DTPA), and sodium tripolyphosphate (STPP) (Araújo, 2004). The efficiency of chelating agents is inversely proportional to the matrix pH, as metal ion solubility increases at low pH (Decker et al., 2010).

2.6 Microalgae and antioxidants

Microalgae are microscopic organisms typically found in freshwater and marine systems FALKOWSKI *et al*., 2004), with size ranging from 5 to 50 μm. Most species are not visible to the naked eye and only become noticeable when they produce large

populations, called blooms, forming cultures with typically black, green, red, or brown colorations (Kumar et al., 2017; Perumal, 2015; Stern et al., 2010).

Traditionally microalgae classification has been based on the composition of photosynthesizing pigments, chloroplast membrane, organization of thylakoids, chemical nature and arrangement of the cell wall (Jacob-Lopes et al., 2020). However, autological systems consider other criteria such as cytological and morphological characteristics, cell wall constitution and the chemical nature of storage products.

There are various methods used to identify and classify microalgae species, including microscopy for morphological observation, molecular biology using small gene sequences (Groendahl et al., 2017), and a more recent and automated approach called flow cytometry, combined with computational methods (Correa et al., 2017).

Currently, there is no consensus among taxonomists worldwide to use a classification system for microalgae. This is because the polyphyletic nature of the group is somewhat inconsistent with traditional taxonomic groupings, making it difficult to classify microalgae (Malcata et al., 2018). However, one of the most recent classification models considers eight main phyla, including Cyanobacteria in the kingdom *Eubacteria, Euglenophyta* in *Protozoa, Cryptophyta, Haptophyta and Hererokontophyta* in *Chromista; Glaucophyta, Rhodophyta, Chlorophyta* in *Plantae* (Ruggiero et al., 2015).

2.7 Microalgae Diversity

Microalgae are an extremely large and diverse group of microorganisms, and poorly explored relative to other microorganisms such as heterotrophic bacteria and fungi. It is estimated that there are about 200,000 to 800,000 species, of which about 50,000 are known (Norton et al., 1996). The genetic and phenotypic diversity of microalgae is evident due, among other factors, to their wide distribution in the biosphere.

Green microalgae generally grow in freshwater and seawater, while other species of microalgae (chlorophytes) grow in extremely saline environments, such as the large Salt Lake in Utah in the United States and the Dead Sea in Israel. Microalgae are widely distributed throughout the water column in aqueous habitats (Massana et al., 2006). These microorganisms are present in virtually any type of terrestrial

environment, including adverse ones, such as the biotic crust of deserts, in Antarctic snow, and in air at an altitude of 2000 m (KIM,2015).

2.8 Microalgal Pigments

Microalgal pigments are colored chemicals that play an important role in photosynthetic metabolism, CO_2 fixation, protection of cells from damage caused by excessive light exposure, macroscopic staining of algal culture. In addition, they exhibit antioxidant, anticancer, anti-inflammatory and neuroprotective effects (**Table 2.8**) (Koller et al., 2014; Levine and Fleurence, 2018).

Three main groups of pigments are found in microalgae: carotenoids, which include carotenes that provide orange coloration and the xanthophylls responsible for the yellowish hue, (0.1-0.2% dry weight); chlorophyll presents green hue (0.5-1% dry weight); and phycobiliproteins, which have blue-red hue (up to 8% in dry weight) (Ambati et al., 2019).

Specifically, these pigments are found in widely organized structures called photosystem I or reaction center (P7000), and photosystem II (FSII), also named as reaction center (P680), which are interconnected through a series of electron transporters (Nascimento et al., 2018). Photosystems are enzyme complexes that use light as a reducing element, thus producing the driving force required for electron transport.

Table 2.8.Microalgal pigments and potential application areas

	Pigment type	Microalgae producers	Application
Carotenoid	β-carotene	*Dunaliella salina,Dunaliella bardawil, Botryococcus brauni*	Provitamin A, antioxidant, egg yolk coloring
Tocopherol	α-tocopherol	*Chlorella sp., Nannochloropsis oculata, Stichococcus bacillaris, Euglena gracilis*	Vitamin E, food additive, antioxidant in cosmetics and food products
Carotenoid	Bixin	*Dunaliella salina*	Food and cosmetic coloring agent
Carotenoid	Violaxanthin	*Botryococcus braunii, Daniela tertiolecta, Nannochloropsis sp*	Food additive, anti-cancer
Xanthophyll	Astaxanthin	*Haematococcus pluvialis, Botryococcus braunii*	Food additive, antioxidant, fish farming (coloring of salmon)
Xanthophyll	Lutein	*Chlorella protothecoides, Chlorella zofingiensis, Botryococcus braunii, Chlorococcum citriforme, Dunaliella salina, Muriellopsis sp., Neospongiococcum gelatinosum*	Food additive, egg yolk coloring, pigmentation of animal tissues

Xanthophyll	Zeaxanthin	*Nannochloropsis oculata, Nannochloropsis gladitana, Botryococcus braunii, Dunaliella salina.*	Food additive, pharmaceutical use (colon cancer)
Carotenoid	Canthaxanthin	*Nannochloropsis oculata, Nannochloropsis salina, Nannochloropsis gladitana*	Animal food additive
Xanthophyll	Phycocyanin	*Arthrospira, Spirulina*	Food coloring (beverages, ice cream, candy), cosmetic. Used in immunofluorescence techniques; markers for antibodies, receptors and other biological molecules
Carotenoids	Phycoerythrocin	*Cionobacteria, Porphyridium*	immunofluorescence techniques; antibody labeling.
Xanthophyll	Chlorophyll a	All algae	Pharmaceuticals and cosmetics

Adapted from Begum et al. (2016), Cuellar-bermudez et al. (2015); Koller et al. (2014); Richmond e Hu (2013; Rizwan et al. (2018).

2.8.1 Carotenoids

Are polyunsaturated hydrocarbons derived from tetraterpenes, composed of eight isoprene units that make up 40 carbon polyene structure. They constitute one of the widest groups of natural pigments, with over 700 described molecules produced by a wide variety of photosynthesizing organisms and microorganisms such as: plants, microalgae, fungi, bacteria, among others (Novoveská et al., 2019)

Despite the wide diversity, less than 30 carotenoids play an important role in photosynthesis. Most are located in the membrane of the thylakoids attached to the photosystems (Gong and Bassi, 2016).

Chemically, carotenoids can be classified into two major groups: hydrocarbon carotenes that have no substitution or oxygen in their structure (α-carotene, β-carotene, lycopene) and xanthophylls (or oxycarotenoids) have oxygen in their structure (astaxanthin, luitein, canthaxanthin) (KIM, 2015). Some common structures of carotenoids are shown in **Figure 2.3**

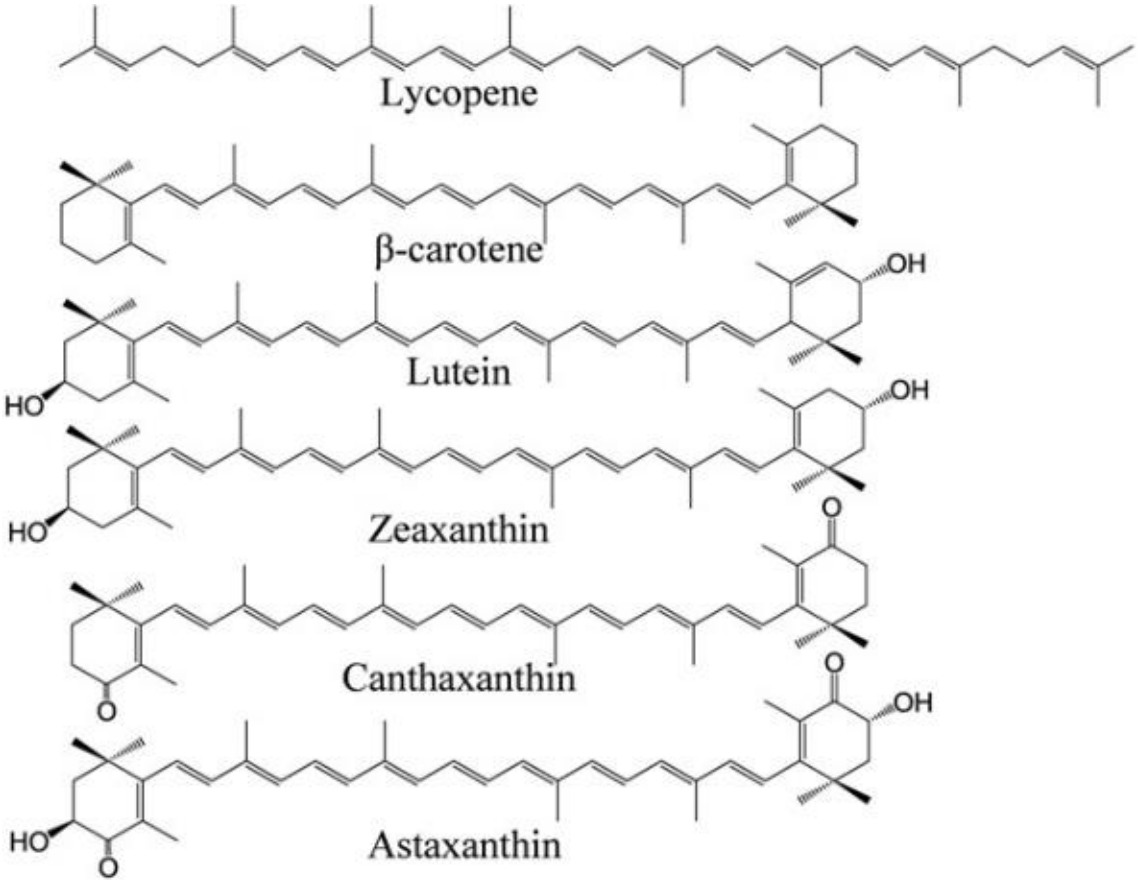

Figure 2.3. Chemical structure of carotenoids found in microalgae (Gong and Bassi, 2016).

These pigments absorb electromagnetic energy in spectral ranges where chlorophylls are unable to absorb light energy, mainly at wavelengths between 400 and 500 nm (Begum et al., 2016; Koller et al., 2014). Lutein, the primary carotenoid, serves as an accessory pigment that transmits absorbed energy to chlorophyll a, thereby expanding the light absorption spectrum (Low et al., 2020).

On the other hand, astaxanthin and canthaxanthin (secondary carotenoids) are produced in the chloroplast and stored in the cytoplasm. In addition, they have a photo-protective function. The mechanism of action involves the formation of a protective layer when cells are exposed to high light intensity condition. Specifically, these carotenoids accept excess energy from chlorophyll and dissipate it. This type of stress provides a pink to red coloration (Novoveská et al., 2019).

Microalgae can synthesize large amounts of carotenoids that act as colorants and food supplements, such as β-carotene, astaxanthin, canthaxanthin, and phycobiliproteins. Thus, microalgae have become an attractive option to be explored in industries as a source of natural colorant and supplement due to the emergence of

health safety problems caused by synthetic products, such as allergic reaction and hyperactivity in humans (Ying et al., 2020).

2.8.2 Chlorophyll

Chlorophyll is a naturally occurring green-colored photosynthetic pigment found in a wide range of organisms, including plants, algae, and cyanobacteria. It primarily absorbs light in the blue region and, to a lesser extent, in the red region of the electromagnetic spectrum, specifically within the range of 430-662 nm (Koller et al., 2014).

Chlorophyll is a complex molecule belonging to the class of porphyrins, characterized by the presence of four pyrrole rings and one isocyclic ring (Levine and Fleurence, 2018). These rings are connected by methylene bridges, and the molecule contains a magnesium atom in the center, coordinated to the rings as shown in **Figure 2.5**. Furthermore, the molecule has a propionic acid chain esterified with phytol (an acyclic diterpene alcohol) at C-17, making it lipophilic (Fernandes et al., 2017).

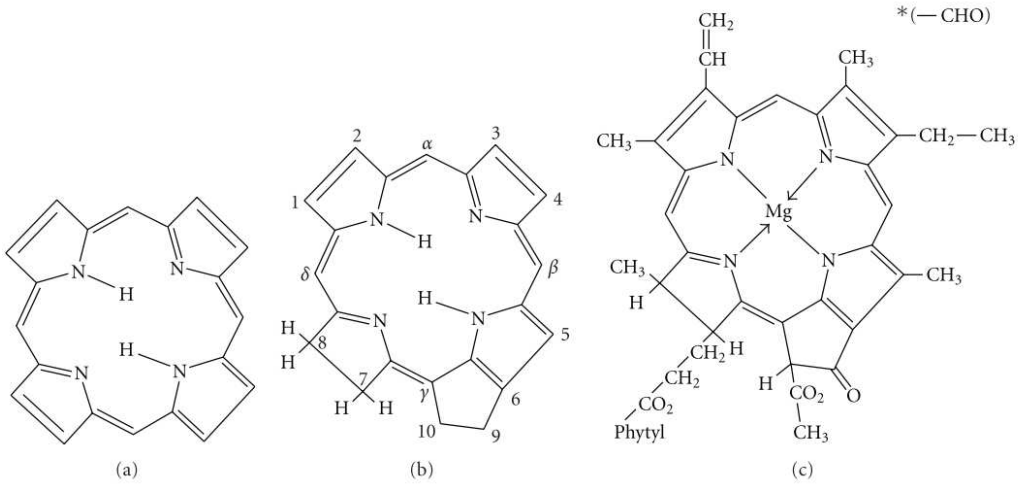

Figure 2.4. Chemical structure of chlorophyll and its constituents. (a) porphyrin macrocycle, (b) phorbine, (c) chlorophyll a (Halim et al., 2010)

Upon receiving a photon, the chlorophyll changes into an excited state (it has more energy than the basic state). This energy can be lost in the form of heat or, if it is not used in some other way, can be radiated as red fluorescent light. Consequently, chlorophyll not only has a specific absorption spectrum, but also a characteristic fluorescence spectrum. In photosynthesis, energy is transported in the form of

chemical potential, allowing electron transfer from one molecule to another (Lehmuskero et al., 2018; Richmond and Hu, 2013). Therefore, chlorophyll is a key molecule in the process of photosynthesis and is vital for plant biomass production.

There are several types of chlorophylls in microalgae: chlorophyll *a, b, c, d,* and *f*, which can be seen in **figure 2.6**. Thus, the different types of chlorophylls exhibit slight differences in absorption spectrum and, consequently, in hue. Chlorophyll a is the most abundant exhibiting a greenish-blue hue present in all microalgae. Chlorophyll b exhibits a bright green hue, unique to the phylum *chlorophyta*. The difference between chlorophyll a and b is that the former has a methyl group at the R2 position, while the other has an aldehyde group (Halim et al., 2010; Levine and Fleurence, 2018).

Chlorophyll c has no phytol chain and there are two types (C_1 and C_2), which are differentiated by the groups at the R3 position, being CH_2-CH_3 and CH-CH_2 respectively. These chlorophylls have a yellow-green hue and are unique to the phylum *rhodophyta* (KIM, 2015). Chlorophyll *d* differs from others due to the presence of -CHO group at the R3 position; this chlorophyll has a bright forest-green hue present in red microalgae. Finally, chlorophyll *f* has an emerald green hue (Levine and Fleurence, 2018).

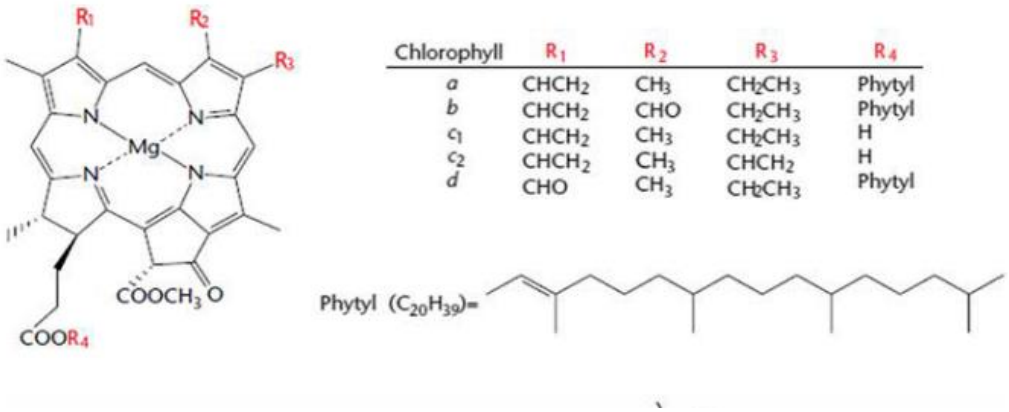

Figure 2.5. Structure of chlorophylls a, b, c, d e f (Levine and Fleurence, 2018).

2.8.3 Phycobiliproteins

Phycobiliproteins or phycobilins are water-soluble of protein nature, present in cyanobacteria (blue-green, prokaryotic algae), rhodophytes (red, eukaryotic algae), cryptomonads (unicellular, biflagellate eukaryotic algae), and cyanellae (endosymbiotic) (Levine and Fleurence, 2018; Sekar, 2008).

These are composed of a subunit heterodimer (α and β), each with a molecular mass of 15 to 20 kDa for 160-165 amino acid (Li et al., 2019). In addition, they are characterized by the presence of a protein group to which linear tetrapyrrole chromophores are covalently attached (as noted in **Figure 2.7**). All phycobiliproteins contain phycocyanocin or phycoerythrobilin chromophores and may also contain one, two or three smaller bilines; each biline presents unique spectral characteristics that can be further modified by interactions of the subunits and the chromophore with the proteic group (Manirafasha et al., 2016).

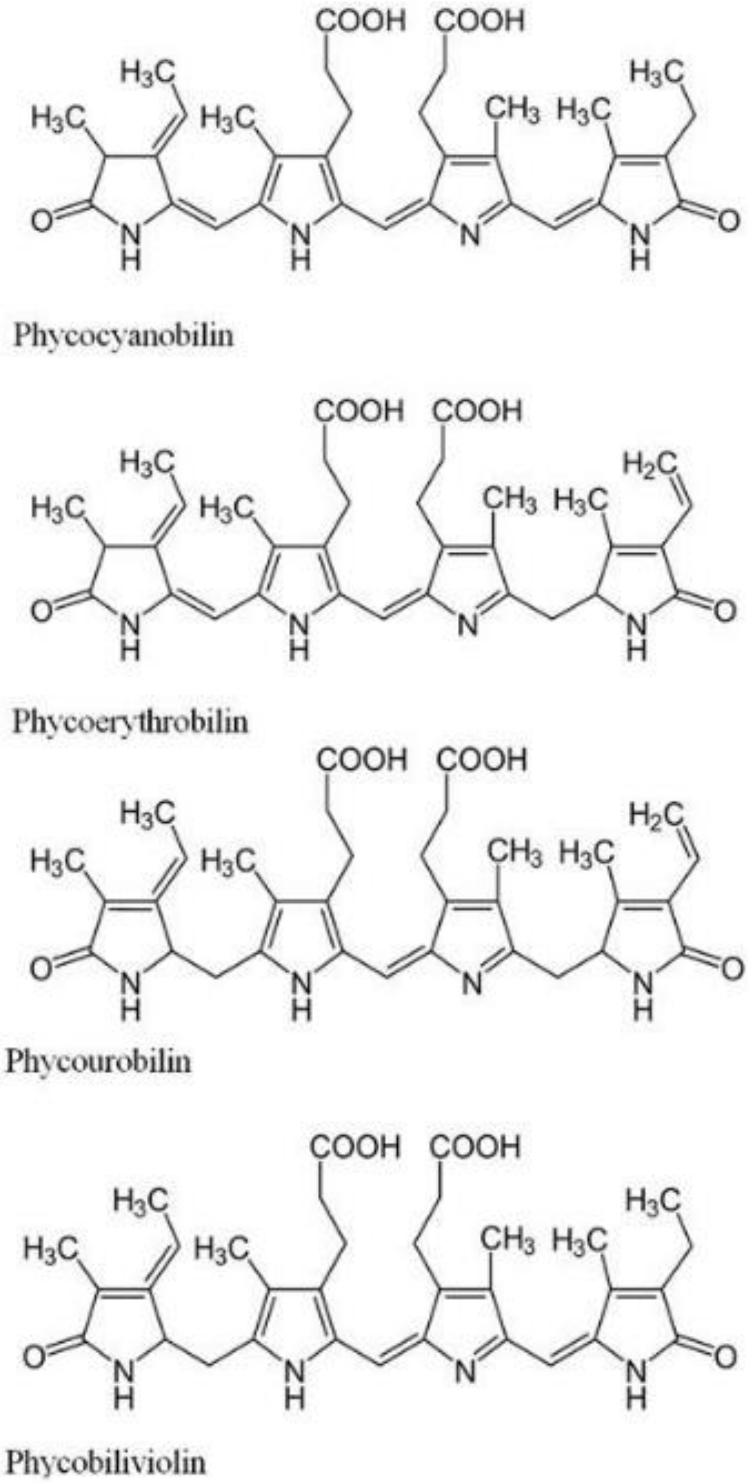

Figure 2.6. Chemical structure of the major phycobiliproteins (Manirafasha et al., 2016).

In many algae phycobiliproteins are organized into sub-cellular complexes called phycobilisomes and located in regular arrays on the outer surface of thylakoid membranes (Pagels et al., 2019). These structures allow the pigments to be geometrically arranged in a way that helps to optimize light capture and energy transfer

(Pagels et al., 2019). All phycobiliproteins directly absorb incident light, but also participate in the energy transfer chain within the phycobilisome. The energy transfer occurs from phycoerythrocin, phycocyanin, allophycocyanin, and finally to chlorophyll *a* (Manirafasha et al., 2016)

Phycobiliproteins can be classified according to their spectral characteristics into four groups: phycoerythrocin, phycoerythrocyanin, phycocyanin, and allophycocyanin (**Table 2.9**).

Table 2.9. Characteristics of phycobiliproteins

Phycobiliproteins	Absorption Spectrum (nm)	Color	Activity
Ficoeritrin	490-570	Red	Pharmaceutical and cosmetics
Phycoerythrocin	560-600	Orange	
Phycocyanin	610-625	Blue	Immunofluorescence, antibody labeling
Allophycocyanin	650-660	Green-blue	food coloring

Adapted by SAINI et al. (2018).

2.8.4 Phycocyanin

Phycocyanin is a blue toned pigment found in cyanobacteria, rhodophytes, and cryptophytes; it is mainly present in *Arthrospira* species, standing out within *Spirulina platensis* and *Spirulina platensis* maximus groups due to the ability to produce high amounts of phycocyanin (Manirafasha et al., 2016). Besides *Arthrospira* species, there are other microalgae that are used to produce phycocyanin, such as *Pyrophyridium* sp., *Synechocystis* sp., *Phormidium ceylanicum, Limnothrix* sp., S*. lividus*.

Two types of phycocyanins are reported in the literature: R-phycocyanin and C-phycocyanin (more widely distributed). Both phycocyanin pigment subunits, α and β, are linked to the phycocyanobilin chromophore (PCB), however, R-phycocyanin also has the phycoerythrobilin chromophore (PEB) linked to the β-subunit (Dumay et al., 2014). Among phycobiliproteins, phycocyanins, specifically C-phycocyanin, stands out for presenting antioxidant, anti-inflammatory, antiviral, anticancer, and radical scavenging properties (Jiang et al., 2017).

In addition, phycocyanine is recognized as safe (GRAS) and used as a colorant in the formulation of not only food products, such as desserts, candies, cake

decoration, milk-shakes, and also in cosmetics, alcoholic beverages, biotechnology products, among others (Mohammadi-gouraji et al., 2019).

The use of phycocyanin is linked to its degree of purity, this being obtained through the ratio of absorbances read at wavelengths A_{620}/A_{280} that correspond to phycocyanin and total protein (Fernández-rojas and Hernández-juárez, 2014; Li et al., 2019). The application usage is defined according to the values obtained by the ratio above, 0.7 for food, 0.7-3.9 for reactive grade and 4.0 for analytic grade (Fernández-rojas and Hernández-juárez, 2014).

2.8.5 Phycoerythrocin

Phycoerythrocyins are primarily responsible for the ability of red seaweeds to grow abundantly in deep water, as they possess an efficient light absorption spectrum of 450 to 570 nm. Therefore, red seaweeds are often found in shallower areas of intertidal region (V. Kumar et al., 2016)

Phycoerythrocin can be divided into four groups according to the absorption spectrum and occurrence in different algal species: β-phycoerythrocin has maximum absorption in the range of 546-565 nm and the estimated market price of $50 per kilogram; R-phycoerythrocin presents maximum absorption peaks at wavelengths of 545,568 and 499 nm; C-phycoerythrocin reaches maximum absorption at 545 nm; and b-phycoerythrocin at 545 nm (Andrade et al., 2020; Levine and Fleurence, 2018). In addition, the purity of phycoerythrocin is obtained using the ratio of A565/A280 wavelengths (Sudhakar et al., 2019).

2.8.6 Allophycocyanin

Allophycocyanin (A-PC) has a blue-green coloration and is present in rhodophyceae, cyanophyceae, and chlorophyceae. Despite having the same applications as C-PC, A-PC has received little attention due to some challenges faced in processing, such as: difficulty in primary extraction, minority constituent in phycobiliproteins, and difficulty in purification (Tavanandi et al., 2019).

2.9 Culture conditions for pigment accumulation

Growth conditions and composition of microalgae depend significantly on the culture system used. Therefore, there are four culture systems in microalgae: photoautotrophic, heterotrophic, myxiotrophic, and photoheterotrophic cultivation (Benavente-valdés et al., 2016) **Table 2.10** shows the main differences between the cultivation systems.

Table 2.10.Cultivation systems employed in microalgae

Cultivation	Power Source	Carbon Source
Photoautotrophic	Light	Inorganic carbon
Heterotrophic	Organic carbon	Organic carbon
Photoheterotrophic	Light	Organic Carbon
Mixotrophic	Light and organic carbon	Organic and inorganic carbon

Source: Benavente-valdés et al. (2016)

The photoautotrophic system is the most commonly cultivation condition used for microalgae growth. Particularly, this type of cultivation occurs when the microalgae use sunlight as energy source and CO_2 as inorganic carbon source, producing chemical energy through photosynthesis. On the other hand, heterotrophic cultivation occurs when microalgae use organic carbon (sugars, organic acids, among others) as source of energy and inorganic carbon as carbon source(Hu et al., 2018; Kamalanathan et al., 2018).

Mixotrophy is a cultivation system where heterotrophic and autotrophic modes work simultaneously, in which inorganic and organic carbon is used in the presence of light. Inorganic carbon is fixed through photosynthesis in the presence of light, while organic carbon is assimilated and metabolized to generate energy and metabolites for biosynthesis through aerobic respiration (Subhash et al., 2017).

On the other hand, photo-heterotrophic cultivation occurs when microalgae require light by using organic compounds as carbon source. The main difference between mixotrophic and photo-heterotrophic cultivation is that the latter requires light as energy source, while mixotrophic cultivation can use organic compounds or sunlight (Zhan et al., 2017) (**Table 2.11).**

Table 2.11.Cultivation system used in the production of pigment of interest in some microalgae

Microalgae	Carbon source	Cultivation systems	Pigment content	References

Chlorella pyrenoidosa	Glucose 30 g L^{-1}	Mixotrophic, fed-batch, tetracycline 100 μ ml^{-1}	218 mg L^{-1} Lutein	Theriault. (1964)
	Glucose 230 g L^{-1}	Heterotrophic, batch, erythromycin 100 μ ml^{-1}	378 mg L^{-1} Lutein	
Haematococcus pluvialis	22.5 mM acetate	Mixotrophic, batch	11 mg g^{-1} cell astaxanthin	(KOBAYASHI et al., 1992)
	22.5 mM acetate	Heterotrophic, batch,	10.5 mg g^{-1} cell astaxanthin	
Galdieria sulphuraria	Glucose 50 gL^{-1}	Heterotrophic, batch	3.6 mg g^{-1} phycocyanin	(Schmidt et al., 2005)
	Fructose 50 gL^{-1}	Heterotrophic, batch	3.4 mg g^{-1} phycocyanin	
	sucrose 50 gL^{-1}	Heterotrophic, batch	4.3 mg g^{-1} phycocyanin	
Spirulina platensis	Glucose 2 gL^{-1}	Heterotrophic, batch	57 mg g^{-1} phycocyanin	(MARQUEZ et al., 1993)
	Glucose 2 gL^{-1}	Mixotrophic	131 mg g^{-1} phycocyanin	
Spirulina platensis	Glucose 2,5 gL^{-1}	Photoheterotrophic	322 mg g^{-1} phycocyanin	(Chen and Zhang, 1997)

2.10 Microalgae in industry

Microalgae industrial usage is due to striking advantages such as: high productivity of lipids and carbohydrates, higher soybean and sugarcane than (conventional energy crops); possibility of growth in different aqueous media, saline, non-potable waters, thus reducing the pressure on water collection for crop irrigation; the composition of the strains can be regulated according to the metabolite of interest through modulation of operational variables and nutritional conditions; production of a wide variety of fascinating secondary metabolites with biological activities such as carotenoids, especially β-carotene, astaxanthin, docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), among others (Colling Klein et al., 2018; Rizwan et al., 2018).

Large-scale commercial microalgae cultivation iniciated in 1960 in Japan, with the culture of *Chlorella* by the Nihon Chlorella company (Taipei, Taiwan). In 1970, a

facility for the cultivation and harvesting of *arthrospira* (spirulina) was established in Lake Texcoco by Sosa Texcoco S.A (Mexico) (Borowitzka, 1999). The first record of microalgae use in aquaculture dates back to 1970, and in 1980 there were already 46 plants in Asia, which produced about one ton of *Chlorella* sp. per month (Borowitzka and Moheimani, 2013). Soon, in 1986, the company Cognis Nutrition and Health (Whyalla, Australia) began commercial production of the microalga *Dunaliella salina* as source of β-carotene. Then, other companies in the United States, Israel, and India followed the same market trends and started the exploration of different microalgae strains as potential commercial (Bahadur et al., 2015).

The current global market for microalgae is estimated at $5.4 billion, of which $2.5 billion is generated by the food and health sectors (Ying et al., 2020). Thus, polyunsaturated fatty acids, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), have a global market of $700 million/year, β-carotene of $261 million/year, astaxanthin of $240 million/year, lutein of $231 million/year, and phycobiliproteins of $60 million per year (Bhalamurugan et al., 2018). Even more, the annual production of microalgae is approximately 7.5 million (Andrade et al., 2020; Mobin and Alam, 2017). Commercial genera and products of interest include *Dunaliella salina* (β-carotene), *Haematococcus* (astaxanthin), *Arthrospira* (Spirulina), *Scenedesmus* sp, and *Chlorella* sp, among others (Andrade et al., 2020; Trentacoste et al., 2014).

2.11 Main used genera of microalgae

2.11.1 Scenedesmus sp

The genus Scenedesmus sp. belongs to the family *Scenedesmaceae*, order *chlorococcales*, class *chlorophyceae*, and division *chlorophyta*. Widely distributed on the planet, they are freshwater species, commonly found in water bodies. Its reproduction is asexual, through the formation of autospores that are arranged in the mother cells with subsequent rupture to form a new cenobium (Richmond, 2004; Richmond and Hu, 2013).

This genus is characterized by the presence of flat-shaped cenobia formed by 2 to 32 cells arranged in one or two rows. In addition, the cells have different shapes, usually small (5-30 µm) elongated, with cell poles that can vary from acute to obtuse. The main pigments present in these genus are chlorophyll *a* and *b*, however, under

stress conditions some species such as *Scenedesmus almeriensis*, can accumulate carotenoids like lutein, neoxanthin, alloxanthin, violaxanthin and β-carotene (Ambati et al., 2019; Cuellar-Bermudez et al., 2015).

2.11.2 *Chlorella* sp

Chlorella (*Chlorophyta*) is a cosmopolitan genus, characterize for having a small globular cells (3-8 μm in diameter), which includes species with high temperature tolerance (15- 40 °C) (Mobin and Alam, 2017). They are considered as promising candidates for commercial lipid production due to their fast growth rate, low nutritional requirement, and robustness for open pond culture, given their potential to resist contaminants. In addition, they are often used as food supplement, in the cosmetic, and pharmaceutical industry (Koller et al., 2014; Molino et al., 2018a; Rizwan et al., 2018).

Chlorella Vulgaris stands out in this genus, because they can accumulate pigments such as chlorophyll *a* and *b*, β-carotene and xanthophylls and its main source of energy reserve is starch (Ambati et al., 2019). In addition, carotenoid accumulation in *C. vulgaris* is affected by several factors, such as light limitation, salinity, nitrogen, and phosphorus (Cecchin et al., 2018; W. Zhang et al., 2015).

2.11.3 *Arthrospira (Spirulina platensis)*

Spirulina is a multicellular, filamentous, planktonic photosynthesizing cyanobacterium belonging to the *Oscillatoriaceae* family that shows a blue-green hue (pigments chlorophyll and C-phycocyanin) (KIM, 2015).

It is characterized by the presence of chains of cells constituting a filament in the form of a spiral, being called trichome. Trichomes are made up of cylindrical cells, short and wide, which are lined by a thin membrane. These can have length of 500 μm and diameter ranging from 6-12 μm, and the helical structures formed by these filaments can have diameter ranging from 30-70 μm, but depending on the species, environmental and nutritional conditions submitted those measurements can vary (Mobin and Alam, 2017).

Spirulina is native from African and Latin American lagoons (Costa et al., 2019). It is recognized for having GRAS certification, ensuring that it can be used as food and medicine, when grown and processed under the required health and safety standards (Leb et al., 2019).

In addition, it is one of the most cultivated microalgae in the world, mainly due to its nutritional biomass value, which has potential for extraction of high value-added biocomposites like carbohydrates, and vitamins such as A, B_1, B_2, B_3, B_6, B_{12}, and potential anti-inflammatory, antioxidant, anticancer, and antiviral activities (**Table 2.12**) (Saini et al., 2018). In addition, carotenoids such as β-carotene, echinenone (4-keto-β-carotene), β-cryptoxanthin, zeaxanthin, β-carotene, 5,6-epoxide, lutein, 3-hydroxy-equinenone, diatoxanthin, canthaxanthin, myxoxanthophyll, and oscillaxanthins have been isolated and identified in this genus (Ambati et al., 2019).

Table 2.12. Pharmacological and biological properties of species of the genus *Arthrospira*

Microalgae	Phycocyanin	Physiological property	Reference
Spirulina platensis	C-Phycocyanin	Antioxidant	CHEWA et al. (2019
Geitlerinema sp	C-Phycocyanin	Antioxidant	PATEL et al. (2018)
Spirulina platensis	C-Phycocyanin	Anti-inflammatory	HAO et al. (2018)
Spirulina platensis	C-Phycocyanin	Anticancer	JIANG et al.(2017) and CZERWONKAA et al.(2018)

Within this genus, the species *Spirulina platensis* and *Spirulina máxima* stand out; they have been widely studied in the field of medicine and food industry (MA et al., 2019). In addition, the main carotenoids identified in *S. platensis* are *β*-carotene, zeaxanthin and *β*-cryptoxanthin. This microalga is the main source of C-phycocyanin (antioxidant). Studies reported that phycocyanin stability range is from pH 5.5 to 6.0 and thermal is 47 °C (Chaiklahan et al., 2012; Martelli et al., 2014).

2.11.4 Haematococcus pluvialis

H. pluvialis, also known as *Haematococcus lacustris* or *Sphaerella lacustre s*, is a green biflagellate microalga from the family *Haematococcaceae*, order *Volvocale*s, and class *Chlorophyceae* (KIM, 2015).

H. pluvialis is a freshwater microalga that exhibits great adaptive capacity to adverse environmental conditions, with high light intensity, presence of reactive oxygen species or nutrient depletion, achieved by the transformation of cells into a non-motile cyst, surrounded by a thick membrane (Orona-navar et al., 2017). In addition, it is widely recognized for accumulating large amounts of astaxanthin (3.8-5%) in dry mass under stress conditions (Mobin and Alam, 2017).

Its life cycle presents four distinct morphological stages: microzoon, macrozoon (zoopores) and palmate, belonging to the vegetative phase, and hematocyst or aplanospore, referring to the red hecystic phase observed in **Figure 2.8.**

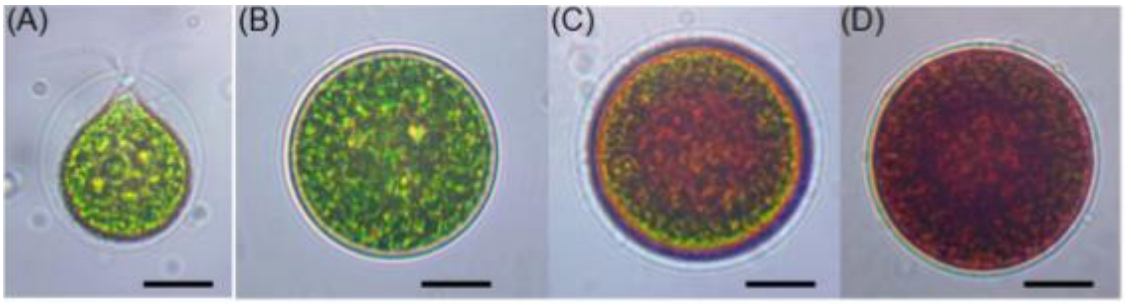

Figure 2. 7. Life cycle of *H. pluvialis*. A) Green vegetative mobile cell, B) *Palmella* cell, C) Palmella cell accumulating astaxanthin, in transition to aplanospore, and D) Hematocysts or aplanospores. Source: Shah et al. (2016).

Macrozoid and microzoid flagellated vegetative cells predominate in the early stages of vegetative growth under favorable cultivation conditions. On the other hand, under unfavorable conditions, macrozooids start to lose their flagella and expand their size; formation of a multi-layered structure begins as they develop into palmately, subsequently, continuous environmental stress interrupts cell division and the palmately progresses to the last morphological stage, transforming into aplanospore, which has a thick, rigid trilaminar sheath and a secondary cell wall of tough material (Khoo et al., 2019). **Table 2.13** shows the composition of *H. pluvialis* biomass.

Table 2.13. *H. pluvialis* composition at the green vegetative stage and hematocyst

Content (% dry weight)	Vegetative	Hematocyst
Protein	29-45	17-25
Total lipids	20-25	32-37
Neutral lipids	59	51,9-53,5
Phospholipids	23,7	20,6-21,1
Glycolipids	11,5	25,7-26,5
Carbohydrates	15-17	36,40

Neoxanthin	8,3	n.d
Violaxanthin	125	n.d
β-carotene	16,7	1
Lutein	56,3	0,5
Zeaxanthin	6,3	n.d
Astaxanthin	n.d	81,2
Adonixanthin	n.d	0,4
Canthaxanthin	n.d	5,1
Equinenone	n.d	0,2
Chlorophyll	1,5-2	0

n.d not detected by the authors. Adapted (Mobin and Alam (2017) and Molino et al. (2018b).

The common culture media for *H. pluvialis* are Bold Basal (BBM), Blue-Green (BG11), KM1 (variant of BBM) and *haematococcus* optimum medium (OHM) (Kobayashi et al., 1993; Rippka et al., 1979). Recently, the common BBM culture medium was develop, adding different reagents such as melatonin and diethylaminoethyl hexanoate, in order to trigger stress-specific effect and hormone expression in *H. pluvialis*, increasing cell density and astaxanthin accumulation (Ding et al., 2019).

Keratogenesis induction occurs when *H. pluvialis* cells are exposed to stress conditions derived from nutrient depletion (nitrogen and phosphorus, mainly), high salinity. Niizawa et al. (2018). Tested nitrogen-limiting medium in *H. pluvialis* and reported a 3.5% (mass) of astaxanthin content, representing two times the concentration obtained when phosphorus was the limiting. Furthermore, it is possible to increase astaxanthin biosynthesis in the hematocyst cell by adding Fe^{3+} (iron sulfate) and acetate. As reported by Hong *et al.* (2016), the addition of 0.45-0.5 m Fe^{3+} is enough for formation of hydroxyl radicals, which promote astaxanthin biosynthesis in hematocyst cells.

Light intensity is the factor that exerts the greatest influence on the cell cycle, morphological changes, and induction of keratogenesis. As described previously*, H. pluvialis* goes through two stages, green mobile cell and red immobile cell, and an optimal light intensity is required to obtain the best cell and astaxanthin yield. Thus, a light intensity of 20-90 μmol-$m^{-2}s^{-1}$ is required to grow green mobile vegetative cells (Ma et al., 2018). Subsequently, when the cells reach the exponential growth phase,

the culture is transferred to the specific medium and exposed to a higher light intensity 100-480 μmol-$m^{-2}s^{-1}$, to transform the cells into hematocysts (Lee et al., 2018).

Astaxanthin produced by *H. pluvialis* is widely used in aquaculture to give the characteristic coloration of salmon (Khoo et al., 2019). The antioxidant properties of astaxanthin inhibit the production of inflammatory compounds, protect against oxidative stress, protein degradation and macular degeneration. In humans, β-carotene is converted into vitamin A, which allows the immune system to function efficiently (Mobin and Alam, 2017). The price of astaxanthin is $2,500 per kilogram (MATOS, 2017).

2.11.5 *Dunaliella* sp

Dunaliella species belong to phylum *Chlorophyta*, order Volvocales, and family *Polyblepharidaceae*, and are unicellular, photosynthetic, motile biflagellate microalgae, standing out morphologically from the rest of the *Volvocales* by the absence of a polysaccharide cell wall (rigid cell wall) (Reza et al., 2017). Depending on the culture conditions and light intensity, cells of *Dunaliella* genus present ovoid, spherical, pyriform, or ellipsoid shapes with size ranging from 5 to 25 μm in length and 3 to 13 μm in width (Tafreshi and Shariati, 2009).

Species of this genus are described as hyper halotolerant microorganisms because they support concentrations high concentration of NaCl (up to 35 %) and temperature range of 0-38 °C. Adaptation to highly saline environments is the result of an osmoregulation mechanism, in which there is production and intracellular accumulation of glycerol, used to balance osmotic pressure and protect enzymes from inhibition and inactivation (Chitlaru and Pick, 1991). Furthermore, glycerol synthesis under hypertonic conditions and elimination under hypotonic conditions are independent of protein synthesis and light photoperiod.

The best-known species of this genus are *Dunaliella salina*, *Dunaliella tertiolecta*, *Dunaliella primolecta, Dunaliella viridis, Dunaliella bioculata, Dunaliella acidophyla, Dunaliella parva, Dunaliella bardawil*, and *Dunaliella* media (KIM, 2015; Richmond and Hu, 2013). In this genus, besides *chlorophyll a* and *b*, there are carotenoids of great importance, such as *α*-carotene, *β*-carotene, voilaxanthin, neoxanthin, lutein, zeaxanthin, and cryptoxanthin (Fu et al., 2013; Raja et al., 2018).

The microalgae *Dunaliella bardawil* and *Dunaliella salina* stand out in this genus due to their ability to accumulate large amounts of *β*-carotene. Particulary, *D. salina*, under specific stress conditions such as high salt concentration, light intensity, temperature, and nitrogen limitation, accumulate between 10-14% (mass) of β-carotene, compared to other microalgae and higher plants this concentration is 0.3% (Macías-sánchez et al., 2009; D. Yang et al., 2013).

β-carotene obtained from *Dunaliella* has a growing demand and a wide variety of market applications: colorant in food industries, component in pharmaceuticals, cosmetics, natural foods, dietary foods, diagnostics, and biomedical research (Ambati et al., 2019).

2.12 Compound extraction in microalgae

2.12.1 Cell disruption

Despite the wide and diverse range of high value-added biomolecules obtained from microalgae such as: pigments, long-chain fatty acids (eicosapentaenoic acid, docohexaenoic acid) small molecules with antimicrobial and anticancer activity, proteins and lipids that can be used for various applications, one of the major difficulties in the recovery process is related to the fact that these compounds are usually produced intracellularly (Yong et al., 2020). Therefore, cell disruption is a necessary step to improve the efficiency in extracting such compounds, because the passive diffusion of solvent through the cell wall is slow (Nee et al., 2018; Sierra et al., 2017).

Hence, the cell wall is one of the main barriers in cell disruption, which has a tensile strength around 9.5 MPa, almost three times higher than the cell wall of plants (Bharte and Desai, 2018). In addition, most microalgae have relatively thick and rigid cell walls (except for *Euglena* and *Dunalielle* which have no cell wall), composed of cellulose, glycoprotein hemicellulose, proteins, carotenoids, tannins, lignin, uronic acid, xylan, mannose, algaenan layers (very resistant biopolymer), and minerals, such as calcium or silicate. This composition can vary greatly among different microalgae species (Youn et al., 2017).

Cell disruption process of some microalgae required a great energy supply, leading to high energy consumption, and in some cases biomolecules degradation

(Yong et al., 2020). Consequently, is necessary a wide and depth study of each microalgae species for choosing the most suitable for cell disruption techniques.

An ideal cell disruption technique is: simple, easy handling, low energy consumption, and high disruption yield in a short period of operation (Nee et al., 2018). There are different techniques used for microalgal cell disruption and they are classified into two main groups, mechanical and non-mechanical, which can be used alone or together (Han et al., 2019; Lee et al., 2012; Youn et al., 2017).

2.13 Conventional extraction techniques (organic solvents)

Conventional extraction of intracellular metabolites (lipids, pigments, proteins) is usually performed from dry biomass, and is based on maceration and extraction using solvent, organic or aqueous, depending on the polarity of the target compound to be extracted (Cuellar-Bermudez et al., 2015).

Lipids are biomolecules soluble in organic solvents but insoluble in water. They can be classified as polar and non-polar based on the chemical structure and polarity of the main group. Non-polar lipids are used by microalgae to form cell membranes, the representatives of which are phospholipids and glycolipids. On the other hand, non-polar lipids or neutral lipids (triacylglycerols) are used as energy source (Han et al., 2019; Sati et al., 2019; Sforza et al., 2017).

Other metabolites of great interest present in microalgae are carotenoids, which exhibit a variety of polarity, stability, and solubility. In addition, most carotenoids have high degree of hydrophobicity, and non-polar solvents such as hexane, dichloromethane, dimethyl ether, and others, are used for extraction. However, acetone and biphasic mixtures of various organic solvents are also used for selective extraction of carotenoids (Alessandro and Antoniosi, 2016; Menegazzo and Fonseca, 2019). A suitable solvent should be selected based on the specific type of carotenoid, allowing for a more selective, efficient extraction with higher purity.

There are several extraction methods that can be used to obtain lipids: with subcritical water, electromagnetic pulse/electrical potential difference, supercritical fluid and extraction via organic solvents, which can be assisted by ultrasound or microwave. Each of these methods can present advantages and good results, as well as disadvantages, since some require high investment in equipment and maintenance,

demanding high energy consumption (Molino et al., 2019). **Table 2.14** presents a summary of the most commonly used solvents for extraction of biocompounds of interest from microalgae.

Table 2.14. Microalgae biocompound extract by organic solvents

Microalgae	Operating conditions	Solvent	Yield (%)	Reference
Acutodesmus obliquus	20 g dry weight at 60 °C for 12 h 60 °C for 2 h	Ethanol: Hexane (1:2)	92 Soxhlet 59 ultrasonic	Escorsim et al.(2018)
Chlorella pyrenoidosa	Ultrasonic pretreatment 25 °C for 15 min to promote cell lysis.	Hexane Chloroform 2-Methyl-tetrahydrofuran Cyclopentyl methyl ether	69,10 74,86 69,76 68,03	Jesus et al. (2019)(Jesus et al., 2019)
Botryococcus braunii	Lipid extraction performed in a tube-type system at a dosage of 10 ml per gram of dry biomass for 5 h	75% v/v chloroform: methanol	98,9	Hidalgo et al.(2016)
Scenedesmus obliquus	10 g dry microalgae to 100 ml solvent mixture were used. Ultrasound-assisted extraction (resonance amplitude 50μm) conducted in a glass reactor took 1.5 h at 25°C	Hexane: isopropanol 4:1	26,63	Ido et al. (2018)
Heterochlorella luteoviridis	Extraction performed with 7% ethanol at 90 V electrical voltage for 50 min 30°C	Ethanol	83	Jaeschke et al. (2016)

In addition, these methods must take into account the possibility of lipids binding to other molecules such as proteins, carbohydrates, and pigments, and the ability of different solvent mixtures to solubilize the target molecule that is to be extracted (Sati et al., 2019; Yong et al., 2020). Thus, neutral lipids are dissolved in non-polar solvents and polar lipids are soluble in polar solvents. Consequently, to achieve efficient extraction it is necessary to have a specific solvent mixture that has sufficient polarity to remove polar lipids while being sufficiently non-polar to dissolve neutral lipids (Boonnoun and Panatpong, 2020; Molino et al., 2020a)

Conventional lipid extraction methodology relies on the use of organic solvents, the more promising are acetone, benzene, chloroform, n-hexane, methanol, ethanol,

butanol, and cyclohexane; however, methanol, chloroform, and hexane are considered the most promising (Jesus et al., 2019).

The Soxhlet is one of the oldest methods, however widely used, which is based on solid-liquid extraction, in which there is continuous contact of the biomass with the organic solvent through reflux for a prolonged period, being operated, generally, at boiling temperature (Mubarak et al., 2015).

Furthermore, the extraction yield is directly related to the solvent's affinity for the compound of interest and time of extraction (number of cycles). However, one of the main disadvantages of this method is high amount of solvent used, high energy consumption, thermal degradation of some lipids and low selectivity (Menegazzo and Fonseca, 2019; Sati et al., 2019; Youn et al., 2017)

The Blight & Dryer (1959) method, despite being initially employed in the determination of lipids in fish tissue, has shown promising results when applied to microalgae, because it allows working with high moisture contents and small amounts of biomass. This method is based on the mixture of two organic solvents: chloroform and methanol 1:2 (v v^{-1}). Using this organic solvent system, residual endogenous water in the microalgae cells acts as a ternary component that allows complete extraction of neutral and polar lipids. Therefore, complete drying of microalgae is not necessary in this system. In addition, once the cellular debris are removed, more chloroform and water are added in order to induce biphasic partitioning. The lower organic phase contains chloroform and most of the lipids (neutral and polar), while the upper organic (non-lipidic) aqueous phase consists of methanol, water, proteins, and carbohydrates (Han et al., 2019; Kumar et al., 2015).

Another solvent system used is hexane isopropyl alcohol 3:2(v/v), which presents itself as a less toxic alternative to chloroform: methanol system. The mixture works similarly to the chloroform: methanol system. After the biphasic separation, the upper organic phase (hexane with some isopropanol) contains most of the lipids (neutral and polar), while the lower aqueous phase (water with some isopropanol) contains most of the non-lipids (proteins and carbohydrates). As for the extraction of lipids in microalgae, hexane:isopropanol mixture is the most selective with respect to neutral lipids compared to chloroform:methanol system (Escorsim et al., 2018; Halim et al., 2012; Sati et al., 2019). Finally, it is important to notice that extraction efficiency, selectivity, and high solvent consumption are the main limiting factors of conventional extraction process.

2.14 Supercritical Fluid Extraction

Supercritical fluids are characterized by having temperature and pressure values higher than the corresponding critical values. Above critical point, there is no longer any surface tension and separation between liquid and gas phases in equilibrium, forming a single supercritical phase whose properties are intermediate between the two states. Below critical point the fluid can present itself as liquid or as vapor (Bharte and Desai, 2018).

Generally, the supercritical fluid used in extraction of natural products is CO_2 because it has some characteristics. Among these, it stands out for being nonflammable, non-toxic, inert, presenting good chemical stability and low cost, and mainly for having a critical point that happens in relatively mild conditions, as can be seen in **Table 2.15** (Manjare and Dhingra, 2019)**.**

Table 2.15.Critical properties of fluids of interest in compound extraction

Fluid	**Temperature (°C)**	**Pressure (atm)**
Carbon dioxide	30.95	72.80
Water	101.00	217.76
Methane	-83.11	45.40
Ethane	32.15	48.10
Propane	96.65	41.90
Ethylene	9.25	49.70
Propylene	91.75	45.40
Methanol	-34.3	79.80
Ethanol	-29.95	60.60
Acetone	234.95	46.40
Nitrous Oxide	36.5	72.5
Xenon	16.60	58.4
Ammonia	132.5	112.5

Adapted from Brondz et al. (2017) and Molinoa et al. (2020).

2.14.1 Effect of temperature and pressure

Extraction temperature and pressure are parameters that play a significant role in solute solubility in the solvent, which mainly depend on the chemical properties of the target compound to be extracted. For example, astaxanthin and lutein are highly thermosensitive biomolecules, so, adaptations in the extraction process are required to avoid their degradation (Molino et al., 2018b; Sanzo et al., 2018).

The efficiency of supercritical CO_2 extraction process is directly related to the use of optimal temperature and pressure values. Thus, the use of high temperatures causes compounds degradation, while high pressures hinder the fluid diffusivity in the matrix. It is also worth noting that temperature and pressure have direct effect on fluid solvation due to their direct action on density, which can be modified or changed by varying these parameters (Manjare and Dhingra, 2019; Molino et al., 2020b)..

Table 2. 16. Effect of temperature and pressure on lipid extraction in microalgae using CO_2.

Microalgae	Pre-treatment	T °C	P MPa	Time (min)	Yield (%)	References
Spirulina platensis	Freeze-drying, ground 0.37 mm. 10 kg h^{-1} CO_2	40	25	60	41	Mendes and Reis. (2006)
		40	40	60	71.7	
		40	55	60	89.9	
		40	70	60	96.4	
Nannochloropsis sp.	Freeze-drying, ground 0.37 mm. 10 kg h^{-1} CO_2	50	40	84	60	Venturi et al. (2005)
		50	55	84	80	
		40	70	84	88	
		55	70	84	92	
Nannochloropsis oculata sp.	Without pre-treatment. CO_2 6 ml min^{-1}	80	20.5	240	71	Bong and Loh, (2013)
Scenedesmus obliquus	Freeze-drying	20	7	540	73.1	Lorenzen et al. (2017)
		20	12	540	92	
		20	15	540	72.06	

Increasing pressure at constant temperature results in increasing both density and solvation of CO_2, therefore, the extraction yield of the process increases. On the other hand, increasing the temperature at constant pressure has a negative effect on the extractive process, as the CO_2 density decreases and, consequently, the solvation decreases as well (Manjare and Dhingra, 2019). Thus, solubility is closely related to CO_2 density and solute properties (molecular mass, polarity, and vapor pressure) (Poojary et al., 2016).

In the work developed by Sanzo et al. (2018), the authors used *Hematococcus pluvialis* to evaluate the effect of increasing extraction temperature in the range of 50-80 °C while keeping constant pressure, resulting in a reduction in extraction yield of lutein and astaxanthin. On the other hand, when keeping constant temperature (65 °C), increasing pressure in the range of 10 to 55 MPa produced an increase in yield of 98 and 52% in astaxanthin and lutein, respectively. A 93.8% extraction of fatty acids at 65 °C and pressure of 55 MPa was also reported. In **Table 2.16 and 2.17** is shown the effect of temperature and pressure on the extraction of biomolecules in microalgae using supercritical CO_2 is presented.

Table 2.17. Effect of temperature and pressure on extraction of biomolecules in microalgae using supercritical CO_2

Microalgae	Pre-treatment	T (°C)	P (MPa)	Time (min)	Yield (%)	Reference
H. pluvialis	Mechanic, CO_2 3,62 g min^{-1}	80	55	120	13.9 Astaxanthin;1.6 lutein	Sanzo et al. (2018)
		65	55	120	36.2 Astaxanthin; 38.4 lutein	
		50	55	120	98.6 Astaxanthin; 52.4 lutein	
		50	40	120	95.8 Astaxanthin; 46.7 lutein	
		50	10	120	0.5 Astaxantina; 1.0 lutein	
Dunaliela salina	Mechanic, CO_2 14,48 g min^{-1}	50	55	110	15.1 β-Carotene	Molino et al. (2019)
		65	55	110	21.5 β-Carotene	
		75	55	110	17.7 β-Carotene	
		65	10	110	1.7 β-Carotene	
		65	40	110	25.5 β-Carotene	
		65	55	110	21.5 β-Carotene	
Scenedesmus almeriensis	Mechanic, CO_2 14,48 g min^{-1}	65	25	120	14.2 lutein	Mehariya et al. (2019)
		65	40	120	50.3 lutein	
		65	55	120	97.6 lutein	
		50	55	120	92.3 lutein	

Pure CO_2 presents some limitations in the extraction process due to its hydrophobic nature. This can undermine the process yield since non-polar and, in some cases, moderately polar biomolecules will be extracted. Thus, pure CO_2 is selective for neutral lipids and does not solubilize polar lipids. For example, when employed in microalgae, the main compounds obtained are mostly triacylglycerols, free fatty acids, sterols, and pigments (Yen et al., 2015).

However, supercritical CO2 in its pure state is rarely used. These limitations can be solved, to some extent, by adding modifiers or co-solvents of polar nature, which broaden the extraction spectrum of biomolecules, increasing the extractive yield (Brondz et al., 2017). Thus, several authors have reported the use of co-solvents such as acetone, butanol, dichloromethane, ethanol, methanol, propanol, toluene, vegetable oil, and water aiming to improve the extraction efficiency of carotenoids and fatty acids from microalgae (Molino et al., 2018b; Poojary et al., 2016; Sivagnanam et al., 2017).

The addition of co-solvent results in microalgae swelling , increasing the surface of contact area. This results in enhanced mass transfer due to the formation of hydrogen bonds with intracellular compounds and, clearly, by increased polarity. Thus,

extraction efficiency depends on the type of co-solvent, biomass composition, and target compound (Sati et al., 2019).

It is necessary to consider the amount of solvent added, because, after a certain value, this addition no longer has a positive effect on the extraction. For example, astaxanthin is a polar biomolecule, so it shows low solubility in supercritical CO_2 (MOLINO et al., 2020).

Nobre et al. (2006) studied the extraction of astaxanthin from the microalga *Haematococcus pluvialis* and observed that the addition of 10% (v/v) ethanol as co-solvent allowed an increase in yield of the extractive process from 58.7 to 91% at 60 °C and 30 MPa.

In another study, Yen et al. (2012) investigated the supercritical CO_2 extraction of lutein from *Scenedesmus* sp. using co-solvents with different polarities (methanol, butanol, acetone, and ethanol). Ethanol stood out among other co-solvents by presenting a good cost benefit and a 62% yield of lutein, with operating conditions of 40% (co-solvent/CO_2 flow rate ratio) at 70 °C and 40 MPa.

Recently, Sivagnanam et al. (2017) studied the extraction of total carotenoid, fucoxanthin from microalga *Saccharina japonica* using vegetable oils (canola, soybean, and sunflower), ethanol and water as co-solvents, at flow rate ranging from 0.5-2.0% (m/m), pressure of 20-30 MPa and temperature of 45-55 °C. The authors reported that the best yield of total carotenoids and fucoxanthin extraction was obtained using sunflower oil as co-solvent at a temperature of 50.62 °C, pressure of 30 MPa and flow rate of 2.00% (CO_2/solvent).

Table 2.18. Effect of different co-solvents on supercritical CO_2 extraction of microalgae biomolecules

Microalgae	Pre-treatment	T (°C)	P (MPa)	Time (min)	Co-solvent (%)	Yield (%)	Reference
***Scenedesmus* sp.**	Freeze-drying and maceration.	70	40	60	40 mol methanol	50.75 lutein	Yen et al. (2012)
	CO_2 1.0 ml min^{-1}	70	40	60	40 mol ethanol	62.20 lutein	
		70	40	60	40 mol propanol	51.55 lutein	
		70	40	60	40 mol butanol	38.68 lutein	
		70	40	60	40 mol acetone	16.91 lutein	
Nannochloropsis oculata	Freeze-drying and maceration 0.14 mm. CO_2 20 ml min^{-1}	50	35	30	10 (v/v) ethanol soybean	63.2 zeaxanthin	Liau et al. (2011)
		50	35	30	10 (v/v) dichloro-methane	40 zeaxanthin	

		50	35	30	10 (v/v) soybean	32.2 zeaxanthin	
		50	35	30	10 (v/v) toluene	20 zeaxanthin	
H. pluvialis	Hydrothermal.CO_2 1.0 ml min^{-1}	40	8	15	20 (v/v) ethanol	31.4 astaxanthin	Cheng et al. (2018)
		55	8	15	20 (v/v) ethanol	98.3 astaxanthin	
		55	8	15	20 (v/v) olive oil	98.6 astaxanthin	
		70	8	15	20 (v/v) ethanol	97.8 astaxanthin	

2.14.2 Effect of extraction time

Among the main advantages of using supercritical CO_2 are extraction time and high purity of the compound. Several papers have demonstrated the importance of extraction time in isolating the target molecule, but few have discussed the effect on the purity of the extracted compound.

As reported earlier, the yield of the process is directly correlated with temperature, pressure and solvent flow rate. Krichnavaruk et al. (2008) optimized extraction time in the isolation of astaxanthin from *Haematococcus pluvialis* at 40 MPa and 70 °C. The authors observed that a higher amount of astaxanthin was extracted in the first 60 minutes of the process, progressively decreasing until 300 minutes. This effect is due to supercritical conditions in which the solvent diffuses rapidly into the cell matrix; thus, intracellular compounds are dissolved and extracted.

In another paper, Sanzo et al. (2018) evaluated the effect of extraction time on the purity of astaxanthin from *Haematococcus pluvialis*, and demonstrated that purity level of 68% was achieved at 50 °C and 40 MPa, while purity of 83% was achieved at 50 °C and 55 MPa, CO_2 flow rate of 14.48 g min-1 and time of 80 min.

2.15 Existing patents

In recent years much interest has been focused on the biotechnological potential of microalgae, mainly due to the identification of several natural compost with high added value for commercial application in various sectors, such as human and animal health nutrition, energy production, chemical and pharmaceutical, among others. Therefore, this interest has directly reflected in the increasing number of patents related to extraction and purification of these high value-added biocompounds, as can be seen in **Table 1.19** (Boonnoun and panatpong, 2020; milos et al., 2016).

Moreover, astaxanthin stands out among these biocompounds given its antioxidant property and safe use as human and animal food additive. (Choi et al., 2002; Gudin and Thepenier, 1993).

Table 2.19. Patents related to extraction of high value biocompounds from microalgae in the last years

Patent Number	Situation	Claim	Reference
WO2002012183A1	Concession	Extraction method of astaxanthin from *haematococcus pluvialis* using solvent mixture of ethanol, methanol and acetone.	CHOI et al. (2002)
EP2658959A1	Concession	Method to improve the extraction capacity of biocompounds of interest in microalgae, such as pigments, carbohydrates, proteins, amino acids, lipids, and minerals.	MILOS et al. (2016)
US8598378B2	Concession	Method for direct extraction of lipids, biopolymers, fat-soluble pigments, and proteins from microalgal biomass using a co-solvent system conformed by an ionic liquid (1-ethyl-3-methylimidazolium ethyl sulfate) and methanol.	COONE and YOUNG, (2013)
CN103880950A	Request	Method for industrial-scale production and extraction of *c-phicocyanin* from the microalgae *Arthrospira platensis.*	Li Meifeng, (2014)
US5179012A	Concession	Method for production and extraction of antioxidants from a culture of photosynthetic microorganisms suspended in liquid medium.	GUDIN and THEPENIER (1993)
CN103613661A	Concession	Method to extract high purity *phycocyanine* from the microalgae *Arthrospira platensis.*	Xiong Wei. et al (2016)
CN104945297A	Request	Method for extracting astaxanthin from pluvial haematococcus, through an acid pre-treatment and subsequent extraction of the compound using the organic solvent ethanol.	Long Xiang et al. (2011)
EP2748144B1	Request	Method to increase the yield of lutein extraction from natural sources (microalgae, fungi and bacteria) using supercritical CO2 and ethanol as co-solvent.	BOONNOUN; PANATPONG, (2020)

2.16 Antioxidant capacity

Evaluation of antioxidant capacity refers to the study of cumulative potential of antioxidants or antioxidant compounds present in an extract or biological sample (Miguel-chávez, 2017).

Antioxidants show varied responses when subjected to different sources of free radicals or oxidizing agents. This variation can be explained by several mechanisms of action such as: the inhibition of antioxidant enzymes, transition metal chelating agents, hydrogen or electron transfer to the radicals, deactivation of single to oxygen, or enzymatic detoxification of reactive oxygen species (ROS) (Becker et al., 2019; Dokki, 2014).

Consequently, no single method is comprehensive enough to determine exactly the total antioxidant potential of an antioxidant compound, so the combination of different methods is needed to have a complete characterization (Alam and Bristi, 2013; Lewoyehu and Amare, 2019)

There are numerous methodologies to determine antioxidant capacity, which differ in several aspects such as: mechanisms of action, target species, reaction conditions and form of expression of the results, making an in-depth comparison between the methodologies difficulties **(Table 19)** (Lewoyehu and Amare, 2019). Furthermore, based on chemical mechanisms of action, these can be divided into two categories: hydrogen transfer (HAT) and electron transfer (ET) (Dontha, 2016).

HAT-based methodologies measure the ability of an antioxidant to sequester free radicals (usually peroxyl) by donating a hydrogen atom. These reactions are independent of pH, solvent, and are usually quite fast, being completed within minutes or seconds. The antioxidant activity is then determined through competition kinetics by measuring fluorescence decay curve of the indicator in both the presence and absence of the antioxidant and integrating the areas under these curves (Alam and Bristi, 2013; Apak et al., 2016; Lewoyehu and Amare, 2019).

On the other hand, methodologies that are based on electron transfer measure the ability of an antioxidant to reduce any compound (metal ions, carboline groups, and radicals) by transferring an electron. These reactions are slower, solvent and pH dependent. Thus, in this methodology, the antioxidant reacts with the fluorescent or colorimetric indicator instead of peroxyl radical. Spectrophotometric assays based on electron transfer measure the ability of an antioxidant to reduce an oxidizing agent, which changes color when reduced, and the degree of the color change (an increase or decrease in absorbance at a given wavelength) is correlated to the concentration of

antioxidants present in the sample (Lewoyehu and Amare, 2019; Miguel-chávez, 2017).

2.17 Methods based on hydrogen atom transfer.

2.17.1 ORAC method (Oxygen Radical Absorbance Capacity)

The ORAC method was developed by Cao et al. (1993) and involves the initiation of lipid peroxidation through the production of water-soluble peroxyl radicals at a constant rate by thermal decomposition of 2,2'-azobis(2-amidinopropyl) hydrochloride (ABAP) at 37°C (Dontha, 2016).

Phycobiliproteins β-phycoerythrins (β-PE) or R-phycoerythrins (R-PE) are used as target molecules for the radicals. These molecules are highly fluorescent, containing a red pigment photoreceptor (34 covalently linked tetrapyrrolic prosthetic groups) (Rocha et al., 2012). These proteins are derived from purple algae and cyanobacterial species and have a weight of 2500,000 daltons. The method is based on the reaction of ROO• radicals with the fluorescent indicator generating a non-flowering product, which is measured by spectrophotometry with maximum fluorescence emission at 575 nm (β-PE) and 578(R-PE) (David et al., 2010; Lewoyehu and Amare, 2019). The added antioxidant reacts rapidly with peroxyl radicals, thus inhibiting the loss of fluorescence intensity.

Antioxidant capacity is obtained by varying the integrated fluorescence intensity from antioxidant addition until its total consumption, as can be seen in **Figure 2.9**. The value obtained is expressed as equivalent of 6-hodroxyl 2,5,7,8-tetramethylchroman-2-oic acid (trolox) which is the water-soluble equivalent of the vitamin that has powerful antioxidant properties (Evrim et al., 2018).

It was observed that β-phycoertrin interacted with phenolic compounds leading to methodology errors. For this reason, Ou et al. (2001) developed and validated a modification of ORAC using fluorescein as a fluorescent marker. The foundation is identical to the traditional one; however, the method adaptation brought some improvements. Fluorescein is a photo and thermo stable compound, which shows homogeneity between different batches after exposure to excitation light and does not react with antioxidant sample (Ou et al., 2001).

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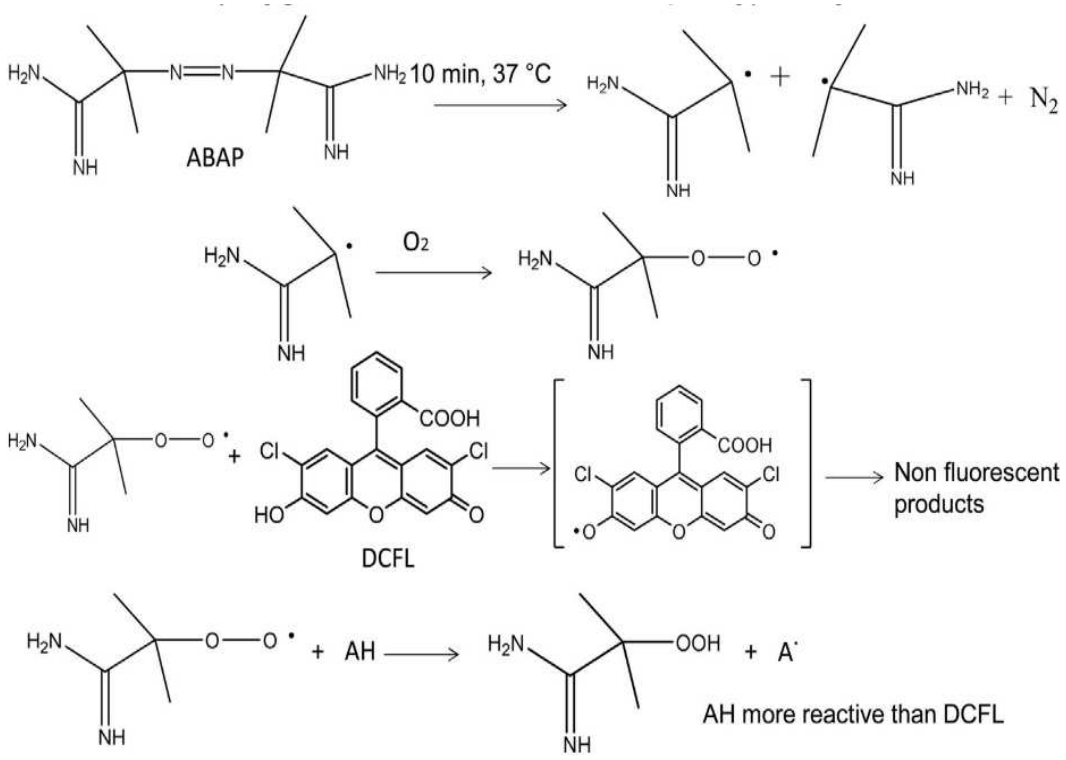

Figure 2. 8 Mechanism of action of the ORAC method (Brizzolari et al., 2016).

This method stands out over the other in determining antioxidant capacity, because fluorescence is used as measure of oxidative damage rather than absorbance like the other methods. Thus, there is less interference from colored compounds present in the samples. In addition, use of peroxyl or hydroxyl radicals as pro-oxidants confers greater biological significance when compared to methods that use oxidants and are not necessarily physiological pro-oxidants (Brizzolari et al., 2016; Dontha, 2016).

2.17.2 Total Radical Trapping Antioxidant parameter (TRAP)

This method was developed by Wayner et al. (1985) and is used to initially determine the total antioxidant potential of plasma samples and subsequently of photochemical extracts. It evaluates the time required for all antioxidants in the sample to be consumed (Cristina et al., 2014).

The method is based on the ability of compounds with antioxidant activity to interfere with the reaction between the peroxyl radical generated by AAPH (2,2'-azobis-2-methyl-propanimidamide dihydrochloride) or ABAP (2,2' azobis-2-amidinopropane

dihydrochloride), with the fluorescent probe R-phycoerythrin. Thus, the probe's oxidation is accompanied by fluorescence (excitation at 495 nm and emission at 575 nm) and results are expressed as Trolox equivalent (Biochem et al., 2011; Huang et al., 2005).

Although the TRAP method is sensitive to all antioxidants, this method has some disadvantages such as high complexity, high time requirement (different endpoints adopted, which makes it difficult to compare between laboratories), and requires a high level of expertise and experience (Badarinath et al., 2010; Miguel-chávez, 2017).

Consequently, the procedure has received adaptations such as: the use of other free radical sources as well as other techniques to control the process speed. Thus, Lissi et al. (2017) have developed chemiluminescent TRAP based on luminol oxidation using ABAP as free radical source. Specifically, the reaction between compounds with antioxidant properties and radicals present inhibits the system's chemiluminescence for a time directly proportional to the additive's total concentration (induction time), and antioxidant capacity is measured in relation to the standard antioxidant, Trolox (Badarinath et al., 2010; Miguel-chávez, 2017).

Figure 2.10 shows the mechanism of action, in which AAP upon thermal decomposition generates two aminopropane radicals that can react with O_2 to produce peroxyl radicals. Aminopropane radicals also react with luminol by abstracting a proton and generating luminol radical. The luminol radical reacts with H_2O_2 producing a short-lived hydroperoxide intermediate (LO_2H^-) that rapidly decomposes to 3-aminophthalic acid. 3-aminophthalic acid loses energy in the form of chemiluminescence generating 3-aminophthalic acid ground state (Balasaheb and Pal, 2015).

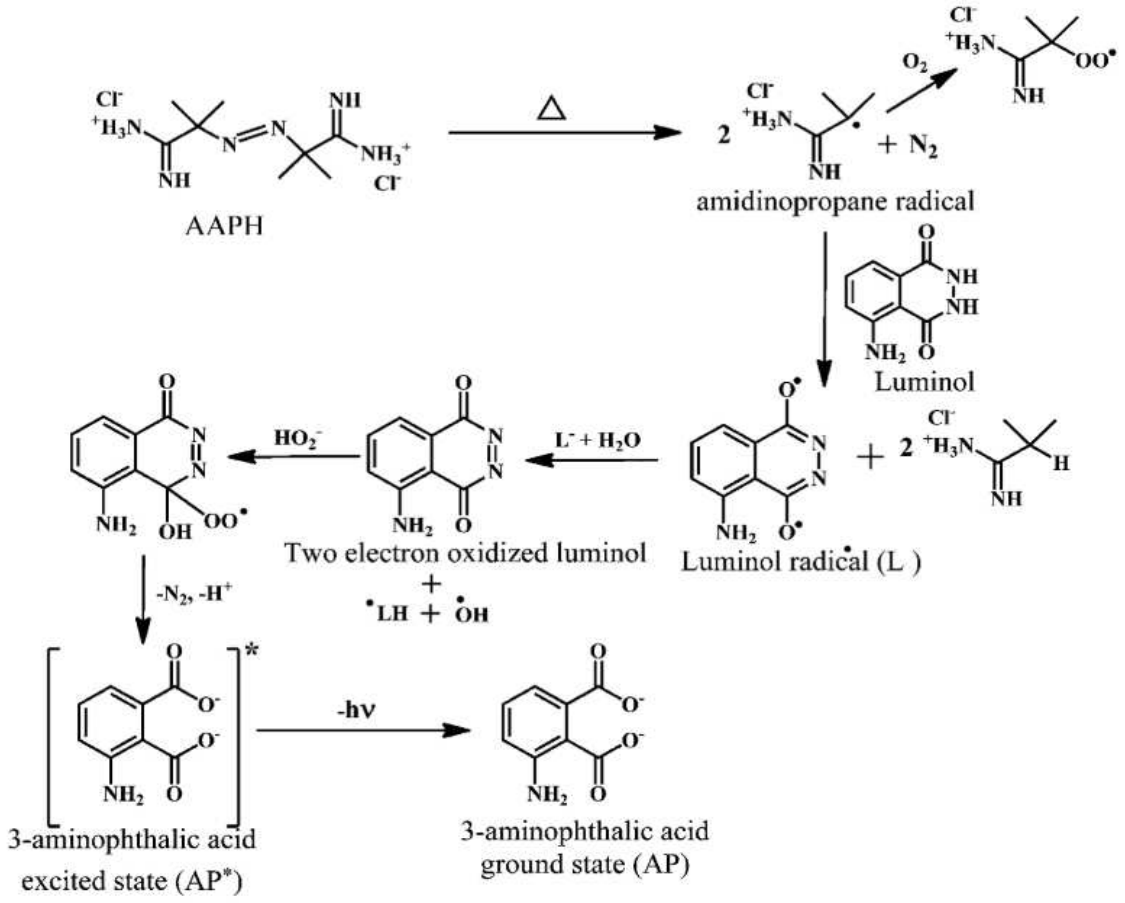

Figure 2.9. Mechanism of luminol AAPH-induced chemiluminescence. (Balasaheb and Pal, 2015).

2.17.3 **Method of β-carotene/linoleic acid co-oxidation**

The β-carotene/linoleic acid co-oxidation method was developed by Marco (1968) and was later modified by Miller (1971). This method evaluates the antioxidant's ability to inhibit the discoloration of β-carotene. This methodology has linoleic acid, polyoxyethylene sorbitan monopalmitate (Tween 40), and β-carotene as reagents. The tween, an emulsifier, aims to completely dissolve the mixture of β-carotene/linoleic acid in chloroform, which, after total solvent removal, is dissolved in water previously saturated with atmospheric oxygen (David et al., 2010; Rocha et al., 2012).

This method is performed in an emulsified medium and evaluates the ability of the antioxidant to inhibit free radicals generated during the peroxidation of linoleic acid caused by light, heat, and oxygen. This methodology is based on spectrophotometric measurement at wavelength of 470 nm, referring to the wavelength of the solution discoloration of β-carotene and linoleic acid, induced by the oxidative degradation products of linoleic acid (Ángel et al., 2013; Dontha, 2016).

Specifically, generated free radicals attack the double bonds of β-carotene, leading to the loss of its chromophore, resulting in discoloration of the orange pigment, characteristic of this solution ((Kumar, 2017). Usually, the synthetic antioxidant butylhydroxydotoluene (BHT) is used as a positive standard for comparison of results (Rocha et al., 2012).

The method is widely used for evaluation of antioxidant activity of food matrices. As it does not occur at high temperatures (50 °C), this method allows the determination of antioxidant power of thermolabile compounds and qualitative evaluation of antioxidant efficacy of plant extracts (Alam and Bristi, 2013).

Thus, the co-oxidation method employing β-carotene/linoleic acid system is a simple and sensitive test but presents some drawbacks. The use of an emulsified medium, for example, interferes in the absorbance values causing low reproducibility, and the interaction of β-carotene with medium oxygen makes it difficult to interpret obtained results (Aminjafari et al., 2016).

2.18 Electron transfer based methods

2.18.1 DPPH Method

DPPH is a colorimetric method developed by Brand-Williams et al. (1995) that is used to determine the antioxidant capacity of a compound to sequester free radicals (DPPH). This method is one of the most widely used because of its speed, practicality, and good stability. 1,1- Diphenyl-2-picrylhydrazyl (DPPH) is an organic nitrogen free radical and is characterized by being stable due to displacement of the unpaired electron throughout the molecule by resonance mechanism, so the molecule does not dimerize as is the case with most other free radicals (David et al., 2010).

The assay is based on the capture of DPPH (purple) radical by the antioxidant compound in a methanol or ethanol solution. The DPPH radical is reduced to diphenyl-picrylhydrazine (yellow) as seen in **Figure 1.11**. The initial DPHH concentrations observed in literature are highly variable (0.0025-0.415 gL^{-1}); time used varies from 30 minutes to the time required to reach equilibrium state (Library, 2015). The decrease in DPPH concentration is accompanied by monitoring reduction in absorbance at wavelength of 517 nm (Miguel-chávez, 2017). Antioxidant capacity results are expressed by the E_{50} parameter, which refers to the amount of antioxidant required to reduce by 50% on the initial DPPH concentration (Dehariya and Dixit, 2018).

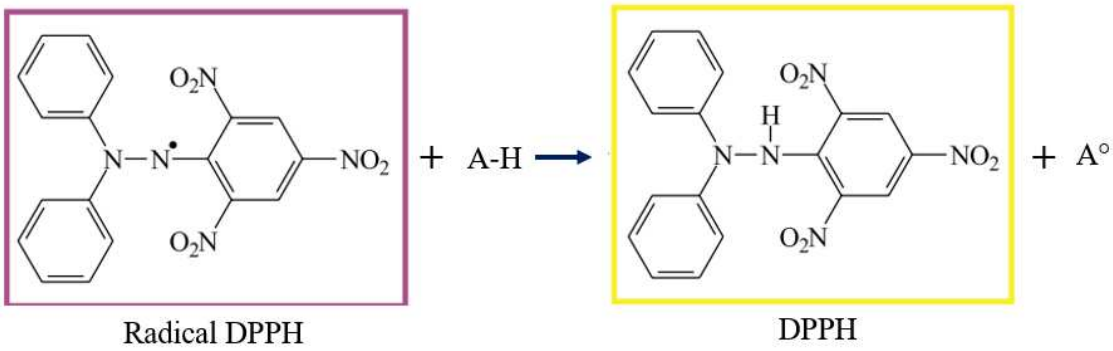

Figure 2. 10 Mechanisms between the DPPH radical and an antioxidant. Adapted Becker et al. (2019).

The advantage of this method is that the free radical, as mentioned earlier, is stable and commercially available, thus avoiding a need to generate different forms, as occurs with ABTS method (Dontha, 2016; Rocha et al., 2012).

This method does not involve drastic temperature and oxygenation conditions. The main disadvantage is due to the reactivity of DPPH that strongly differs from the reactivity of biologically important radicals (pro-oxidants such as the peroxyl radical). Thus, this method determines only the reducing power of the analyzed compounds. In addition, antioxidant compounds that react rapidly with reactive radicals, such as the peroxyl radical, may show low reactivity or may not react with the stable DPPH radical due to its steric hindrance (Miguel-chávez, 2017).

2.18.2 Folin-Ciocalteu

This colorimetric method was developed by Folin and Ciocalteu (1927) and is used in the determination of total polyphenol content in natural products. Specifically, the mechanism of action of Folin-Ciocalteu is based on the reduction in alkaline medium (Na2CO3 being the most suitable base) of phosphotungstic acid-phosphomolybdic acid complex by phenolic hydroxyls (Dontha, 2016).

In this complex molybdenum is at oxidation state VI (yellow color) and in the presence of deprotonated phenolic compounds (due to the alkaline medium) it is reduced to oxidation state V and, concomitantly, blue molybdenum-tungsten complexes are formed (**Figure 2.12**) (Alam and Bristi, 2013; Oliveira et al., 2009).

The intensity of coloration is related to the concentration of phenols present in the sample, and is determined by spectrophotometry at wavelength of 765 nm (Miguel-

chávez, 2017). The results are expressed as gallic acid equivalent, generally used as standard.

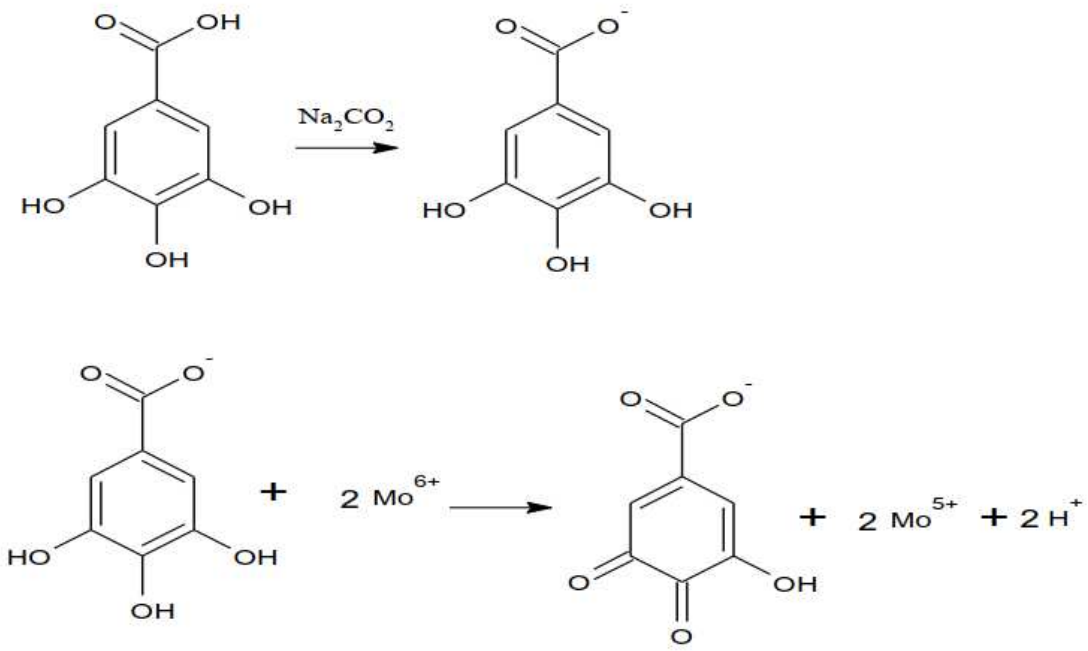

Figure 2.11. Reaction of gallic acid with molybdenum VI, leading to molybdenum formation (Oliveira et al., 2009).

This method has some disadvantages, such as: the Folin-Ciocalteu reagent is not specific for phenolic compounds and can be reduced by some non-phenolic compounds like aromatic amines, ascorbic acid, Cu(I), Fe(II), and amino acids. This is due to the fact that the method's basic mechanism is an redox reaction (Dontha, 2016).

2.18.3 Trolox equivalent antioxidant capacity (TEAC)

This method was developed by Cano et al. (1998) and later modified by Villano et al. (2004). It is based on the capture of ABTS radical (chemically stable and with high water solubility) in a neutral medium that shows a blue-green coloration. As the antioxidant is mixed into the solution, the ABTS radical is reduced, consequently causing loss of color in the reaction medium **Figure 2.13**.

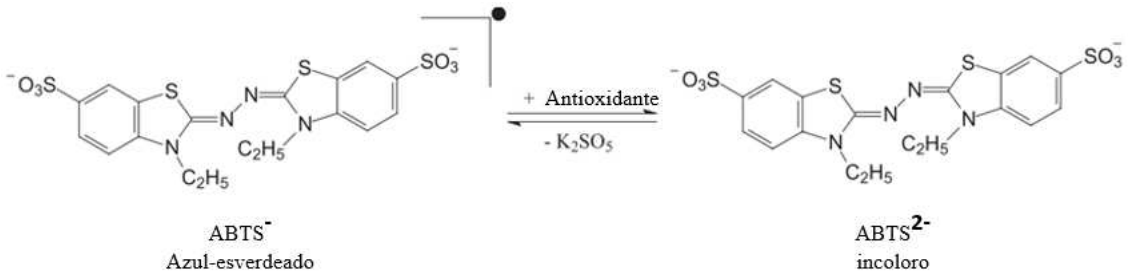

Figure 2.12. Mechanisms between the ABTS radical and an antioxidant. Adapted from Litescu et al. (2014).

Specifically, ABTS radicals can be generated through different processes such as: enzymatic (peroxidases), chemical (magnesium dioxide), or even electrochemical (Alam and Bristi, 2013; Miky et al., 2017). However, the traditional methodology is based on the oxidation of 2,2'-azinobis (3-ethylbenzothiazoline-6-acidosulfonic acid) by the addition of potassium persulfate $K_2S_2O_8$ (Litescu et al., 2014).

The degree of decoloration in the reaction medium reflects the decrease kinetics of ABTS radical concentration and can be characterized by an absorbance maximum at 415, 645, 734 and 815 nm. Among them, 415 and 734 nm are adopted for spectrophotometric monitoring of the reaction (Miguel-chávez, 2017). Results are expressed in trolox equivalent (standard subjected to the same analysis conditions).

This method stands out due to its simplicity, which allows application in routine analyses (Rocha et al., 2012). In addition, ABTS radical is soluble in both water and organic solvents, so it can be used for both water-soluble and fat-soluble samples, which gives it an advantage over other methods.

It has been used on foods, for instance, tomatoes and soy sauce, wines and beers, and biological samples. However, a disadvantage of the method is related to the fact that the result obtained expresses the sample's ability to react with the ABTS radical and not its ability to inhibit an oxidative process (Dontha, 2016).

2.18.4 FRAP method (Ferric Reducing Antioxidant Power)

FRAP is a simple and automated method used to measure the ferric reducing power in different samples, such as plasma, phenolic strata, food or pure compounds (Pérez-Cruz et al., 2018). This method is based on the ability of antioxidants to reduce in acidic medium (pH~3.6) the ferric 2,4,6-trypyridyl-s-triazine (TPTZ) complex from $[Fe^{3+}\text{-}(TPZT)_2]^{3+}$ to $[Fe^{2+}\text{-}(TPZT)_2]^{2+}$ (**Figure 2.14**), which shows intense blue coloration in acidic medium (pH~3.6) and wavelength of 593 nm (Miguel-chávez, 2017).

Furthermore, FRAP value is calculated by determining the solution's absorbance (593 nm) by comparing it to the absorbance of a standard solution of ferrous ions or standard solutions of antioxidants, such as Trolox (Jones et al., 2018; Pérez-Cruz et al., 2018).

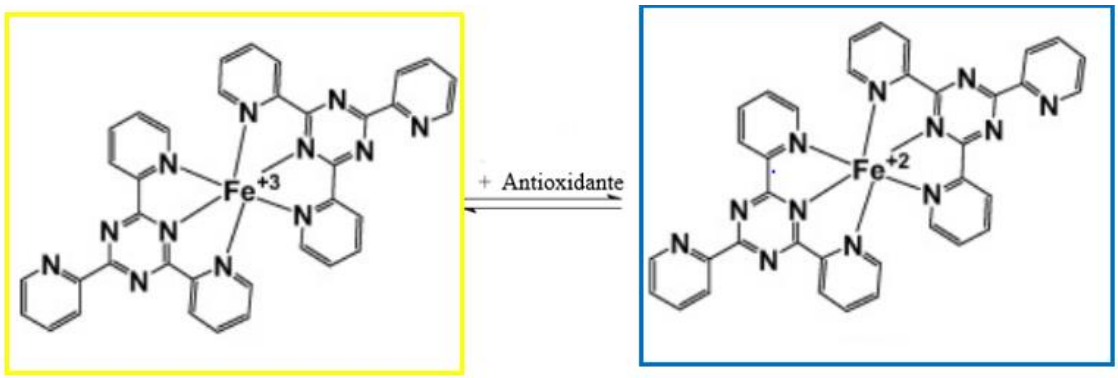

Figure 2. 13. Mechanisms between $[Fe^{3+}\text{-}(TPZT)_2]^{3+}$ and an antioxidant. Adapted from Pérez et al. (2018).

This method has some limitations; for example, any compound without antioxidant property can reduce Fe^{3+} to Fe^{2+}, overestimating the antioxidant capacity. In addition, another factor to consider is that Fe^{2+} can contribute to the formation of hydroxyl radicals resulting in the same problem (Dontha, 2016).

The most critical point of this method is that it assumes that maximum reaction time is between 4 and 6 min, but, in the case of phenolic compounds, including acids such as caffeic, tannic, and ferulic, the reaction can last more than an hour (Miguel-chávez, 2017).

2.18.5 Cupric reducing antioxidant power method (CUPRAC)

The CUPRAC method was developed by Apak et al. (2004) and is based on the reduction of $Cu^{2+}$to Cu^{+} (in hydroethanolic medium, pH 7 with ammonium acetate buffer) by the action of reducing agents (antioxidants) in presence of neocuproin (2,9-dimethyl-1,10-phenanthroline) or batocuproin (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline) that form complexes with Cu^{+} with absorption peaks at 450 and 490 nm, respectively (**Figure 2.15**). Thus, the increase in absorbance is proportional to the concentration of antioxidants present in the sample (Akar and Burnaz, 2019; Miguel-chávez, 2017).

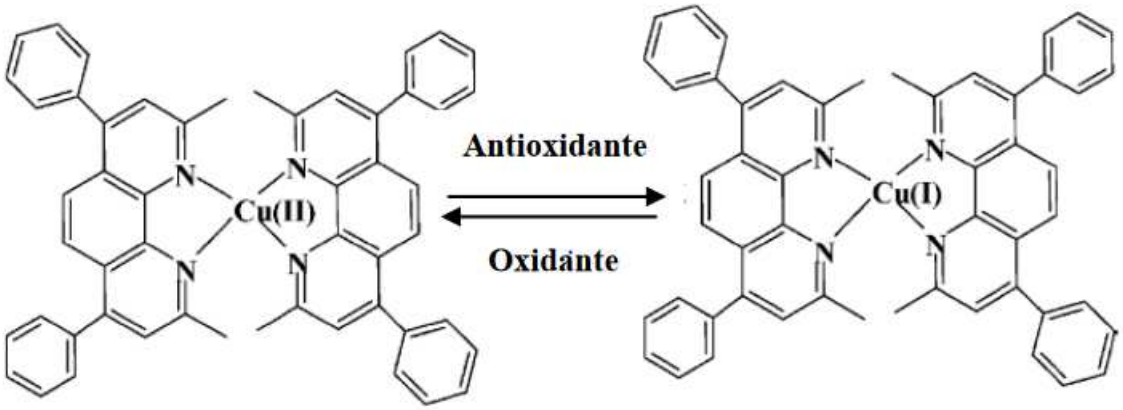

Figure 2. 14. Mechanism of the CUPRAC method. (Huang et al., 2005).

2.19 Electrochemical techniques

Antioxidants are reducing agents that easily oxidize at the electrode surface. Thus, electrochemical methods have great potential in characterizing electro-active compounds by studying their redox properties at the electrode surface. Thus, the possibility of characterizing redox mechanisms make these methods powerful analytical tools for the evaluation of antioxidant capacity and determination of various physicochemical parameters (Hoyos-arbeláez et al., 2017).

In recent years, these methods have emerged as a promising alternative to conventional spectrophotometric and fluorimetric methods in determining the capacity of antioxidants (food, beverage, and photochemical extract). This is due to several advantages when compared to other methods, such as high sensitivity, good selectivity, rapidity, simplicity, use of portable equipment, and a low cost and consumption of reagents, which implies a lower environmental impact (Biochem et al., 2011; Muhammad et al., 2019; Tyurin et al., 2015).

These methods offer a large number of ways for analysis, which are classified according to the electrical properties used for quantification of the analyte; among them can be mentioned: Potentiometry (measurement of potential generated by changing the concentration of a species in an electrochemical cell); coulometry (measurement of the amount of charge consumed for the conversion (oxidation or reduction) of a given species); and voltammetry/amperometry (measurement of the current generated

by oxidation or reduction reaction of a chemical species on the surface of an electrode) (Muhammad et al., 2019).

Among the most widely used techniques for antioxidant determination are voltammetric and amperometric. Voltammetry is based on the application of an electric potential difference (potential sweep) to an electrode, with the consequent measurement of the electric current obtained by the transfer of electrons during a redox process. Within this method, cyclic voltammetry and differential pulse voltammetry stand out (Hoyos-arbeláez et al., 2017).

The total antioxidant capacity of the sample is a function of the combination of two analytical parameters. The first is oxidation potential (anodic Ea and cathodic Ec), which specifically reflects the compound's reducing power; and the second is the intensity of generated current (I), reflecting components concentration.

This method has some advantages compared to the other methods, more specifically to FRAP. The advantage of using Cu^{2+} over Fe^{3+} is that all classes of antioxidants, including thiols, will be detected with little interference from free reactive radicals and reduction kinetics of Cu^{2+} is faster than that of Fe^{3+} (BADARINATH et al., 2010; BIOCHEM; PISOSCHI; NEGULESCU, 2011; HUANG; OU; PRIOR, 2005).

Furthermore, Cu^{2+}, free or complex, has low redox potential when compared to F^{3+}.Thus, sugars and citric acid, common interferents in the FRAP method, will not be oxidized as the reaction is more selective (Güçlü et al., 2008; Gupta, 2015; Miguel-chávez, 2017).

The CUPRAC method is fast and can happen within minutes for compounds such as: ascorbic acid, uric acid, gallic acid, and quercetin, but requires a longer time (30-60 min) for more complex molecules (Akar and Burnaz, 2019). The result is expressed in the form of uric acid equivalent (used as standard).

Table 2. 20.Measurement of antioxidant capacity in photochemical extracts

Antioxidant	Extract source	Method	Analysis conditions	Antioxidant capacity	Reference
C phicocyanin	*Spirulina máxima* (dry biomass)	TEAC	Pretreatment with supercritical CO_2 60 C 24.13 Mpa batch. Sample was diluted in H2O (50% weight/volume), filtered on 45µm filter. Flow injection system was used 1.2 ml L^{-1}	241,1 µmols Trolox mg^{-1}	DEJSUNGKRANONT; CHEN, 2017
Astaxanthin	*Haematococcus pluvialis*	TEAC	Extraction with super critical CO_2	3,01mmol g^{-1} Trolox	RÉGNIER et al., 2015
	Haematococcus pluvialis	TEAC	Sonic pre-treatment 90 s, then extraction with Dimethylsulfoxide.	0,18 mmol trolox g^{-1} extract	
Astaxanthin (powder)	*Haematococcus pluvialis*	TEAC	Extraction performed with supramolecular octanoic acid-based solvent	2,92 µM α-TE	SALATTI-DORADO et al., 2019
Astaxanthin (powder)	*Haematococcus pluvialis*	TEAC	Extraction with pressurized hexane 10,34 MPa 20 minutes at 10,34 MPa	0,196 mmol trolox g^{-1} extrato	CIFUENTES et al., 2010
Phycocyanin (crude extract)	*Arthrospira sp.*	β-carotene linoleic acid	Extraction was used hexane for 2 h at 6 ºC.BHA was used as a positive standard to compare the results.	63,28 % a 5mg L^{-1}	CHENTIR et al., 2018
Total Phenols	Ginger *(Zingiber officinale R.*)	DPPH	Super critical extraction 30M Pa 30 °C CO_2 flow rate of $1.42x10_{-4}$ Kg s_{-1}	E_{50} 145 mM trolox g^{-1}	JUSTO et al., 2008
	Rosemary (*Rosmarinus officinalis L.*)	DPPH	Super critical extraction 25 M Pa 40 °C, CO_2 flow rate $1,13x10^{-4}$ Kg s-1	E_{50} 80 mM trolox g^{-1}	
Total Phenols	Tamarind Seeds (*Tamarindus indica* L)	DPPH	Extraction of the wet samples was performed with methanol, 70% acetone. The solvents were removed using evaporation and subsequently lyophilized.	Metanol E_{50} 39,05 mg ddph g^{-1} Acetone E_{50} 41,45	SIDDHURAJU, 2007
		ABT		Metanol 3,327 mmol trolox g^{-1} Acetona 2,863 mml trolox g^{-1}	
Total Phenols	*Valeriana dioscoridis Sp*	FRAP	Sample was macerated for 24 h, then extracted with ethyl acetate.	E_{50} 2,03 mg ml	(Sarikurkcu et al., 2020)
		CUPRAC		E_{50} 3,45 mg ml	

Total Phenols	*Stachys schtschegleevii*	DPPH	Powdered samples (leaves) were extracted with methanol in a Soxhlet extractor for 8h. After complete extraction, the methanol solvent was evaporated by rotary evaporator at 45 °C reduced pressure and dried for 24h in a vacuum oven	E_{50} 3,09 mg ml	(Nasrollahi et al., 2019)

2.20 Conclusion

The vulnerability of biodiesel to oxidation is mainly related to the presence of polyunsaturated fatty acid chains in the ester molecules, which react easily with oxygen when exposed to air. Thus, the addition of antioxidants is generally necessary to achieve the minimum limit of oxidative stability for commercialization. The use of natural antioxidant substances derived from plants, microalgae, and fruits are an alternative to synthetic ones, given their renewable and less polluting nature.

Microalgae stand out as a promising source of antioxidants since strains composition can be regulated to the metabolite of interest by modulating operational variables and nutritional conditions. Therefore, these strains can synthesize different types of pigments that have important biological activities and are of great commercial interest. Among the pigments with antioxidant activity are the phycocyanins, *β*-carotene, lutein, and astaxanthin, produced specifically by the cyanobacteria *Spirulina platensis, and* microalgae *Dunaliella salina, Scenedesmus almeriensis,* and *Heamatocaccus pluvialis*, respectively.

However, despite the wide and diverse range of high value-added biomolecules obtained from microalgae, one of the major difficulties in the recovery process is related to the fact that these compounds are usually produced intracellularly. Consequently, cell disruption is necessary to improve the extraction efficiency of compounds since the passive diffusion of solvent through the cell wall is slow.

Among the various extraction methodologies, the use of supercritical CO_2 stands out because of the possibility of continuous adjustment of solubility power and solvent selectivity through the choice of processing parameters. Despite the known advantages of the process, the use of supercritical technology has an economic constraint due to the high initial investment inherent in process facilities.

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3. Chapter 2 - Potential antioxidants for biodiesel from natural extracts of microalgae *Scenedesmus obliquus* and *Haematococcus pluvialis*, turmeric, annatto, and coffee husk

Abstract

Biodiesel oxidation process causes changes in the chemical and physical properties of biodiesel, resulting in losses of fuel quality. Antioxidants addition has shown economical and viable approach to overcome biodiesel oxidative instability. This study evaluated the effect of ethanolic extracts from microalgae *Scenedesmus obliquus* and *Haematococcus pluvialis* (Cultivated – *H. pluvialis*, and commercial – C. *H. pluvialis*), and from turmeric (*Curcuma* sp.), annatto (*Bixa orellana*), and coffee husk (*Coffea arabica*) as antioxidant stabilizers for biodiesel from soybean (*Glycine max*), canola (*Brassica napus*), and macauba (*Acrocomia aculeata*). The potential antioxidant activity of the extract was determined by DPPH (1,1- Diphenyl-2-picrylhydrazyl). The most effective extract was turmeric (556.88 μM Trolox g^{-1}), followed by annatto (159.96 μM Trolox g^{-1}), *H. pluvialis* (56.35 μM Trolox g^{-1}), C. *H. pluvialis* (50.41 μM Trolox g^{-1}), C. husk (44.99 μM Trolox g^{-1}) and (42.99 μM Trolox g^{-1}). All the natural extracts increased the stability for all types of biodiesel studied, and the effects were dose dependent.

Keywords: Biodiesel storage. Microalgae. Natural antioxidants. *Tetradesmus obliquus* Coffee waste.

3.1 Introduction

Human population had experienced a dramatic increased over the past decades, reaching 8 billion in 2022 and is projected to increase 9.7 billion in 2050, and 10.4 billion in 2100 (UNITED-NATIONS, 2022). Global energy matrix is based mainly in non-renewable energy sources as such as: natural gas, coal, crude gasoline, gasoline, diesel and kerosene (Suparmaniam et al., 2019). Thus, the unregulated, disorganized and over exploration has caused several problems, such as: fossil fuel depletion (energy crisis), diseases associated with ecosystem degradation, environment deterioration (Kazemi et al., 2019). Sustaining population growth and economic development while minimizing environmental deterioration, global warming, and climate change, will require the use of a variety of biomass and technologies (Dar and Asif, 2023; Tan et al., 2018).

Biofuels stand out as a promising alternative to fossil fuels due to the renewable and economic importance. A plethora of feedstock are available for biofuels, such as animal and vegetable byproducts, biomasses, and waste materials. The characteristics of those renewable sources include to be non-toxic, reduce CO_2 emission and atmospheric pollutant during combustion (Mahmud et al., 2022; Moreira et al., 2022).

Biodiesel, a liquid biofuel, is composed of alkyl esters of long-chain carboxylic acids, produced from the transesterification or esterification of fatty materials (Agência Nacional do Petróleo, 2019). Moreover, it can be produced using a wide variety of feedstocks, edible (first generation), non-edible (second generation), and waste oils (third generation) (Yuan et al., 2020). However, the biodiesel main drawback is its susceptibility to oxidation, exacerbated on long storage period. Generally, oxidative stability of biodiesel is measured using the Rancitmat method, developed by Hadorn e Zürcher (1974) and which is based on the principle that the degradation rate increases linearly with the temperature increasing (Bär et al., 2018). The current Brazilian standard for biodiesel (APN Resolution No 798/219) establish a minimum of 12 hours stability for the commercialization of biodiesel.

The oxidative stability of mono alkyl-esters is mainly related to the presence, number and position of double bonds in the fatty acid chain (allylic and bis-allylic site), which easily react with oxygen radical species (Jemima Romola et al., 2021; Varatharajan and Pushparani, 2018). The oxidation causes changes in the chemical and physical properties of biodiesel, resulting in the loss of fuel quality due to the

formation of oxidation products such as aldehydes, alcohols, carboxylic acids, gums, and insoluble sludge that results in fouling problems and shorten life of engines (Kumar, 2017).

Biodiesel oxidative instability can be overcome by adding antioxidants (natural or synthetic) (R. Kumar et al., 2016; Varatharajan and Pushparani, 2018). Among the synthetic antioxidant, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), propylgallate (PG), oxytol gallate (OG), dodecyl gallate (DG), pyrogallol (PY) and ethoxyquin (EQ) are widely used (Chandra and Sharma, 2020; Sundus et al., 2017). Besides synthetic antioxidants, many natural compounds present antioxidant capacity and they are investigated due to their renewable and less polluting nature. Among the plant-based antioxidant commercially produced and on a large scale are tocopherols, carotenoids, lycopene, zeaxanthin, canthaxanthin, astaxanthin, gallic acid, caffeic acid, vanillin, sinapic acid, ferulic acid, protocatechuic acid, p-coumaric acid, eugenol, sesamol, vanillic acid (Makwana et al., 2015; Singh et al., 2020).

Microalgae is another potential source of natural antioxidants, which its production in 2020 achieved 7.5 million tons (Andrade et al., 2020; Mobin and Alam, 2017). Among the commercial microalgae genera and products of interest are *Dunaliella salina* (*β* carotene), *Haematococcus pluvialis* (astaxanthin), *Arthrospira platensis* (Spirulina), *Scenedesmus* sp. and *Chlorella* sp., among others (Andrade et al., 2020). In addition, microalgal pigments are colored chemicals that play an important role in photosynthetic metabolism, CO_2 fixation, protection of cells against damage caused by excessive exposure to light (Ambati et al., 2019; Koller et al., 2014). Moreover, they exhibit antioxidant, anticancer, anti-inflammatory, and neuroprotective effects (Levine and Fleurence, 2018).

Except for tocopherols, few studies have been carried out testing these natural antioxidants as additives for biodiesel. The purpose of the present study was to evaluate the antioxidant effect of ethanolic extract from *Scenedesmus obliquus* (*S. obliquus*), *Haematococcus pluvialis* (*H. pluvialis*), turmeric (*Curcuma* sp.), seeds annatto (*Bixa orellana)*, Coffee husk (*Coffea arabica*) and commercial *Haematococcus pluvialis* (*C.H. pluvialis*) in soybean and canola, and macauba biodiesel. The actions of the antioxidants were monitored by iodine, peroxide, and acid value over a period of 56 days.

3.2 Material and methods

3.2.1 Antioxidant biomass preparation

Strains of *Scenedesmus obliquus* BR003 and *Haematococcus pluvialis* BR040 was obtained from the Collection of Cyanobacteria and Microalgae of the Department of Plant Biology, at Federal University of Viçosa (Minas Gerais, Brazil). *S. obliquus* and *H. pluvialis* were maintained in BG11 (Andersen, 2005) and BBM (Harry William Bischoff; Harold C Bold, 1963) media, respectively. The *S. obliquus* was cultivated using L4-m culture medium (Rocha et al., 2019) in a 4.000 L open raceway tank using with an average daily radiation intensity of 752.1 µmol photons m^{-2} s^{-1}, and temperature ranged from 20 to 30 °C (Rocha et al., 2020). *H. pluvialis* cultivation was carried out a 250 ml Erlenmeyer flask containing 140 ml of BBM medium, under growth conditions at temperature 22±1 °C, photo period 12/12 hours (light/dark), under light intensity 100 µmol photons m^{-2} s^{-1} for five days for green vegetative stage. Astaxanthin production was induced (aplanospore stage) by increasing light intensity to 250 µmol photons m^{-2} s^{-1} for 14 days. After reaching the stationary phase, microalgal cells were centrifuged at 4000 g for 10 minutes, supernatant was discarded, and the pellet was frozen at -20 °C for 48 hours. Afterwards, the microalgae biomasses were freeze-dried under vacuum (lyophilizer L101,liobras, Brazil) for 48 hours (Zhang et al., 2022).

Fresh turmeric rhizome, annatto seeds (*Bixa Orellana)* were purchased form the local market of Viçosa, Minas Gerais State, Brazil. *Bixa Orellana*, coffee husk and Turmeric (cut in cylinder slices) were frozen at -20 °C for 24 hours and freeze-dried at -50 °C for 48h (Chumroenphat et al., 2021). Finally, freeze-dried biomasses were crushed using a mortar pestle and then sieved using a 100 mesh and stored -20 °C before analysis (Bharti and Singh, 2020)

Coffee husks were kindly provided by Colibri & Jatobá farm, located in Paula Cândido, Minas Gerais State, Brazil. Commercial astaxanthin was bought from Hhwish company (China).

3.2.2 Extraction of antioxidants

Extraction was performed by weighting 10 g of freeze-dried samples in a conical flask with 200 extraction solvent for 2 days at room temperature (22 ± 1 °C) with

continuous stirring at 2000 rpm (**Table 3.1,** Akter et al., 2019; Buosi et al., 2016). Flasks were covered whit aluminum foil to avoiding oxidation and protection from sunlight. After the extraction time, extracts were centrifuged at 10000 g for 10 minutes, supernatant was filtrated and concentrated on rotatory evaporator at 0,1 MPa and 40 °C (Devi et al., 2018). Residual biomass was re-extracted under the same conditions until it became colorless, approximately 4 times more, totally 8 days of extraction. Then, the extracts were dried under nitrogen and the yield was determinate by gravimetry (Equation 1). Finally the extracts were resuspended in ethanol 99 % at 30 mg mL $^{-1}$ (Stirk et al., 2020) and store -20 °C until used.

Table 3.1. Extraction solvent for each biomass

Sample	Solvent	References
Scenedesmus obliquus	Ethanol 99%	Mtaki et al. (2020)
Haematococcus pluvialis	Ethanol 99%	Zou et al. (2013)
Curcuma sp. (turmeric)	Ethanol 99%	Akter et al. (2019)
Bixa Orellana (Annatto)	Ethanol 99%	Quintero Quiroz et al. (2019)
Coffee husk (Coffea arabica)	Ethanol 99%	Silva et al. (2020)
Commercial *Haematococcus pluvialis*	Ethanol 99%	Mtaki et al. (2020)

Extraction yields of each extract were calculated using the equation 3.1.

$$Yiled\ (\%) = 100\frac{M_1}{M_2} \qquad \text{(Eq. 3.1)}$$

Where M_1 is extract weight after evaporation and dried under nitrogen, and M_2 is dry weight of freeze-dried samples.

3.2.3 Antioxidant activity DPPH assay

The DPPH scavenging assay done at deem light as reported by Brand-Williams et al. (1995) and adapted by Assunção et al. (2017). DPPH stock solution 1 mmol L^{-1} was prepared in ethanol. This solution was stored at 5 °C and protected from light. Afther, DPPH working solution was prepared 0,06 mmol L^{-1}

This solution was stored at 5 °C and protected from light. In brief, 200 µL of each extract were add to 1,8 ml DPPH working solution, mixed and vortex vigorously for 1 min. The mixture solution was incubated for 40 minutes, protected from light, at room temperature 25 °C followed by absorbance reading at 515 nm. Because of intrinsic characteristic of the extract that absorbs at 515 nm, a blank sample was prepared with ethanol and the extract (Maadane et al., 2015). DPPH radical scavenging activity (SA) were calculated using the equation 3.2.

$$SA = 100\left(1 - \frac{A_s - A_b}{A_c}\right) \quad \text{(Eq. 3.2)}$$

where A_c denotes the initial absorbance of DPPH 0,06 mM without test samples or standard, A_b is the absorbance of the blank sample and A_s is the absorbance of ethanolic extract or standard with DPPH. Trolox was used as standard at concentration from 8 to 1000 µ mol L^{-1} (Li et al., 2007), and scavenging activity was expressed as µM Trolox equivalent per gram of extract.

3.2.4 Phenolic content of the extracts

Total phenolic content was estimated by Folin-Ciocalteu method (Singleton et al., 1999) adapted by Martínez-Sanz et al. (2020). In brief, 0.2 ml of extract at concentration of 5 mg·L^{-1} in ethanol were added to 1 mL of folin-Ciocalteu working solution, diluted as 1:10 in water (v v^{-1}), and incubated at room temperature for 4 minutes. Then, 0.8 mL of aqueous Na_2CO_3 75 mg·mL^{-1} was added and the solution was incubate at 50 °C for 30 minutes, followed by absorbance measurement at 750 nm. Gallic acid was used as standard (0 - 500 µg/ml) (Morowvat and Ghasemi, 2016a) and the result were expresses as mg gallic acid per gram of extract.

3.2.5 Chlorophyll a, Chlorophyll b and carotenoids in the extracts

Pigments were quantified from freeze-dried biomass using dimethyl sulfoxide (DMSO) according to Griffiths et al. (2011). In brief, 2 mg of biomass was added in 2 mL DMSO pre-heated at 60 ºC and incubated for 10 minutes. Afterwards, samples were centrifugate as 10000g for 10 minutes. The supernatant was collected and diluted

in DMSO until the absorbance became below 1. After cooling the sample at room temperature, the absorbance were measured at 480 nm (A_{480}), 649 nm (A_{649}), and 665 nm (A_{665}) for the quantification of carotenoids, chlorophyll *a* and *b* respectively (Eq. 3.3 to 3.5), Wellburn, 1994), expressed in mg·L^{-1}.

$$Chorophyll\ a\ (chl\ a)(\ mg\ L^{-1}) = 12{,}47(A_{665}) - 3{,}62\ (A_{649}) \quad \text{(Eq. 3.3)}$$

$$Chorophyll\ b\ (chl\ b)(\ mg\ L^{-1}) = 25{,}06(A_{649}) - 6{,}5\ (A_{665}) \quad \text{(Eq. 3.4)}$$

$$Carotenoids\ (\ mg\ L^{-1}) = \frac{1000(A_{480}) - 1{,}29(Chl\ a) - 53{,}78(Chl\ b)}{220} \quad \text{(Eq. 3.5)}$$

Astaxanthin extraction and quantification was performed according to Li et al. (2012). In brief, 2 mg of freeze-dried *Haematococcus pluvialis* was suspended in DMSO pre-heated at 70 ºC and incubated for 10 min. Afterwards, samples were centrifuged at 10000g for 10 minutes. The extraction procedure was repeated until supernatant become colorless. Supernatant for each extraction was diluted in DMSO until the absorbance became lower than 1. After the sample cooling to room temperature, the absorbance at 530 nm (A_{530}) was measured for quantification of astaxanthin content (Equation 6), expressed in in mg·L^{-1}.

$$Astaxanthin\ (C_a)(\ mg\ L^{-1}) = \frac{A_{530} - 0{,}0107}{0{,}01556} \quad \text{(Eq. 3.6)}$$

3.2.6 Spectrophotometric profile of the extracts

UV-visible spectrum of ethanolic extract profile of *Scenedesmus obliquus, Haematococcus pluvialis*, turmeric, annatto, coffee husk, and commercial *Haematococcus pluvialis* were determined in a 96-well quartz microplate. The spectrophotometer profile was performed from 220 to 750 nm with a measurement interval of 1 nm in a microplate reader (Multiskan GO, Thermo Scientific, Germany).

3.2.7 GC-MS analysis of the extracts

The chemical composition of natural extracts were determinate by gas chromatography coupled to mass spectrometry using a Shimadzu GCMS-QP5050A equipment. In brief, the analysis were performed using a SPB-5 capillary column (30

m x 0.25 mm, 0.25 mm); Helium as carrier gas at 1.6 mL min^{-1}; sample volume 1.0 mL; split ratio 1:3; injector temperature 300 °C; detector temperature 300 °C; oven temperature from 80 °C for 5 min, increase rate of 4 °C min^{-1} until 300 °C and kept for 45 min; Scan mode (35 to 700 m/z); ionization by electron impact mode at 70 eV. Before analysis, oil sample of 3 were subjected to derivatization with 100 mL of *N,O-bis* (trimethylsilyl trifluoroacetamide (BSTFA), and 60 mL of anhydrous pyridine in a conical reaction vial. The mixture was kept at 70 °C for 30 min and then subjected to CG-MS analysis (Rocha et al., 2020).

3.2.8 Feedstock for biodiesel production

Soybean and canola oil, free from antioxidants, were purchased at the local market in Viçosa, Minas Gerais State, Brazil. Macauba pulp oil obtained from cold mechanical press, was kindly donated by Experimental Station of Araponga CVT (Centro Vocacional Tecnológico de Araponga) at the Department of Agronomy of Federal University of Viçosa.

3.2.9 Oil refining

The refining of oils were performed according to Crexi et al., (2010) and adapted for (Moraes et al., 2020) with some modification. The refining process comprised of degumming, neutralization, washing and bleaching.

Degumming was carried out using 1% phosphoric acid (85% v v^{-1}) in relation to the crude oil mass at 80 °C for 30 minutes in a magnetic stirrer at 500 rpm. The gums were removed by centrifugation at 7000 g for 10 minutes. Neutralization step was performed by adding aqueous solution of sodium hydroxide 20 % (w w^{-1}), using 4% of excess to the acid value after the degumming step at 40 °C for 20 minutes and magnetic stirred at 500 rpm. Soap formed was removed by centrifugation at 7000 g for 10 minutes.

Washing step was carried out by adding 10% (w w^{-1}) water at 95 °C, in relation to the oil mass, at 50 °C for 10 minutes and magnetic stirred at 500 rpm. This step was repeated tree times. The oil sample was dried in a rotatory evaporator under reduced pressure at 40 °C for 1 hour (Devi et al., 2018).

The bleaching step was performed by adding 2% (w w^{-1}) of adsorbents (activate earth and activated coal at a 9:1 ratio) at 70 °C for 20 minutes under magnetic stirring at 40 rpm. The oil sample was filtrated in a Buchner funnel with a filter paper of 0.22 µm (Whatman 1820-047 GF/A) under 700 mmHg of vacuum.

3.2.10 Biodiesel synthesis

Acid esterification was performed on refined macauba oil, prior to transesterification, in order to reduce the fatty acid content to less than 5 mg KOH g^{-1}. The process was performed according to Khan et al. (2010) and Damanik et al. (2017) with modifications. The oil was pre-heated at 65 °C for 5 minutes and add concentrated H_2SO_4 0.5 % (w w^{-1}) and methanol at molar ratio of 10:1 in relation to the oil mass. The reaction was kept under reflux at 65 °C for 3 h and magnetic stirred at 500 rpm. After the reaction time, the products were settled in two phases during 4 hours using a separation funnel (Sebastião et al., 2018). Washing was carried out by adding 10% (w w^{-1}) water at 50 °C, in relation to the oil mass, for 10 minutes and magnetic stirred at 500 rpm. Finally, the washed sample was dried in a rotatory evaporator (lyophilizer L101,liobras, Brazil) under reduced pressure at 40 °C for 1 hour (Devi et al., 2018).

Biodiesel production of refined oils were carried out through transesterification reaction with homogeneous alkali catalysis. The reaction was performed in round bottom flash equipped with a thermometer, reflux condenser to avoid methanol losses, a water bath and magnetic stirred. In brief, the refined oil was pre-heated at 65 °C for 5 minutes and added by 1% of KOH premixed with methanol (molar ratio oil: methanol of 1:6). The reaction was kept at 60 °C for 60 minutes under magnetic stirring at 500 rpm (Nogueira et al., 2020a). After the reaction time, the mixture was poured into a separator funnel and let to settle for 4 hours, resulting in crude methyl ester (upper phase), methanol excess, glycerol and catalyst (lower phase). The methyl ester phase was washed using 10% water (w w^{-1}), in relation to the oil mass, at 50 °C, this process was repeated 3 times or until neutral pH (Buosi et al., 2016). Finally, the ester phase was dried using rotatory evaporator under reduced pressure at 40 °C for 1 hour (Devi et al., 2018) and filtrated using a Buchner funnel whit a filter paper 0.22 µm carried at a vacuum 700 mmHg.

3.2.11 Characterization of biodiesel

3.2.11.1 Fatty acid profile composition of biodiesel

Fatty acid methyl ester composition of biodiesel were defeminated according to Ichihara and Fukubayashi (2010), and Guihéneuf et al. (2015) by gas chromatography (Shimadzu, GC-2010, Japan) coupled to flame ionization detector (GC-FID) equipped with a 100 m x 0.25 mm capillary column (SP-2560, Sigma-Aldrich, USA). The analyses were carried out by dissolving samples in and hexane and direct injection of 1 µl. Gas Helium was used as carried at a flowrate of 1 mL min^{-1}, linear heating ramp from 60 to 330 °C at a heating rate of 20 °C min^{-1}. The identification of the peaks was confirmed by comparison with the standard mixture of FAME (SupleCo 37 FAME mix).

3.2.11.2 Moisture content

The determination of moisture content and volatile compounds was based on the AOCS Ca 2c-25 standard (AOCS, 2007). The standard determines the percentage of water and organic matter that volatilizes up to a temperature of 130°C. Sample of 1 g in a crucible or petri dish previously dried and tared. The container with sample was oven dried at 130 °C for a period of 30 minutes. Then, the set was removed from the oven, and placed in a desiccator until cooling to room temperature and weighted. The weighting process was repeated until the variation in the mass was below 0.05%.

3.2.11.3 Density 20 °C

Density was measured according to ASTM D-4052 standard (ASTM, 2008).The determination was performed using a 10 ml pycnometer, previously calibrated, into a thermostatic bath. The calibration of the pycnometer was performed using water at 20 °C. Therefore, the difference in weight, measured on an analytical balance, between the mass of the full and empty pycnometer, is the mass of water, using its density it was possible to determine the real volume of the pycnometer. The determination of the density of the samples began by carefully pouring the sample into the pycnometer to avoid the formation of bubbles. Subsequently, the set was immersed in a thermostatic bath at 20 °C and kept for 10 minutes. Finally, the set was removed and weighed, and the density calculated.

3.2.11.4 Free fatty acid and Acid Value

The acid value (FFA) was determined by titration following the AOCS Ca 5a-40 standard (AOCS, 2007). A sample of 1 gram was weighed in a 125 ml Erlenmeyer flask. A volume of 25 ml of a neutral solution of ether: ethyl alcohol (2:1 v v^{-1}) was added to the flask, and two drops of phenolphthalein indicator were added. Finally, the sample was titrated with 0.1 mol L^{-1} NaOH, until the appearance of a persistent pink color for at least 30 s. The results were expressed % free fatty acid as oleic (FFA, equation 7).

$$FFA = \frac{28{,}2\ f\ M\ (A-B)}{m} \quad \text{(Eq. 7)}$$

where *A* and *B* are the volumes (mL) of titrating for the sample and the blank, respectively, f is the correction factor, M is the molarity of the titrant solution (0.1 mol·L-1 NaOH), and *m* is the mass (g) of the sample. The free fatty acid content can be also expressed in terms of acid value (mg KOH g^{-1}). To convert percentage of free fatty acid (as oleic acid) to acid value, is necessary to multiply for 1,99.

3.2.11.5 Iodine Value

The iodine content (II) was determined by the Wijs method following the EN14111 standard (EN 14111, 2003). According to the standard, 0.25 g of the sample was weighed in a 250 ml Erlenmeyer flask. Then, 20 ml of a solution of cyclohexane and glacial acetic acid 1:1 (v/v), and 25 ml of Wijs reagent were added. The Erlenmeyer flask was kept at rest and protected from light for one hour. Then, 20 ml of a 10% KI solution (100 g L^{-1}) were added. Finally, the system was titrated using 0.1 mol·L^{-1} sodium thiosulfate, previously standardized, until the yellow color almost disappeared. Then, 2 ml of 1% starch indicator suspension was added. The titration continued until the indigo color disappeared. The results were expressed in g 100 g^{-1}, according to equation 8.

$$II = \frac{12{,}69\ f\ M\ (B-A)}{m} \quad \text{(Eq. 8)}$$

where *A* and *B* are the volumes (mL) of titrating for the sample and the blank, respectively, f is the correction factor, M is the molarity of the titrant solution (0.1 mol·L-1 sodium thiosulfate), and *m* is the mass (g) of the sample.

3.2.11.6 Peroxide value

The peroxide index (PI) was determined following the AOCS Cd 8–53 standard (AOCS, 2007). A sample of 1 g was weighed in a 125 ml Erlenmeyer flask and dissolved in 30 ml of a 3:2 (v v^{-1}) glacial acetic acid: chloroform solution. Then, 0.5 ml of saturated KI solution was added and left to rest for 1 minute and protected from light. Subsequently, 30 ml of deionized water was added, and titration was performed using 0.1 mol L^{-1} of sodium thiosulfate, previously standardized, until the yellow color almost disappeared. Finally, 1 ml of 1% (w v^{-1}) starch suspension was added and the titration continued until the indigo color disappeared. Peroxide index (PI) was calculated using equation 9.

$$PI = \frac{1000\ f\ N\ (A-B)}{m} \quad \text{(Eq. 9)}$$

where *A* and *B* are the volumes (mL) of titrating for the sample and the blank, respectively, f is the correction factor, M is the molarity of the titrant solution (0.1 mol·L^{-1} sodium thiosulfate), and *m* is the mass (g) of the sample.

3.2.12 Determination of stability time

Oxidative stability was analyzed in a Rancimat equipment in accordance with EN14112 standard (EN 14112, 2003). Rancimat is an accelerated ageing method, in which the oxidation is induces by bubbling steam air at a flowrate of 10 L h^{-1} through 3 grams of biodiesel sample maintained at a 110 ºC. Highly volatile secondary reaction oxidation products, mostly formic acid, are transfer by the airflow to a measuring vessel containing 50 ml demineralized water and an electrode for measuring the electrical conductivity. Values of electrical conductivity (µS) as a function of time (h) were then related in a chart, and the inflection point of the curve represented the induction time. Biodiesel samples with natural extracts (*Scenedesmus obliquus, Haematococcus pluvialis*, turmeric, annatto, and coffee husk) at different concentrations were analyzed.

3.2.13 Screening of potential natural antioxidant for biodiesel

In order to assess the individual effect of each extract of *Scenedesmus obliquus*, *Haematococcus pluvialis,* turmeric*, annatto,* and coffee husk at specific concentration optimized on Rancitmat (higher stability time) on soybean, canola and macauba biodiesel stability time were tested on concentration of 500 to 3000 ppm. Butylated hydroxytoluene (BHT) at concentration 1000 ppm was used as positive control (Jemima Romola et al., 2021; Varatharajan and Pushparani, 2018), and negative control was defined as biodiesel samples without additive.

3.2.14 Long term storage stability test

After verifying the antioxidant potential of the extracts and the positive impact on the oxidative stability, a long-term storage test was conducted for soybean, canola, and macauba biodiesel to evaluate the oxidation behavior over the time. The experiment was performed for 56 days according to ASTM-D4625 (ASTM, 2003). at 43 °C in glass bottled wrapped with aluminum foil. The lid of the sample container was semi-closed to allow gaseous exchange. Peroxide, acid, and iodine value and induction time were monitored at every 8 days to monitored biodiesel degradation.

3.2.15 Statistical analysis

Experiments were performed in triplicate and values are represent as means ± standard deviation. One-way ANOVA was used to determinate significance differences between means ($p < 0,05$) followed by post hoc Tukey test.

3.3 Results and discussion

The fatty acid composition of soybean, canola, and macauba biodiesel synthetized are given in **Table 3.2.** The physical-chemical properties (density, kinematic viscosity, cetane number) of biodiesel are strongly influenced by the individual characteristics of the methyl ester profile, which depend on the fatty acid content of the raw material (Nor et al., 2015; Yaşar, 2020b). In addition, the vulnerability of biodiesel to oxidation is mainly related to the presence, number and position of double bonds in the alkyl chain (Kumar, 2017; Varatharajan and Pushparani, 2018).

However, saturated compounds have good oxidation stability, but negatively impact on biodiesel properties, such as viscosity and cloud point, especially at low temperatures (Bing et al., 2020; Signori et al., 2018).

Table 3.2. Biodiesel fatty acid composition %

Fatty acid esters	Composition	Soybean	Canola	Macauba
Palmitic	C16:0	1.5	4.6	22.19
Palmitoleic	C16:1			4.99
Estearic	C18:0	4	2.0	2.4
Oleic	C18:1n9c	28.8	61.0	60.53
Linoleic	C18:2n6c	51.1	18.2	8.98
Linolenic	C18:3n6c	5.6	6.5	
Total				
∑ Saturated (C16:0 + C18:00)		14.5	6.6	24.59
∑ Monounsaturated (C18:1)		28.8	61.0	60.53
∑ Polyunsaturated (C18:2+ C18:3)		56.7	24.7	8.98

Thus, results for fatty acid compositions found for biodiesel studied here indicates that soybean biodiesel is more sensitive to oxidation (**Table 3.2**) due to the high concentration of polyunsaturated and monounsaturated fatty acids, followed by and Macauba.

3.3.1 Physicochemical characterization biodiesel

The physicochemical parameters of produced biodiesel are presented in **Table 3.3**. Nearly all values are within requirements of Brazilian National Agency of Petroleum, Natural Gas and Biofuels (ANP), except for acidity of Macauba biodiesel and oxidation stability of all biodiesel. Particularly, ANP resolution No 798/219 establish a minimum of 12 hours stability time and maximum acidity 0,5 mg KOH g^{-1}.

The physical-chemical characterization of feedstocks is an important step in biodiesel manufacture because it determines the more suitable conversion process (acid esterification or alkaline transesterification). For instance, water content and acid value are the most crucial parameter. Moreover, higher acid values (0.5 mg KOH g^{-1}) and water content (200 mg kg^{-1}) negatively affect the conversion yield and quality of biodiesel (Agência Nacional do Petróleo, 2019; Ramos et al., 2019).

The high acidity value of macauba oil is related to hydrolysis of triglycerides, and consequently formation of free fatty acid (Souza et al., 2016). Because of the high

acidity value of macauba oil (74.040 ± 0.50 mg KOH g^{-1}), it was necessary to perform an acid esterification prior to alkaline transesterification, leading to an esterified oil acid value of 4.41 ± 0.007 mg KOH g^{-1}.

In the study, all biodiesel fell short to meet ANP specification of stability time, which can be attributed to the high content of polyunsaturated fatty acids demonstrating the need for the addition of antioxidants, that can prevent or delay biodiesel oxidation. Particularly, biodiesel's oxidative stability was: 4.93, 6.02 and 1.98 h for soybean, canola and macuaba biodiesel respectively. Comparable result for soybean biodiesel 3.77 and 4.53h were found by Sousa et al. (2021) and by Nogueira et al. (2020b) respectively. In addition, Moser (2011) found equivalent stability time for canola biodiesel 6.4 h. Aguieiras et al. (2014) worked with macauba biodiesel found similar stability time of 0.95 h.

Table 3.3.Physicochemical proprieties of soybean, canola and macauba biodiesel

‘		Biodiesel			Limit
	Unit	Soybean	Canola	Macauba	ANP
Acidity Value	mg KOH g^{-1}	0.17±0.00	0.17±0.00	0.54±0.01	max 0,5
Peroxide Value	meq Kg^{-1}	2.97±0.02	3.31±0.03	1.47±0.01	n.s*
Iodine value	g I2 100 g^{-1}	112.62±0.62	107.59±0.64	83.51±0.75	n.s*
Oxidation Stability (110 °C)	h	4.93±0.05	6.02±0.28	1.98±0.00	min 12
Ester content (wt)	%	97.33	96.89	97.19	96,5
Water content	mg kg^{-1}	141.56±0.00	126.56±0,00	180.50±0.00	max 200
Density	Kg m^{-3}	864.11±1.36	861.51±0,60	867.95±0,27	860-900

*Not specified.

3.3.2 Phenolic compounds

The total phenolic content of ethanolic extracts was measured using Folin-Coicalteu assay and the results are presented in **Table 3.4**. Phenolic content in this study showed a wide variation, *H. pluvialis* 9.65 ±1.69 to turmeric 115.26 ± 5.56 mg GAE g^{-1} extract.

Table 3.4. Determination of extraction yield, and phenolic content in the natural extracts

Extract	**Extraction yield (%)**	**Total phenol content (mg GAE g^{-1} extract)**
S. obliquus	29.89	17.60 ± 1.76ab

H. pluvialis	25.17	9.65 ± 1.69a
Turmeric	54.08	115.26 ± 5.56c
Annatto	31.55	28.08 ± 2.00b
C. husk	6.89	16.30 ± 0.00ab
C H. pluvialis	18.21	23.46 ± 1.51ab

*Values containing different letters differ from each other by Tukey's test at 5%.

The highest phenolic content was observed for turmeric extract 115.26 ± 5.56 mg GAE g^{-1}. This value is in agreement with those found by Akter et al. (2019) in their study on the phenolic an antioxidant activity of different species and varieties of turmeric (*Curcuma* spp) Turmeric (*Curcuma* sp.) is an herb originally from the southeast Asia, belonging to the Zinzaberaceae (Rodrigues et al., 2020). It had been reported to have antioxidant anti-inflammatory, anti-angiogenic, antibacterial, antifungal (Akter et al., 2019). The major phytochemicals in *Curcuma* sp. responsible for these properties are mainly curcuminoids (curcumin, demethoxycurcumin, bisdemethoxycurcumin and cyclocurcumin), followed by phenolic acids, and flavonoids (Chumroenphat et al., 2021).(Freitas et al., 2019).

..

Extracts from annatto seeds showed the second higher value of phenolic content (Table 3.4). Equivalent result were found by Oliveira et al. (2022) that assed the antioxidant and antimicrobial activity seed of *Bixa orellana*, in different solvents, found 30.10 ± 0.05 mg GAE g^{-1} extract in methanolic extract. Also, Ahmed et al. (2020) performed a comparative study of aqueous, ethanolic and methanolic extract on annatto seeds for in vitro phytochemicals, antioxidant, and antibacterial activities. The authors found a higher content of phenolics of 46,34 mg GAE g^{-1} in the extract. Annatto seeds are widely used as a coloring and flavoring agent, supplement feed in animal and human, cosmetic, and pharmaceutical industry (Ahmed et al., 2020). Several carotenoids had been detected in the in the annatto seeds and extract, with bixin accounting for 80% of the total carotenoid content of these seeds (Quintero Quiroz et al., 2019) (Oliveira et al., 2022)..

Phenolic content in *S. obliquus* is also close to those found for species of the *Scenedesmus* genus, e.g *Scenedesmus bijuga* (20.62 ± 1.18 GAE g^{-1}Santhakumaran et al., 2020), *Scenedesmus* sp. (12.23 GAE g^{-1}, Bulut et al., 2019), and *S. obliquus* (11.2 GAE g^{-1}, Silva et al., 2021).

Coffee cherry processing into cherry bean can be done by wet or dry routes, generation different types of residues. The most used method in Brazil is drying, which generates as a principal byproduct coffee husks yielding approximately 50% of weight of the coffee beans (Silva et al., 2020). Coffee husk is comprised of skin, pulp, mucilage and part of the silverskin (Rebollo-Hernanz et al., 2021). According to results in **Table 3.4**, the phenolic content of coffee husk was 6.30 ± 0.00 mg GAE g^{-1} in the extract. Rebollo-Hernanz et al. 2021 modulated a optimize a green sustainability method to extract phenolic compounds from coffee husk, that study reported similar value of total phenolic 5.45 ± 0.12 mg GAE g^{-1} in the extract.

3.3.3 Pigments

Total pigment, chlorophylls, and carotenoid content of ethanolic extracts are presented in **Table 3.5**. Annatto displayed the highest carotenoid content. Similar value (49.0 ± 2.0 mg g^{-1}) was reported by Albuquerque and Meireles (2012) reported analogous carotenoid content of in annatto seeds.

Table 3. 5. Pigment content in extracts

	Chlorophyll		**Carotenoid**		
Extract	***Chl* a**	***Chl* b**	**Total carotenoid**	**Astaxanthin**	**Total pigment (mg g^{-1})**
S. obliquus	29.09±0.13a	14.33±0.75a	2.50±0.17a		46.40±0.64a
H. pluvialis	3.76±0.11b	4.44±0.32b	10.08±0.10b	10.02±0.06a	18.62±0.48b
Turmeric	2.92±0.11c	6.80±0.35c	38.29±0.14c		48.01±0.31a
Annatto	2.82±0.13c	6.34±0.27c	48.11±1.11d		57.27±1.45c
C. husk	2.82±0.14d	3.17±0.08d	0.17±0.03e		4.86±0.10d
C H. pluvialis	2,82±0,15d	4.52±0.17b	15.37±1.13f	9.85±0.82a	21,56±1.30e

*Values containing different letters differ from each other by Tukey's test at 5%.

In this study the second higher carotenoid content was found in turmeric extract (38.29 ± 0.14 mg g^{-1}).Turmeric rhizome carotenoid content is mainly related to curcumin where its concentration varied from 20 to 60 mg g^{-1} (Degot et al., 2021; Monton et al., 2016).

H. pluvialis is a freshwater microalga that exhibits a great adaptive capacity to adverse environmental conditions, with high light intensity, presence of reactive oxygen species or nutrient depletion, achieved by the transformation of the cells into a

non-motile cyst, surrounded by a thick membrane (Orona-navar et al., 2017). *H. Pluvialis in* red cyst, carotenoid content are mainly related to astaxanthin. Astaxanthin is a secondary carotenoid that has a protective function against photodamage, and is stored into oil droplets in the microalga cytoplasm (Gong and Bassi, 2016). In this study, astaxanthin recorded a concentration of 10.08 ± 0.1 mg g^{-1} in *H. pluvialis* and 15.37 ± 1.13 mg g^{-1} in commercial *H. pluvialis, contents in agreement with* Niizawa et al., (2018) that found 12 mg g^{-1} of astaxanthin. On a recent study, León-Vaz et al., (2023) observed a carotenoid content of 8.20 ± 0.78 mg g^{-1} in *Haematococcus pluvialis*.In addition, it is widely recognized for accumulating large amounts of astaxanthin in stress conditions.

S. obliquus in the present work had low carotenoid content (2.50 ± 017 mg g^{-1}), compared to 5.4 ± 0.34 mg g^{-1} (Banskota et al., 2019) and 5.0 mg g^{-1} (Almendinger et al., 2021), and *Scenedesmus almeriensis* 3.0 ± 0.5 mg g^{-1} (Molino et al., 2018). The low carotenoid content observed may be due to carotenoid degradation, due they are sensitive to light, temperature and oxygen, therefore, preserving it physicochemical properties along the time is a complex task (Gong and Bassi, 2016).

3.3.4 Antioxidant activity

Antioxidant activity of astaxanthin is related to chemical structure, presence hydroxyl and keto group bounded in an ionone ring (Liu and Osawa, 2007). Differences in antioxidant activity of astaxanthin reported in literature are related to isomers composition present in the extract. Therefore, the isomer 9-cis and 13 cis are known for having higher antioxidant activity compared with all-trans isomers (Aye Myint et al., 2022; Liu and Osawa, 2007).

The phytochemical compounds present in the extract are responsible for the distinct antioxidant activity in the two *H. pluvialis* under study (**Table 3.6**). Antioxidant activity of *H. pluvialis* and commercial *H. pluvialis* extract were 56.35 ± 0.69 and 50.41 ± 0.11 µM Trolox g^{-1}, respectively. Coherent results (45.5 ± 1.4 µM Trolox g^{-1}) were observed by Aye Myint et al. (2022) on ethanolic extract. *S. obliquus* presented an antioxidant capacity of 42.99 ± 2.68 4 µM Trolox g^{-1} .Similar value 68.68 ± 5.95 were related by (Morowvat and Ghasemi, 2016b). Goiris et al. (2012) studied the phenolic content and antioxidant activity of 32 microalgae. The authors performed a multiple

regression analysis and concluded that both phenolic and carotenoid significantly contribute to the antioxidant activity.

Table 3.6.Determination of antioxidant activity (DPPH) in natural extracts

Extract	**(DPPH)** **(μM Trolox g^{-1})**
S. obliquus	42.99 ± 2.68a
H. pluvialis	56.35 ± 0.69b
Turmeric	556.88 ± 1.74c
Annatto	159.9 6± 0.65d
C. husk	44.99 ± 2.47ae
C H. pluvialis	50.41 ± 0.11be

*Values containing different letters differ from each other by Tukey's test at 5%.

Turmeric presented the highest antioxidant activity among all ethanolic extract (556.88 ± 1.74 μmol L^{-1}Trolox g^{-1}. Lower antioxidant activity was found by Manaois et al. (2020) that found 303 ± 9.0 μmol L^{-1} Trolox g^{-1}. Curcumin acts as primary antioxidants interrupting initiation and delaying propagation oxidation reaction promoted by radicals that donate hydrogen to free radicals (J. S. Rodrigues et al., 2020; Varatharajan and Pushparani, 2018).

Annatto antioxidant activity was 159.96 ± 0.65 μmol L^{-1} Trolox g^{-1} (**Table 3.6**). Other study using antioxidant assay reported 140 μmol μM Trolox g^{-1} for annatto ethanolic extract (Cardarelli et al., 2008). Bixin and phenolic contents are directly related to the antioxidant activity of annatto seed extract. Those compounds have conjugated double bonds that quench singlet oxygen and extinguish singlet triple state of sensitizers (Quintero Quiroz et al., 2019)

Coffee husk presented an antioxidant activity of 44.99 ± .47 μmol L^{-}1Trolox g^{-1}. Coffee husk is source high value phytochemical, phenolic compounds (Chlorogenic, protocatechuic, and gallic acids are the main phenolic), caffeine and tannins, responsible for the antioxidant, antimicrobial, and anticarcinogenic activities of coffee husk (Rebollo-Hernanz et al., 2021).

Comparation of carotenoid, phenolic content and antioxidant activity in literature, need to take in consideration phytochemical composition is directly related to culture condition (temperature, photo period, nutrient availability) (Maadane et al., 2015). Moreover, choosing the extraction solvent is crucial, since the solvent polarity

significantly influence the pool of extracted products, such as ethanol that extract more polyphenols than water or hexane (Gallego et al., 2018).

3.3.5 GC-MS analysis of extract

GC-MS analysis was carried out to identify the organic compound in the natural extract. Natural extract analysis (**Table 3.7**) showed a plethora of chemical compound (classified in 7 groups) with varying relative content (%). The striking difference between the natural extract were higher relative content of phenolic compounds (aromatic), and carboxylic acids. Thus, according to the results presented in **Tables 3.4** and **3.6,** phenolic content and antioxidant activity are correlated. As reported by Goiris et al. (2012) the phenolic content and antioxidant activity of 32 microalgae were strictly related to the antioxidant activity. Therefore, among the natural extract turmeric stand out with 30.95% of phenolic content, followed by 7.43% in coffee husk, 1.69% in *H. pluvialis*, 1.67% in annatto, 0.7% in *S. obliquus,* and 0.68% in C. *H. pluvialis*.

Table 3.7. Relative content (%) of major organic compounds identified by GC-MS analysis of bio-oil from slow pyrolysis, after derivatization by sylilation

Compound group			Natural extract			
	S. obliquus	*H. pluvialis*	Turmeric	Annatto	C. Husk	*C. H. pluvialis*
Aromatic	0.7	1.69	30.95	1.67	7.43	0.68
Hydrocarbons	5.75	2.2	1.7	3.03	0.64	0.59
Carboxylic acids	46.84	56.46	1.3	9.61	34.29	62.78
Esters	10.17	28.58	4.22	19.61	30.51	31.09
Alcohols	9.16	2.09	0.41	37.44	3.45	1.06
Others	2.32	0	26.51	0	6.75	0

3.3.6 Stability time screening of additivated biodiesel

Soybean, canola and macauba biodiesel samples free from antioxidant and doped with extracts (*S. obliquus*, *H. pluvialis*, turmeric, annatto, C. husk, C. *H. pluvialis* and BHT) ragging from 500 to 3000 ppm were subject to oxidation stability according to EN14112 standard (EN 14112, 2003). Natural extract had positive effect on the stability time in comparation to neat soybean biodiesel (4,93 h, **figure 3.1**). It is possible to see a trend of higher antioxidant concentration result in increasing stability time. Thus, concentration of 3000 ppm increased stability time in 36.04% (6.70 ± 0.04 h) for *S. obliquus*, 23.35% (6.08 ± 0.08 h) for *H. pluvialis*, 35.14% (6.66 ± 0.01 h) for turmeric,

28.52% (6.33 ± 0.10 h) for annatto, 8.83% (5.36 ± 0.03h) for C. husk, and 33.90% (6.60 ± 0.09h) for C. *H. pluvialis*.

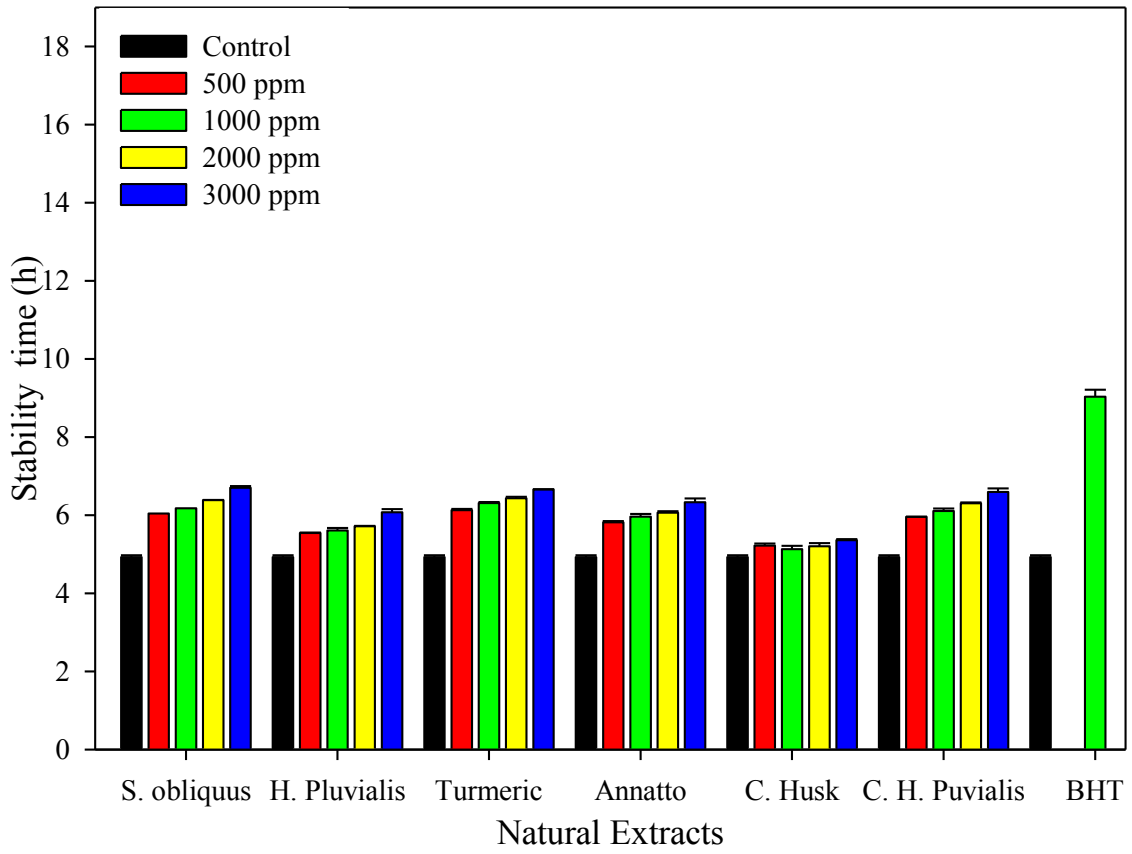

Figure 3. 1. Stability time of soybean biodiesel doped with natural antioxidants extracts.

Figure 3.2 illustrated the oxidative stability of control and additive samples of canola biodiesel as function of the antioxidant at concentration. Natural extracts had positive effect on the stability time in comparation to neat canola biodiesel (6.02 ± 0.28 h, **Figure 3.2**). It is possible to see a trend of higher antioxidant concentration result in increasing stability time. Thus, concentration of 3000 ppm increased stability time in 46.68% (8.83 ± 0.00 h) for *S. obliquus*, 19.85% (7.22 ± 0.08 h) for *H. pluvialis*, 51.3% (9.14 ± 0.08 h) for turmeric, 35.71% (8.17 ± 0.03 h) for annatto, 21.51% (7.32 ± 0.009 h) for coffee husk, and 46.18% (8.80 ± 0.14h) for C. *H. pluvialis*.

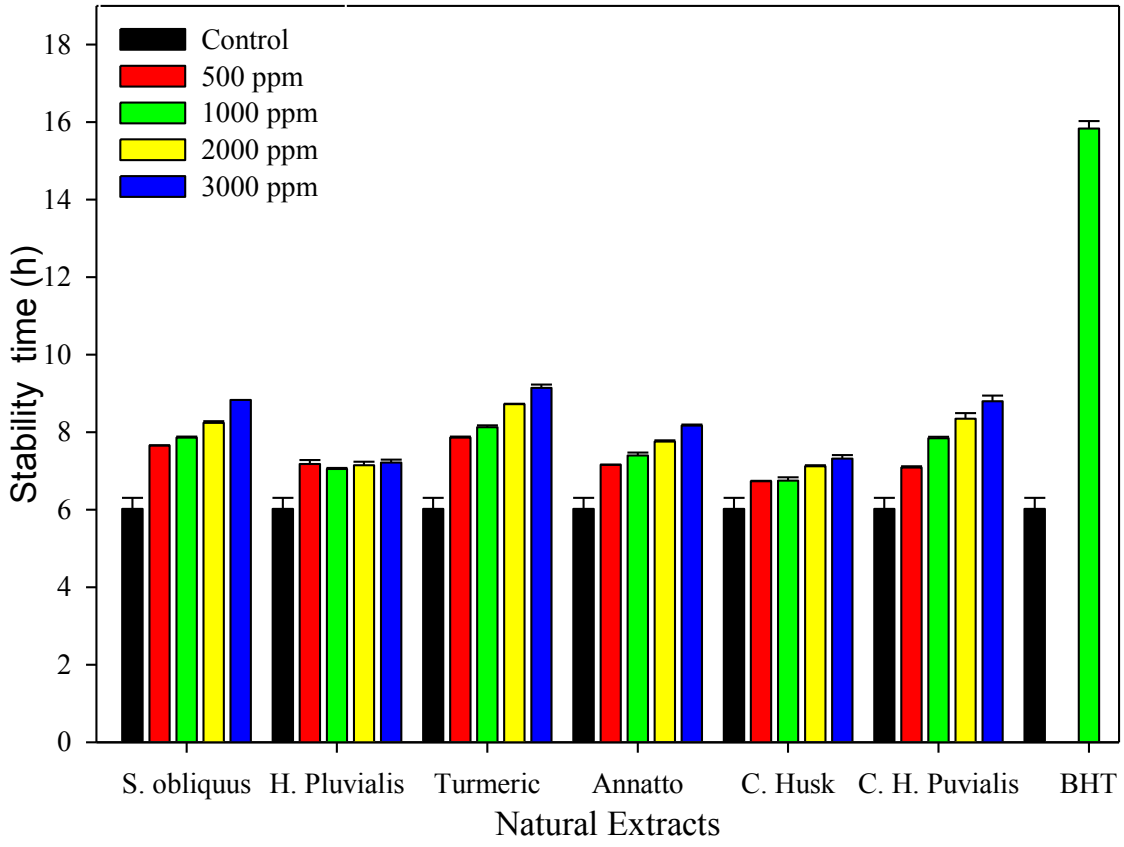

Figure 3. 2. Stability time of canola biodiesel dopped with natural antioxidants.

Macauba biodiesel free from antioxidant presented the lower stability time (1,98 ± 0,01 h, **Figure 3.3**). As reported in previous **Figures 3.1** and **3.2**, stability time for macauba biodiesel followed the same trend, higher natural extract concentrations produced higher stability times. Thus, concentration of 3000 ppm increased stability time in 122.86% (4.44 ± 0.15 h) for *S. obliquus*, 52.26% (3.03 ± 0.01 h) for *H. pluvialis*, 338.44% (8.73 ± 0.12 h) for turmeric, 100.00% (3.98 ± 0.07 h) for annatto, 43.47% (2.86 ± 0.01 h) for C. husk, and 90.70 % (3.80 ± 0.01 h) *C. H. pluvialis*.

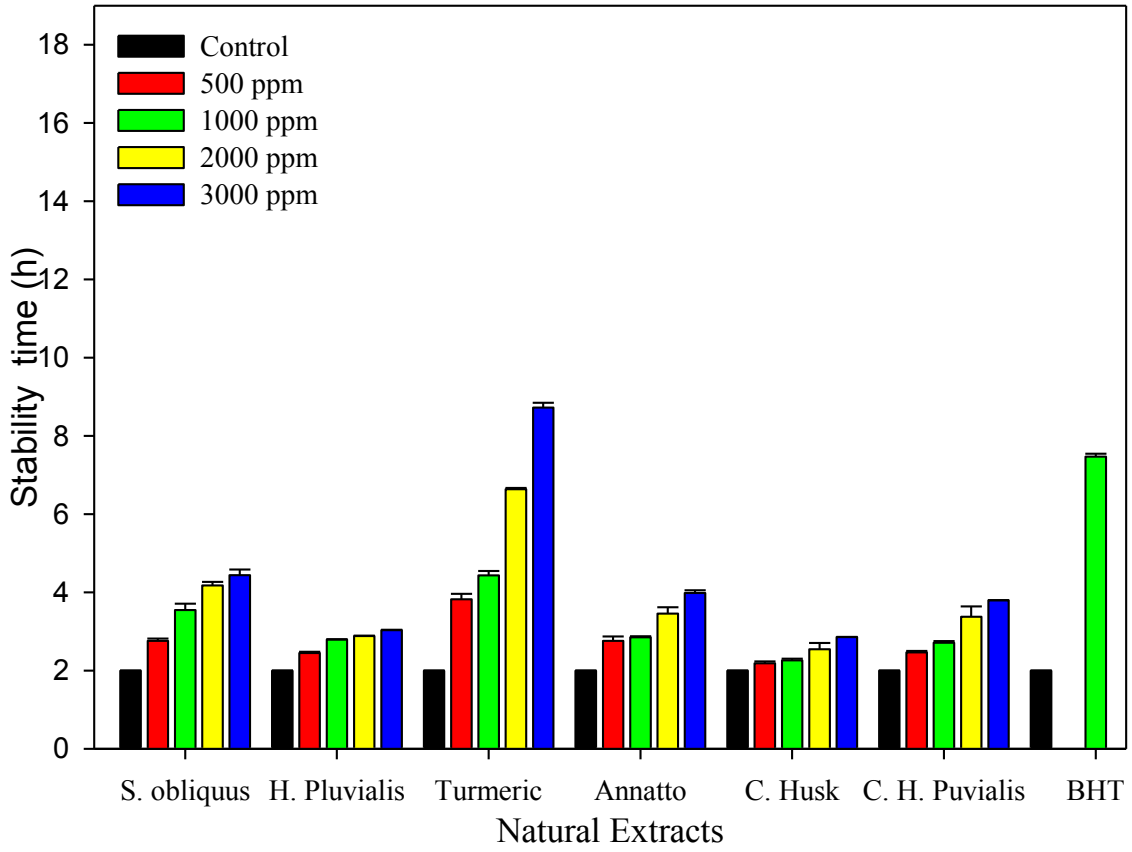

Figure 3. 3. Stability time of macauba biodiesel dopped with natural antioxidants.

A variety of works had been presented in literature using natural and synthetic antioxidants on different biodiesel feedstocks, reporting a significantly relation on antioxidant concentration an increase on oxidative stability. For instance, Sousa et al. (2014) tested curcumin extract at 1500 ppm on soybean biodiesel and promoted in an increased in stability time of 83.29%. Valenga et al. (2019) used barley waste and *Moringa oleifera* leaves as antioxidant on soybean biodiesel and reported an increased in stability time of 202.47% (from 4.04 to 8.18h). In addition, Rial et al. (2019) tested dichloromethane ginger extract at 4000 ppm produced an increase of 57% (5.24 to 8.26 h) stability time in soybean biodiesel. França et al. (2017), using a commercial biodiesel (30% soybean, 30% tallow and 40% cotton) evaluated the antioxidant potential of ethanolic extract of leaves at 3000 ppm, which resulted on an increase of 63.70% of stability time (5.51 to 9.02 h). Another study evaluated the effect of the ethanolic extract of *Platymiscium floribundum* on soybean biodiesel. At 1000 ppm, the extract produced an increase of 91.16% on the stability time (4.53 to 8.66 h, Nogueira et al., 2020b). The study performed by Damasceno et al. (2013) on soybean biodiesel used caffeic acid at 1000 ppm produce an increased 62.79 % (4.5 to 7.0 h) in the stability time. Ahanchi et al. (2018) evaluated pistachio hull extract on canola biodiesel.

Those authors observed a linear positive correlation between the antioxidant concentration and the stability time, in which the concentration of 5000 ppm produced an improvement of 422% (1.53 to 8 h) in the stability time. Finally, Rodrigues et al., (2020) evaluated the Biodiesel produced form tilapia oil doped with turmeric extract at 500 ppm, which increased the stability time from 1.98 to 10.09 h (450%).

3.3.7 Long term storage stability test

S. obliquus, turmeric and annatto extracts were chosen for the long-term storage experiment, due to the higher phenolic content, that directly influence in the high antioxidant activity. Hence, a concentration of 3000 ppm was chosen, due to the higher stability time produced on the screening test. In addition, BHT at 1000 ppm concentration was used as a positive control.

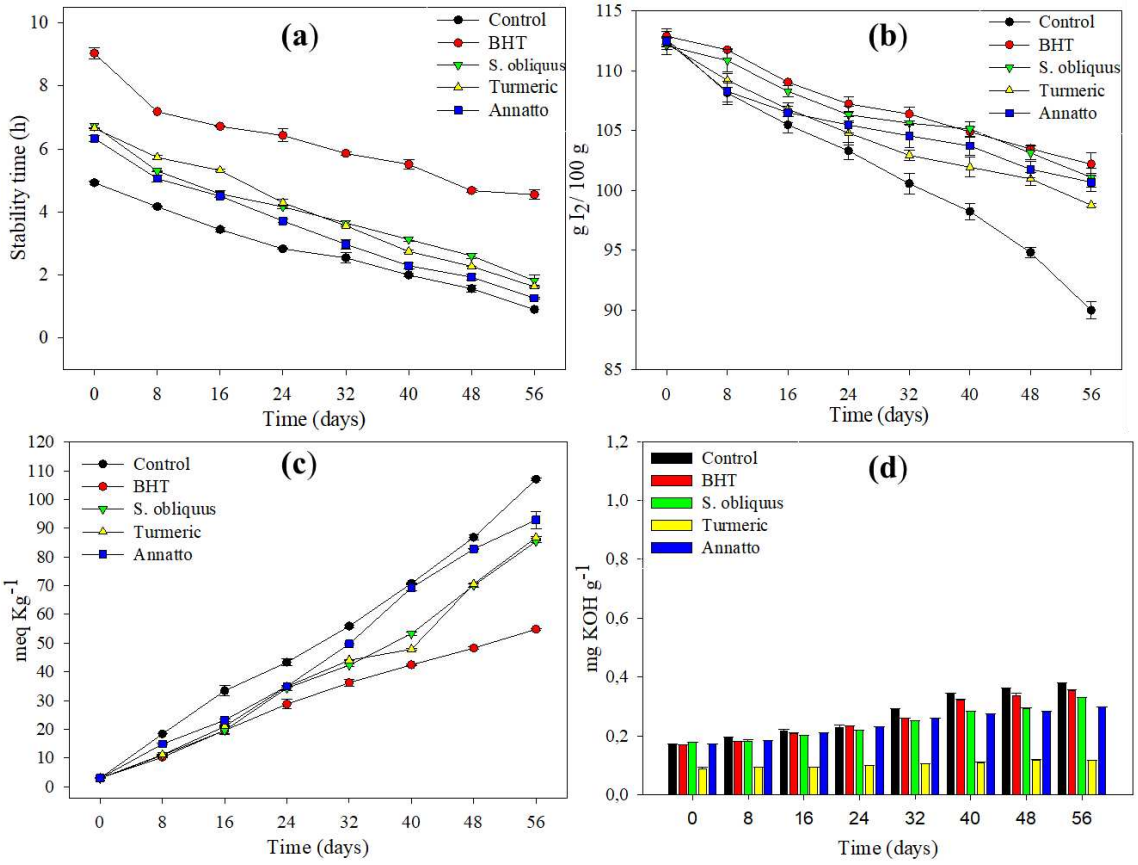

Figure 3. 4. Physicochemical parameters of soybean biodiesel at 43°C during 56 day of storage time. A) stability time, b) Iodine value, c) peroxide value and d) acid value.

During storage test, as expect, the stability time of soybean biodiesel decreased (**Figure 3.4a**), attributed to the high level of monounsaturated and polyunsaturated fatty acids 56.7%. At the initial time, the stability time of neat biodiesel were 4.93 ± 0.04

h. Moreover, neat soybean biodiesel is more prone to oxidation, confirming on previous result acid, peroxide, and iodine value. Particularly initial and eddying stability time of doped samples were: 4.93 ± 0.04 to 0.90 ± 0.00 h free from antioxidants (decrease of 81.73%), 9.04 ± 0.17 to 4.55 ± 0.14 h for BHT (decrease of 49.70%), 6.73 ± 0.00 to 1.82 ± 0.17 h for *S. obliquus* (decrease of 73.03%), 6.66 ± 0.00 to 1.63 ± 0.03 h for turmeric (75.59 % of reduction) and 6.33 ± 0.09 to 1.26 ± 0.02 h for annatto (decrease of 80.09%). Even though doped biodiesel show great oxidation stability results, they cannot fulfill the requirement of the Brazilian standard (ANP resolution No 798/219) of 12 h as the minimum stability time.

In this study, the iodine value found was 112.62 ± 0.62 soybean biodiesel free from antioxidants. In other studies, analogous result were reported: (103.35 gI^2 /100 g, Silva de Sousa et al., 2021), (113.6 gI^2 /100 g, Lapuerta et al., 2012), and (110.9 gI^2 /100 g, De Sousa et al., 2014). As was expected all samples undergo a decline in the iodide value over the times, due to oxidation, polymerization reaction. As shown in **Figure 3.4b** Soybean biodiesel free from antioxidants experience markable decreased in iodine value 20.10% (112.62 ± 0.62 to 89.97 ± 0.73 gI^2 /100 g). Particularly initial and eddying iodine values of doped samples were: 112.88 ± 0.64 to102.19 ± 0.99 gI^2 /100 g for BHT (decrease 9.47%), 112.08 ± 0.78 to 101.08 ± 0.80 gI^2 /100 g for *S. obliquus* (decreased 9.81%), 112.23 ± 0.47 to 98.77 ± 0.12 gI^2 /100 g for turmeric (decreased of 12.00%), 112.45 ± 0.37 to 100.68 ± 0.74 gI^2 /100 g for annatto (decreased of 10.46%).

The peroxide value concentrations of neat and dopped soybean biodiesel is displayed in **Figure 3.4c**. Peroxide values of all samples at time zero were closely to zero, 2,97 meq Kg^{-1}, confirming that the was not appreciable formation peroxides and hydroperoxides after biodiesel synthesis. During storage biodiesel free from antioxidants were more susceptible to peroxide formation, presented a linear incrementation, ending with a peroxide value of 107.06 ± 0.40 meq Kg^{-1}. In contrast, all the natural extract and BHT samples presented a lower peroxide value, therefore, showing greater resistance to oxidation, preventing the formation peroxide and hydroperoxide, initial degradation products. Concretely, initial, and eddying peroxide of doped samples were: 2.97 ± 0.15 to 54.80 ± 0.22 meq Kg^{-1} for BHT, 2.89 ± 0.03 to 85.37 ± 0.39 meq Kg^{-1} for *S. obliquus*, 2.95 ± 0.07 to 86.76 ± 0.38 meq Kg^{-1} for turmeric, 2.97 ± 0.06 to 92.90 ± 3.04 meq Kg^{-1} for annatto. The lower peroxide value in dopped biodiesel confirm the antioxidant activity of the phenolic compound present in the

extract, that act as primary antioxidant, quenching the free radical interrupting the propagation of lipid oxidation, by forming a more thermochemical stable product.

Acidity value of soybean biodiesel is shown on **Figure 3.4d**. initial, and eddying acid value of samples were: 0.17 to 0.38 mg KOH g^{-1} for neat (increase of 122.98%), 0.17 to 0.35 mg KOH g^{-1} for BHT (increase of 109.00%), 0.17 to 0.33 mg KOH g^{-1} for *S. obliquus* (increase of 86.30%), 0,08 to 0,11 mg KOH g^{-1} for turmeric (increased of 33.18%), and 0.17 to 0.29 mg KOH g^{-1} for annatto (increase of 73.73%). Therefore, all biodiesel samples, neat and dopped, were within requirements, 0,5 mg KOH g^{-1} ANP resolution No 798/219.

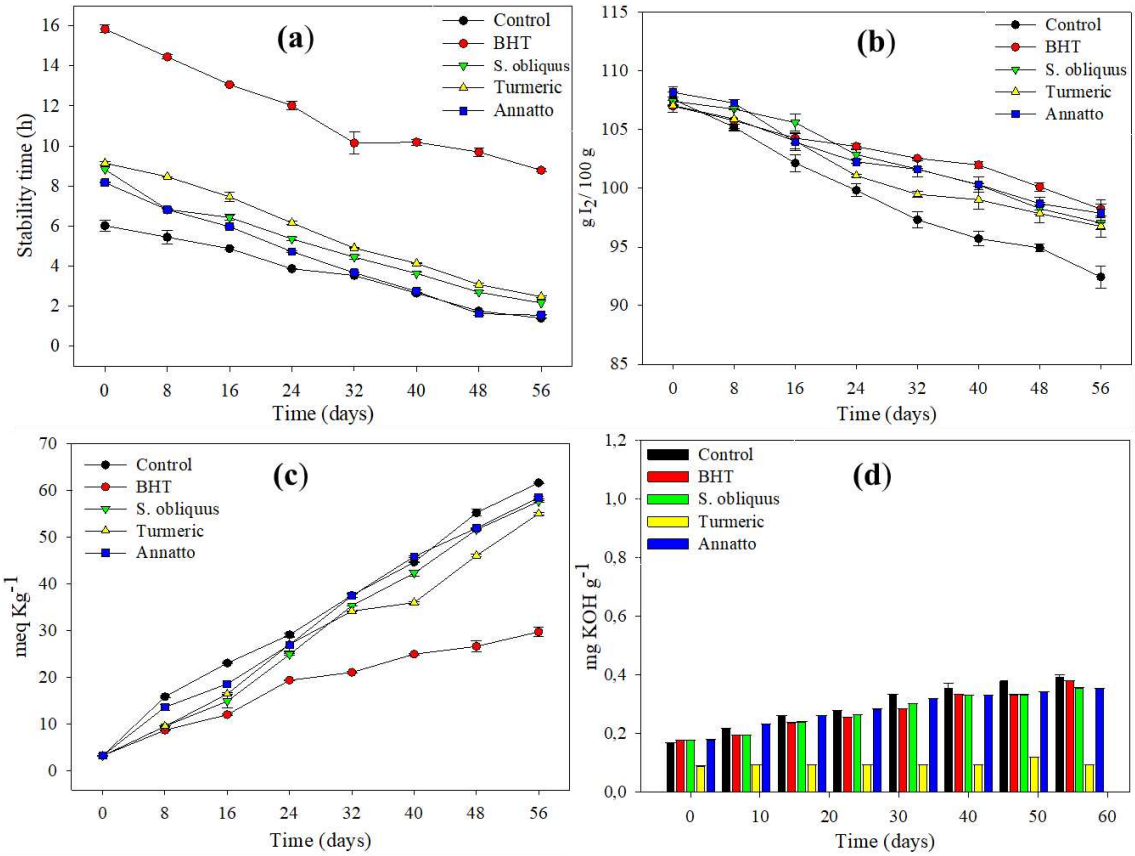

Figure 3. 5. Physicochemical parameters of canola biodiesel at 43°C during 56 day of storage time. A) stability time, b) Iodine value, c) peroxide value and d) acid value

As shown on **Figure 3.5**, the physicochemical parameter evaluated on the storage on canola biodiesel. Variation of stability time in canola biodiesel neat and doped samples during storage test is presented in **Figure 3.5a**. All samples experience a decrease in the stability through the storage test. Greater reduction stability time were observer on neat biodiesel 6.04 ± 0.28 to 1.40 ± 0.14, a decreased of 76.74% confirming the necessity of adding an antioxidant.

Moreover, dopped samples presented a better resistance to oxidation, as expected. Particularly initial and eddying stability were: 15.84 ± 0.19 to 8.79 ± 0.07 h for BHT (decreased 44.52%), 8.83 ± 0.00 to 2.16 ± 0,00 h for *S. obliquus* (decrease of 75.54%), 9.14 ± 0.08 to 2.48 ± 0.03 h for turmeric (decrease of 72.92%), and 8.17 ± 0.02 to 1.55 ± 0.028 h (decrease of 81.03%). Even though, natural extract increased stability time and showed a great resistance to oxidation, samples were not able to reach the minimum stability time (12 h) establish by ANP resolution No 798/219. Only BHT samples were able to satisfy ANP resolution. BHT greater stability time is related to its chemical characteristic of been a pure compound. On the other hand, natural extracts are a complex matrix of substances (phenolic compound, pigments, carbohydrates, lipids) and may contain some interference that reduce the antioxidant effect.

The iodine value of canola biodiesel is shown on **Figure 3.5b**. As was expected all samples undergo a decline in the iodide value over of storage test, due to oxidation. Therefore, canola biodiesel was prone to oxidation, because of its chemical composition, 68% monounsaturated and 24% polyunsaturated fatty acid, as shown on **Table 3.2**. Furthermore, neat canola biodiesel experienced a significant variation in iodine value (107.59 ± 0.64 to 92.44 ± 0.95 gI^2/100g), declined of 14.08%, On the other hand, canola biodiesel dopped with natural antioxidant and BTH, experienced a lower variation. Particularly, initial and eddying iodine value of samples were: 107.01 ± 0.50 to 98.23 ± 0.76 gI^2/100g for BHT (decreased of 8.20%), 107.41 ± 0.36 to 97.05 ± 0.60 gI^2/100g for *S. obliquus* (decrease of 9.65%), 107.06 ± 0.06 to 96.75 ± 0.93 gI^2/100g (decrease of 9.63%), and 108.18 ± 0.43 to 97.88 ± 0.79 gI^2/100g for annatto (decrease of 9.52%).

Peroxide concentration on canola biodiesel samples is presented on **Figure 3.5c**, at day 0 peroxide value were closely to zero, therefore there was not significant concentration of primary oxidation products (hydroperoxide and conjugated dines). During storage canola biodiesel free from antioxidants were more susceptible to peroxide formation, presented a linear increase, 3.31 ± 0.03 to 61.63 ± 0.13 meq Kg^{-1}. Particularly initial and eddying peroxide values of doped samples were: 3.32 ± 0.05 to 29.78 ± 0.92 meq Kg^{-1} for BHT, 3.27 ± 0.07 to 57.64 ± 0.26 meq Kg^{-1} for *S. obliquus*, 3.30 ± 0.07 to 55.02 ± 0.26 meq Kg^{-1} for turmeric, and 3.31 ± 0.03 to 58.49 ± 0.33 meq Kg^{-1} for annatto.

Acidity value of canola biodiesel is shown on **Figure 3.5d**. initial, and eddying acid value of samples were: 0.16 to 0.39 mg KOH g^{-1} for neat biodiesel (increase of 134.23%), 0.17 to 0.37 mg KOH g^{-1} for BHT (increase of 113.26%), 0.17 to 0.355 mg KOH g^{-1}) for *S. obliquus* (increase of 100.86%), 0.08 to 0.09 mg KOH g^{-1} for turmeric (increase of 6.84%), and 0.17 to 0.35 mg KOH g^{-1} for annatto (increase of 99,83%). Therefore, all biodiesel samples, neat and dopped, were able to maintain acid value within 0.5 mg KOH g^{-1} establish on ANP resolution No 798/219.

In another study, Yang et al. (2013) evaluated the oxidation stability of soybean canola and animal fat biodiesel, reported that acid value increased sharply on storage time, exceeding 0.5 mg KOH g^{-1}. Moreover, Moser (2011) evaluated the acid value, iodine, and storage time on canola methyl ester over a period d 12 month at different temperatures (-15, 20 and 40 °C). Similar outcomes were found on neat canola biodiesel, acid value increased to 0.26 mg KOH g^{-1} at 60 days of experiment, a drastically reduction of the stability time from 6.4 to 0.8 h, an iodide decreased as well from 107 to 104 gl^2/100g.

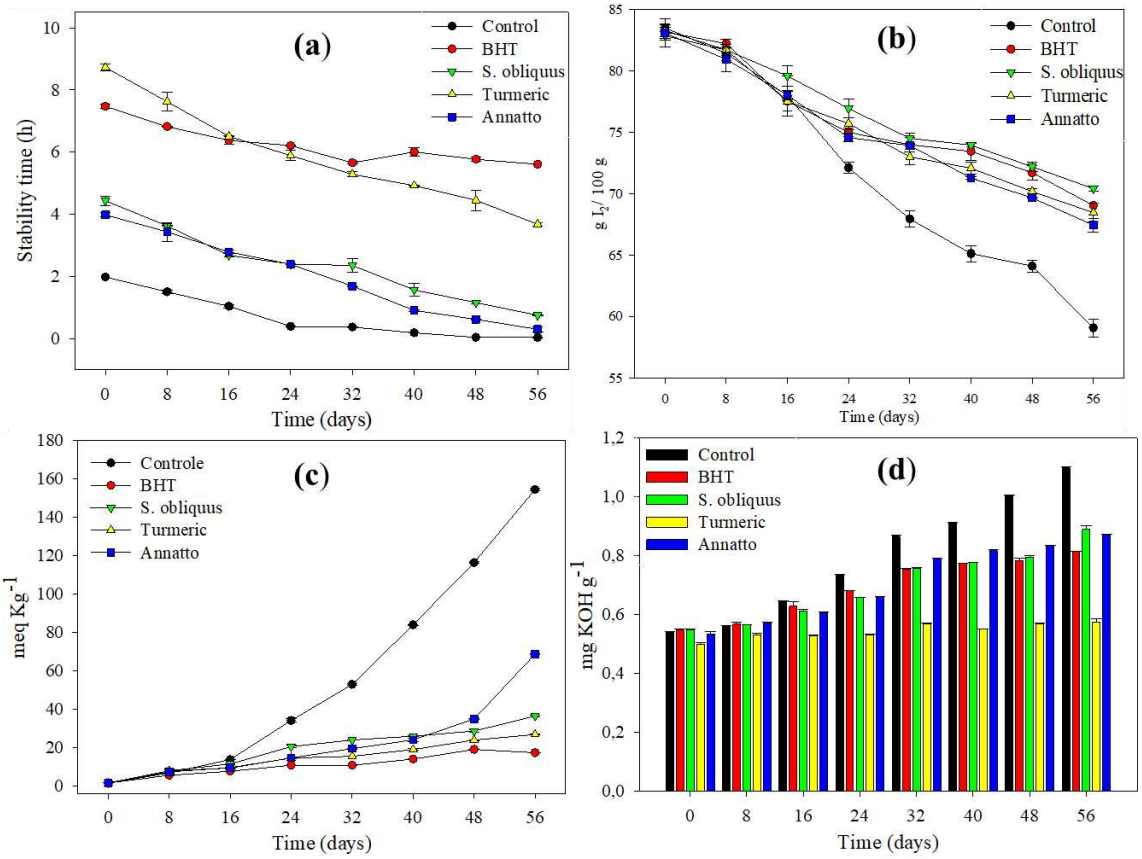

Figure 3. 6.Physicochemical parameters of macauba biodiesel at 43°C during 56 day of storage time. A) stability time, b) Iodine value, c) peroxide value and d) acid value

As shown on **Figure 3.6**, the physicochemical parameter evaluated on storage macauba biodiesel. Stability time of macauba biodiesel is display on **Figure 3.6a**. Stability time of all samples decreased in function on the storage time. A more prominent reduction was record on neat macuaba biodiesel 1.98 ± 0.00 to 0. 04 ± 0.00 h (decrease of 98.10%), correlating to lower acid, peroxide and iodine value having the worst performance over the storage time, been therefore very predisposed to oxidation. Particularly, initial, and eddying stability times of samples were: 7.47 ± 0.07 to 5.61 ± 0.00 h for BHT (decreased of 24.90%), 4.44 ± 0.14 to 0.75 ± 0.01 h for *S. obliquus* (decrease of 83.09%), 8.73 ± 0.12 to 3.68 ± 0.05 h for turmeric (decrease of 57.82%), and 3.98 ± 0.71 to 0.30 ± 0.08 h for annatto (decrease of 92.46%). Nevertheless, all the natural extract was able to increase the stability time and maintain during storage time, not were able to satisfy the minimum stability time of 12 required by ANP resolution.

The iodine value of macauba biodiesel is shown on **Figure 3.6b**. In this study, the initial iodine value macauba biodiesel prior to alkaline transesterification were 83.51 gI^2 /100 g, consistent value to the fatty acid composition presented on **Table 3.2**. Similar iodine value 76.94 gI^2 /100 g, were reported by Souza et al. (2016). During storage time macauba biodiesel, were more prone to oxidation, confirmed in the greater reduction iodine value 83.51± 0.75 to 59.09 ± 0.72 gI^2 /100 g a decrease of 29.24%. Particularly, initial, and eddying stability times of samples were: 83.18 ± 0.68 to 69.05± 0.00 gI^2 /100 g for BHT (decrease of 16.99%), 82.86 ± 0.91 to 70.43 ± 0.19 gI^2 /100 g for *S. obliquus* (decrease of 14.99%), 83.28 ± 0.10 to 68.47 ± 0.75 gI^2 /100 for turmeric (decrease of 17.78%), 83.09 ± 0.44 to 67.45 ± 0.56 gI^2 /100 for annatto (decrease of 18.82).

Peroxide concentration on macauba biodiesel samples is presented on **Figure 3.6c**. Peroxide values of all samples at time zero were closely to zero. During storage neat biodiesel were more susceptible to oxidation confirmed by the continuous increase, ending with a peroxide value of 154.51 ± 0.88 meq Kg^{-1}. In contrast, all the natural extract and BHT samples presented a lower peroxide value, therefore, showing greater resistance to oxidation. Particularly initial and eddying peroxide values of doped samples were: 1.48 ± 0.02 to 17.26 ± 0.00 meq Kg^{-1} for BHT, 1.45 ± 0.00 to 36.39 ± 0.42 meq Kg^{-1} for *S. obliquus*, 1.49 ± 0.01 to 26.78 ± 0.05 for turmeric, and 1.45 ± 0.01 to 68.67 ± 0.07 for annatto.

Acidity value of macauba biodiesel monitored over the storage test is shown on **Figure 3.6d.** Biodiesel processing quality and quantity is strongly affected by high degree of free fatty acid content on the feedstocks (Ramos et al., 2019). Macauba pup oil as reported early on **Table 3.3** presented an extremely high acidity. Therefore, acid esterification was used to transform free fatty acid into ester, lowering the acidity value and increase transesterification yield.

In addition, right after transesterification macauba biodiesel displayed an acid value 0.54 ± 0.01 mg KOH g^{-1} slightly over ANP resolution regulation on 0.5 mg KOH g^{-1}. In special, similar trend as previously reported on soybean and canola biodiesel, acid value on neat macauba, were susceptibility to oxidation, confirmed by significantly increased on the acid value 0.54 ± 0.00 to 1.09 ± 0.00 mg KOH g^{-1} an increase of 102.71%. Particularly initial and eddying acid values of doped samples were: 0.54 to 0.81 mg KOH g^{-1} for BHT (increase of 48.69%), 0.55 to 0.89 mg KOH g^{-1} for *S. obliquus* (increase of 61.89%), 0.50 to 0.57 mg KOH g^{-1} for turmeric (increase of 15.37%), and 0.53 to 0.87 mg KOH g^{-1} for annatto (increase of 63.29%).

3.4 Conclusion

This study assessed the effect of alternative natural antioxidant additives on soybean, canola and macauba biodiesel. The potential antioxidant activity of the extracts was confirmed, more pronounced for turmeric (556.35 μM Trolox g^{-1}), followed by annatto, *Haematococcus pluvialis*, commercial *Haematococcus pluvialis* , Coffee husk, and *S. obliquus*. Moreover, on the screening of antioxidant concentration, all natural extracts presented a significative effect in increasing the stability time in all biodiesel. However, natural extract performance was dosage and matrix dependent. Higher stability time increased was found for turmeric extract (35.15% in soybean, 51.83% in canola and 338.44% in macauba), followed by *S. obliquus* (36.04% in soybean, 46.68% in canola, and 122.86% in macauba), commercial *H. pluvialis* (33.91% in soybean, 46.18% in canola, and 90.70% in macauba), and for annatto (28.53% in soybean, 35.71% in canola, and 100.00% in macauba).

During the long storage test, neat biodiesel were susceptible to oxidation, as demonstrated on the stability time decreasing along the 56 days. Moreover, decreasing ware quantified in 81.73, 76.74, and 98.11% for soybean, canola and macauba biodiesel, respectively. On the other hand, turmeric and *Scenedesmus obliquus* extract

shown very promising alternative as natural antioxidants, due to protective effect on delaying lipid oxidation, confirmed on lower iodine value, acid value, peroxide value and stability time decreasing over the storage time. Particularly, decreasing value were verified for turmeric (75.65, 72.92 and 57.82% in doped soybean, canola and macauba biodiesel, respectively), and *S. obliquus* (73.03, 75.54, and 83.09% in doped soybean, canola and macauba biodiesel, respectively).

Stability time of doped biodiesel were not able to accomplish all parameters of Brazilian standards (ANP resolution No 798/219) of stability time of 12 h. Even though, *Scenedesmus obliquus* and turmeric extracts presented strictly benefits, like sustainable, biodegradable, non-toxic over common synthetic antioxidants like BHT, used as positive control in the experiment.

3.5 References

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4. Chapter 3 Potential of bio-oil from slow pyrolysis of crude glycerol and depigment *Scenedesmus obliquus* as antioxidant for biodiesel

Abstract

The low oxidation stability of biodiesel is an important drawback to be considered during long storage periods. The oxidation process causes changes in the chemical and physical properties of biodiesel, resulting in fuel quality loss. This study assessed the antioxidant effect of the organic phase of bio-oil form slow pyrolysis of analytic glycerol, crude soybean, canola, and macauba crude glycerol, and from *Scenedesmus obliquus* depigment biomass as an alternative source of antioxidants to increase the stability time of soybean, canola and macauba biodiesel. The potential antioxidant activity of the bio-oils was determined by capture ABT 2,2'-azinobis (3-ethylbenzothiazoline-6-acidosulfonic acid) and DPPH radicals (1,1- Diphenyl-2-picrylhydrazyl). Significative positive effect was presented by *S. obliquus* bio-oil 1.25 ± 0.01 mM Trolox g^{-1}. In addition, from the screening assay demonstrated that antioxidant performance was dosage (4000 ppm) and biodiesel matrix dependent. Higher stability time increase was produced by *S. obliquus* bio-oil for soybean (116.85% or 13.21h), canola (194.52 % or 17.73 h) and (563.57% or 13.21h) macauba biodiesel. Consequently, the addition of *S. obliquus* bio-oil fulfils the requirement of ANP resolution No 798/219 of minimum stability time 12 hours on biodiesel.

Keywords: Biodiesel storage. Oxidation stability. Pyrolysis antioxidant. Bio-oil. *Tetradesmus obliquus.*

4.1 Introduction

The development of clean, efficient, and high-performance technologies is essential for population growth to impact the environment as little as possible. In addition, growing concerns about climate change and over-reliance on fossil fuels have shifted investment and public interest towards more sustainable and renewable biofuels (Soccol, 2016) .

Because their potential to reduce greenhouse gas and pollutants emission, biofuels have gained increasing attention as an alternative to replace fossil fuels (Reen et al., 2022). Among the various biofuels, biodiesel stands out as a promising alternative energy source, especially because it is non-toxic, results in lower pollution emission during combustion, presents lower sulfur and aromatic compounds content, is highly biodegradable, and has higher flash point (Aghbashlo et al., 2018). Biodiesel can be produced through a wide variety of raw materials, so the availability of raw materials and the economic aspect of the country represent crucial factors for the production of biodiesel (Chyuan and Silitonga, 2020).

Biodiesel is a mixture of fatty acid methyl or ethyl esters (FAME or FAEE), mostly commercially produced by the transesterification of triglycerides from renewable sources with short-chain alcohols, mainly methanol (Singh et al., 2020). Moreover, the main by-product crude glycerol is produced in large quantities and does not have the purity required for commercialization, becoming an important liquid contaminant of the process (Arumugam and Sankaranarayanan, 2020).

Physicochemical properties of biodiesel are strongly influenced by the individual characteristics of the methyl esters that compose it, which depend on the fatty acid content of the feedstock used (Nor et al., 2015). Among those properties, the low oxidation stability of biodiesel is an important drawback to be considered during long storage periods (Devi et al., 2019). The oxidation process causes changes in the chemical and physical properties of biodiesel, resulting in fuel quality loss (Kumar, 2017). The low oxidative stability of biodiesel is a problem which has not been satisfactorily resolved and represents the main barrier to expansion in the market.

Hence, a promising approach to improve the oxidative stability of biodiesel is by the addition of an antioxidant. Antioxidants, natural or synthetic, are chemical substances that are capable of delay the oxidation reaction, specifically acting in the initial stage of lipid peroxidation, by quenching reactive oxygen species from the

medium (Rial et al., 2020). Commercial synthetic antioxidant are synthetized from non-renewable sources, which is considered as a drawback for its use in biodiesel (Chandra and Sharma, 2020; Rial et al., 2020; Sundus et al., 2017). Furthermore, natural antioxidants have not achieved significant commercial success, mainly due to their high costs when compared to synthetic compounds (Kumar, 2017; Lau et al., 2022).

Another promising source of antioxidant compound is the thermochemical processing of biomasses and wastes (Wu et al., 2018). Pyrolysis is a thermochemical process of biomass breakdown at high temperatures (400-800 °C) in the absence of oxygen and at atmospheric pressure, leading to the production of a solid product (biochar), a condensate liquid (aqueous phase and biocrude or bio-oil), and a gaseous product (syngas) (Xia et al., 2022). Slow pyrolysis enhances the production yield of non-condensable gases and principally biochar in detriment of bio-oil, because of the slower heating rates, 5-20°C min^{-1}, and longer retention times 30-60 min (Patra et al., 2021).

The purpose of the present study is to evaluate the antioxidant effect of organic non-aqueous phase (bio-oil) produced from slow pyrolysis of analytic glycerol, crude glycerol from soybean, canola, and macauba biodiesel synthesis, and *Scenedesmus obliquus* depigmented biomass. The antioxidant effect will be compared to a synthetic additive (BHT) in different biodiesel, in order to identify alternative sources of additives. The effectiveness of antioxidants were monitored by iodine, peroxide, and acid value over a period of 56 days of storage (7weeks).

4.2 Material and methods

4.2.1 Feedstock for biodiesel production

Soybean and canola oil, free from antioxidants, were purchased at the local market in Viçosa, Minas Gerais State, Brazil. Macauba pulp oil obtained from cold mechanical press, was kindly donated by Experimental Station of Araponga CVT (Centro Vocacional Tecnológico de Araponga) at the Department of Agronomy of Federal University of Viçosa.

4.2.2 Oil refining

The refining of oils were performed according to Crexi et al., (2010) and adapted for (Moraes et al., 2020) with some modification. The refining process comprised of degumming, neutralization, washing and bleaching.

Degumming was carried out using 1% phosphoric acid (85% v v^{-1}) in relation to the crude oil mass at 80 °C for 30 minutes in a magnetic stirrer at 500 rpm. The gums were removed by centrifugation at 7000 g for 10 minutes. Neutralization step was performed by adding aqueous solution of sodium hydroxide 20 % (w w^{-1}), using 4% of excess to the acid value after the degumming step at 40 °C for 20 minutes and magnetic stirred at 500 rpm. Soap formed was removed by centrifugation at 7000 g for 10 minutes.

Washing step was carried out by adding 10% (w w^{-1}) water at 95 °C, in relation to the oil mass, at 50 °C for 10 minutes and magnetic stirred at 500 rpm. This step was repeated tree times. The oil sample was dried in a rotatory evaporator under reduced pressure at 40 °C for 1 hour (Devi et al., 2018).

The bleaching step was performed by adding 2% (w w^{-1}) of adsorbents (activate earth and activated coal at a 9:1 ratio) at 70 °C for 20 minutes under magnetic stirring at 40 rpm. The oil sample was filtrated in a Buchner funnel with a filter paper of 0.22 µm (Whatman 1820-047 GF/A) under 700 mmHg of vacuum.

4.2.3 Biodiesel synthesis

Acid esterification was performed on refined macauba oil, prior to transesterification, in order to reduce the fatty acid content to less than 5 mg KOH g^{-1}. The process was performed according to Khan et al. (2010) and Damanik et al. (2017) with modifications. The oil was pre-heated at 65 °C for 5 minutes and add concentrated H_2SO_4 0.5 % (w w^{-1}) and methanol at molar ratio of 10:1 in relation to the oil mass. The reaction was kept under reflux at 65 °C for 3 h and magnetic stirred at 500 rpm. After the reaction time, the products were settled in two phases during 4 hours using a separation funnel (Sebastião et al., 2018). Washing was carried out by adding 10% (w w^{-1}) water at 50 °C, in relation to the oil mass, for 10 minutes and magnetic stirred at 500 rpm. Finally, the washed sample was dried in a rotatory evaporator (lyophilizer L101,liobras, Brazil) under reduced pressure at 40 °C for 1 hour (Devi et al., 2018).

Biodiesel production of refined oils were carried out through transesterification reaction with homogeneous alkali catalysis. The reaction was performed in round bottom flash equipped with a thermometer, reflux condenser to avoid methanol losses, a water bath and magnetic stirred. In brief, the refined oil was pre-heated at 65 °C for 5 minutes and added by 1% of KOH premixed with methanol (molar ratio oil: methanol of 1:6). The reaction was kept at 60 °C for 60 minutes under magnetic stirring at 500 rpm (Nogueira et al., 2020a). After the reaction time, the mixture was poured into a separator funnel and let to settle for 4 hours, resulting in crude methyl ester (upper phase), methanol excess, glycerol and catalyst (lower phase). The methyl ester phase was washed using 10% water (w w^{-1}), in relation to the oil mass, at 50 °C, this process was repeated 3 times or until neutral pH (Buosi et al., 2016). Finally, the ester phase was dried using rotatory evaporator under reduced pressure at 40 °C for 1 hour (Devi et al., 2018) and filtrated using a Buchner funnel whit a filter paper 0.22 µm carried at a vacuum 700 mmHg.

4.2.4 Physicochemical characterization biodiesel

4.2.4.1 Fatty acid profile composition of biodiesel

Fatty acid methyl ester composition of biodiesel were defeminated according to Ichihara and Fukubayashi (2010), and Guihéneuf et al. (2015) by gas chromatography (Shimadzu, GC-2010, Japan) coupled to flame ionization detector (GC-FID) equipped with a 100 m x 0.25 mm capillary column (SP-2560, Sigma-Aldrich, USA). The analyses were carried out by dissolving samples in and hexane and direct injection of

1 µl. Gas Helium was used as carried at a flowrate of 1 mL min^{-1}, linear heating ramp from 60 to 330 °C at a heating rate of 20 °C min^{-1}. The identification of the peaks was confirmed by comparison with the standard mixture of FAME (SupleCo 37 FAME mix).

4.2.4.2 Moisture content

The determination of moisture content and volatile compounds was based on the AOCS Ca 2c-25 standard (AOCS, 2007). The standard determines the percentage of water and organic matter that volatilizes up to a temperature of 130°C. Sample of 1 g in a crucible or petri dish previously dried and tared. The container with sample was oven dried at 130 °C for a period of 30 minutes. Then, the set was removed from the oven, and placed in a desiccator until cooling to room temperature and weighted. The weighting process was repeated until the variation in the mass was below 0.05 %.

4.2.4.3 Density 20 °C

Density was measured according to ASTM D-4052 standard (ASTM, 2008). The determination was performed using a 10 ml pycnometer, previously calibrated, into a thermostatic bath. The calibration of the pycnometer was performed using water at 20 °C. Therefore, the difference in weight, measured on an analytical balance, between the mass of the full and empty pycnometer, is the mass of water, using its density it was possible to determine the real volume of the pycnometer. The determination of the density of the samples began by carefully pouring the sample into the pycnometer to avoid the formation of bubbles. Subsequently, the set was immersed in a thermostatic bath at 20 °C and kept for 10 minutes. Finally, the set was removed and weighed, and the density calculated.

4.2.4.4 Free fatty acid and Acid Value

The acid value (FFA) was determined by titration following the AOCS Ca 5a-40 standard (AOCS, 2007). A sample of 1 gram was weighed in a 125 ml Erlenmeyer flask. A volume of 25 ml of a neutral solution of ether: ethyl alcohol (2:1 v v^{-1}) was added to the flask, and two drops of phenolphthalein indicator were added. Finally, the sample was titrated with 0.1 mol L^{-1} NaOH, until the appearance of a persistent pink

color for at least 30 s. The results were expressed % free fatty acid as oleic (FFA, equation 4.1).

$$FFA = \frac{28{,}2\ f\ M\ (A-B)}{m} \quad \text{(Eq. 4.1)}$$

where *A* and *B* are the volumes (mL) of titrating for the sample and the blank, respectively, f is the correction factor, M is the molarity of the titrant solution (0.1 mol·L-1 NaOH), and *m* is the mass (g) of the sample. The free fatty acid content can be also expressed in terms of acid value (mg KOH g^{-1}). To convert percentage of free fatty acid (as oleic acid) to acid value, is necessary to multiply for 1,99.

4.2.4.5 Iodine Value

The iodine content (II) was determined by the Wijs method following the EN14111 standard (EN 14111, 2003). According to the standard, 0.25 g of the sample was weighed in a 250 ml Erlenmeyer flask. Then, 20 ml of a solution of cyclohexane and glacial acetic acid 1:1 (v/v), and 25 ml of Wijs reagent were added. The Erlenmeyer flask was kept at rest and protected from light for one hour. Then, 20 ml of a 10% KI solution (100 g·L^{-1}) were added. Finally, the system was titrated using 0.1 mol·L^{-1} sodium thiosulfate, previously standardized, until the yellow color almost disappeared. Then, 2 ml of 1% starch indicator suspension was added. The titration continued until the indigo color disappeared. The results were expressed in g 100 g^{-1}, according to equation 4.2.

$$II = \frac{12{,}69\ f\ M\ (B-A)}{m} \quad \text{(Eq. 4.2)}$$

where *A* and *B* are the volumes (mL) of titrating for the sample and the blank, respectively, f is the correction factor, M is the molarity of the titrant solution (0.1 mol·L-1 sodium thiosulfate), and *m* is the mass (g) of the sample.

4.2.4.6 Peroxide Value

The peroxide index (PI) was determined following the AOCS Cd 8–53 standard (AOCS, 2007). A sample of 1 g was weighed in a 125 ml Erlenmeyer flask and

dissolved in 30 ml of a 3:2 (v v^{-1}) glacial acetic acid: chloroform solution. Then, 0.5 ml of saturated KI solution was added and left to rest for 1 minute and protected from light. Subsequently, 30 ml of deionized water was added, and titration was performed using 0.1 mol L^{-1} of sodium thiosulfate, previously standardized, until the yellow color almost disappeared. Finally, 1 ml of 1% (w v^{-1}) starch suspension was added and the titration continued until the indigo color disappeared. Peroxide index (PI) was calculated using equation 4.3.

$$PI = \frac{1000\ f\ N\ (A-B)}{m} \quad \text{(Eq. 4.3)}$$

where *A* and *B* are the volumes (mL) of titrating for the sample and the blank, respectively, f is the correction factor, M is the molarity of the titrant solution (0.1 mol·L^{-1} sodium thiosulfate), and *m* is the mass (g) of the sample.

4.2.4.7 Determination of stability time

Oxidative stability was analyzed in a Rancimat equipment in accordance with EN14112 standard (EN 14112, 2003). Rancimat is an accelerated ageing method, in which the oxidation is induces by bubbling steam air at a flowrate of 10 L h^{-1} through 3 grams of biodiesel sample maintained at a 110 ºC. Highly volatile secondary reaction oxidation products, mostly formic acid, are transfer by the airflow to a measuring vessel containing 50 ml demineralized water and an electrode for measuring the electrical conductivity. Values of electrical conductivity (µS) as a function of time (h) were then related in a chart, and the inflection point of the curve represented the stability time. Biodiesel samples with soybean, canola, macauba and *S. obliquus* bio-oils at different concentrations were analyzed.

4.2.4.8 Biomasses and waste

Strain of *Scenedesmus obliquus* BR003 was obtained from the Collection of Cyanobacteria and Microalgae of the Department of Plant Biology, at Federal University of Viçosa (Minas Gerais, Brazil). *S. obliquus* were maintained in BG11 (Andersen, 2005) in the Collection, but was cultivated using a low-cost culture medium (L4-m, Rocha et al., 2019) in a 4.000 L open raceway tank using with an average daily

radiation intensity of 752.1 µmol photons m^{-2} s^{-1}, and temperature ranged from 20 to 30 °C (Rocha et al., 2020). After reaching the stationary phase, microalgal cells were centrifugate at 4000 g for 10 minutes, supernatant was discarded, and the pellet was frozen at -20 °C for 48 hours. Afterwards, the microalgae biomasses were freeze-dried under vacuum (lyophilizer L101,liobras, Brazil) for 48 hours (Zhang et al., 2022).

The extraction of *Scenedesmus obliquus* pigments was performed by weighting 100 g of freeze-dried samples in a 2000 ml conical flask covered with aluminum foil (to avoid oxidation by light). An amount of 500 mL of ethanol was added to the sample and stirred at 500 rpm for 2 days at room temperature (22±1 °C) (Akter et al., 2019; Buosi et al., 2016). After the extraction time, the extract was centrifugate at 10000*g* for 10 minutes. The supernatant was filtrated (Whatman 1820-047 GF/A) and concentrated on rotary evaporator at 0,1 MPa and 40 °C (Devi et al., 2018). The residual microalgae biomass was re-extracted under the same conditions until it became colorless, approximately 4 times more, totally 8 days extraction. Finally, residual biomass was stored -20 °C until used.

Crude glycerol (wastes) were obtained from biodiesel homogeneous alkali catalysis (KOH 1%) transesterification reaction of refined soybean, canola and macauba oil.

4.2.5 Protein content in depigment *Scenedesmus obliquus* biomass

Alkaline protein extraction was performed from freeze-dried biomass. In brief, 2 mg biomass was added 400 µL of 0.1 mol L^{-1} NaOH, incubated for 60 minutes at 95°C with occasionally shaking. Thus, a second incubation was performed for 5 minutes at -20 °C. Afterwards, the sample was centrifugate as 10000*g* for 10 minutes.at 4°C. The supernatant was used for quantification of total proteins according to Bradford (1976). The colorimetric pipetting reaction was performed with 5 µL of supernatant and 250 µL of Bradford's reagent. After 5 minutes, absorbance was measured at 595 nm in the microplate spectrophotometer (Multiskan GO, Thermo Scientific, Germany). A standard curve of bovine serum albumin (BSA) with concentrations between 8 and 500 µg mL^{-1} was used to estimate the protein concentration.

4.2.6 Carbohydrates content in depigment *Scenedesmus obliquus* biomass

The carbohydrate extraction was performed from freeze-dried biomass using H_2SO_4 1 mol L^{-1}. In brief, 2 mg of sample was added 2 ml of H_2SO_4 1 mol L^{-1}, incubated for 30 minutes at 80 °C with occasional shaking. Afterwards, samples were centrifuged at 10000*g* for 10 minutes at 4 °C. The supernatant was used to determination of the total neutral carbohydrates contents according to the methodology proposed by Masuko et al. (2005), which is s based on the phenol-sulfuric acid method described by Dubois et al. (1956). The colorimetric assay was performed by pipetting 100 µL of diluted supernatant, 60 µL of 5% (w v^{-1}) solution of phenol and 300 µL of concentrated H_2SO_4. After 30 minutes, the absorbance was measured at 490 nm in a microplate spectrophotometer (Multiskan GO, Thermo Scientific, Germany). A standard curve of D-glycose at concentrations between 10 and 200 µg mL^{-1} was used to estimate the total neutral carbohydrates.

4.2.7 Pyrolysis

Pyrolysis of dried residual *Scenedesmus obliquus* biomass and glycerol wastes were performed in a reactor. The apparatus consists on a stainless-steel tube, 60 cm long and 6.5 cm diameter, allocated inside an electric kiln (LF2312, Jung, Brazil). Pyrolysis of samples were performed according (Rocha et al., 2020) and (Delgado et al., 2013). In brief, before pyrolysis initiation, the samples and the inner atmosphere of the reactor was purged by 5 minutes using nitrogen at a flowrate of 10 L h^{-1}. The slow pyrolysis condition consists on heating rate of 20 °C min^{-1}, beginning from room temperature 22 ± 1 °C to a final temperature of 550 °C under a constant nitrogen flowrate of 10 L h^{-1}. Condensable products were collected using an ice bath trap and stored in a 500 mL Kitassato flask, previously weighted. In addition, the weight of biochar was quantified as the residue that remained in the reactor at the end of pyrolysis. The amount of gas produced was quantified by difference.

The liquid products (Lp) were separated according the methodology proposed for Bordoloi et al., (2016). Diethyl ether was poured on the Kitassato trap and transferred to a separation funnel. The organic phase was separated and dried using anhydrous sodium sulfate, filtered using a 0,22 µm (Whatman 1820-047 GF/A) and the solvent was removed by heating the liquid sample at 40°C. The aqueous (Ap), organic

(Op), solid (Sp) and gas (Gp) phases yield was calculated according to equations 4.4 to 4.8.

$$p = 100\frac{m_L}{m_B} \quad \text{(Eq. 4.4)}$$

$$Ap = 100\frac{m_A}{m_B} \quad \text{(Eq. 4.5)}$$

$$Op = 100\frac{m_O}{m_B} \quad \text{(Eq. 4.6)}$$

$$Sp = 100\frac{m_S}{m_B} \quad \text{(Eq. 4.7)}$$

$$Gp = 100 - Sp - Lp \quad \text{(Eq. 4.8)}$$

LWhere m_L, m_A, m_O and m_S are the weight (kg) of the liquid, aqueous, organic, and solid phases, respectively, and m_B is the sample weight (kg).

4.2.8 GC-MS analysis of bio-oils

The chemical composition of natural extracts were determinate by gas chromatography coupled to mass spectrometry using a Shimadzu GCMS-QP5050A equipment. In brief, the analysis were performed using a SPB-5 capillary column (30 m x 0.25 mm, 0.25 mm); Helium as carrier gas at 1.6 mL min^{-1}; sample volume 1.0 mL; split ratio 1:3; injector temperature 300 °C; detector temperature 300 °C; oven temperature from 80 °C for 5 min, increase rate of 4 °C min^{-1} until 300 °C and kept for 45 min; Scan mode (35 to 700 m/z); ionization by electron impact mode at 70 eV. Before analysis, oil sample of 3 were subjected to derivatization with 100 mL of *N,O-bis* (trimethylsilyl trifluoroacetamide (BSTFA), and 60 mL of anhydrous pyridine in a conical reaction vial. The mixture was kept at 70 °C for 30 min and then subjected to CG-MS analysis (Rocha et al., 2020).

4.2.8.1 DPPH assay

The DPPH scavenging assay done at deem light as reported by Brand-Williams et al. (1995) and adapted by Assunção et al. (2017). In brief, DPPH stock solution 1 mmol L^{-1} was prepared in ethanol. This solution was stored at 5 °C and protected from light. After, DPPH working solution was prepared 0,06 mmol L^{-1}. For each extract 5 different concentrations were tested by diluting in ethanol (0.05 ,0.09, 0.18, 0.45, 0.9,

2.25, 4.50, 9, 18, 27 mg/ml). In brief, 200 µl of each extract were add to 1,8 ml DPPH working solution, mixed and vortex vigorously for 1 min. The mixture solution was incubated for 40 minutes, protected from light, at room temperature 25 °C followed by absorbance reading at 515 nm in spectrophotometer (Multiskan GO, Thermo Scientific, Germany). Because of intrinsic characteristic of the extract that absorbs at 515 nm, a blank sample was prepared with ethanol and the extract (Maadane et al., 2015). DPPH radical scavenging activity (SA) were calculated using the equation 4.9.

$$SA = 100\left(1 - \frac{A_s - A_b}{A_c}\right) \quad \text{(Eq. 4.9)}$$

where A_c denotes the initial absorbance of DPPH at 0,06 mM without test samples or the standard, A_b is the absorbance of the blank sample and A_s is the absorbance of ethanolic extract or standard with DPPH. The scavenging activity was expressed as IC50 (50% scavenging effect) concentration of the extracts were calculated using regression equation between the concentration and the scavenging activity for each extract. The IC50 represent the concentration required to decreased DPPH concentration by 50%.

4.2.8.2 Trolox Equivalent Antioxidant Capacity assay (TEAC)

TEAC scavenging assay was performed at deem light according to Cano and Herna (1998), and later adapted by (Santhakumaran et al., 2020) In brief, the ABTS 2,2'-azinobis (3-ethylbenzothiazoline-6-acidosulfonic acid) radical solution was generate by mixing 7 mmol L^{-1} $abts^{+}$ and 2,45 mmol L^{-1} $K_2S_2O_8$. Thus, the mixture was incubated overnight at 25°C. Afterwards, ABTS working solution was prepared by diluting in water until an absorbance of 0.7 at 734 nm. The reaction mixture was performed by adding 190 µl ABTS working solution and 10 µl sample or standard, with vigorous mixing for 1 min. The mixture was incubated for 60 min. protected from the light at room temperature 25 °C, followed by absorbance reading at 734 nm in spectrophotometer (Multiskan GO, Thermo Scientific, Germany). Because of intrinsic characteristic of the extract that absorbs at 734 nm, it was necessary to prepare a control for each sample (sample and solvent only). Trolox was used as standard at 8

to 1000 mmol L^{-1} (Li et al., 2007), and scavenging activity was expressed as μM Trolox equivalent per gram of extract.

4.2.9 Phenolic content

Total phenolic content was estimated by folin-ciocalteu method (Singleton et al., 1999) and adapted by Martínez-Sanz et al. (2020). In brief, 0,2 ml of extract at concentration of 5 $mg{\cdot}L^{-1}$ in ethanol were added to 1 mL of folin-ciocalteu working solution, diluted as 1:10 (v v^{-1}) in water, and incubated at room temperature for 4 minutes. After, 0.8 ml of Na_2CO_3 (75 $mg{\cdot}mL^{-1}$) was added and incubate at 50 °C for 30 minutes, followed by absorbance measurement at 750 nm. Gallic acid was used as standard (0 - 500 μg ml^{-1}) (Morowvat and Ghasemi, 2016a) and the result were expresses as mg gallic acid per gram of extract.

4.2.10 Screening of potential of bio-oils for biodiesel

In order to assess the individual effect of bio-oils from depigmented *S. obliquus*, and soybean, canola and macauba glycerol at specific concentration optimized on Rancitmat (higher stability time) on soybean, canola and macauba biodiesel, the stability time was tested on concentration from 500 to 4000 ppm. Butylated hydroxytoluene (BHT) at concentration of 1000 ppm was used as positive control (Jemima Romola et al., 2021; Varatharajan and Pushparani, 2018), and negative control was defined as biodiesel samples without additive.

4.2.11 Long term storge stability test

After verifying the antioxidant potential of teste bio-oil and the positive impact on the oxidative stability, a long-term storage test was conducted for soybean, canola an macuaba biodiesel, to evaluate the oxidation behavior over the time. The experiment was performed for 56 days according to ASTM-D4625 (ASTM, 2003) at 43 °C in glass bottled wrapped with aluminum foil. The lid of the sample container was semi-closed to allow gaseous exchange. Peroxide, acid, and iodine value, and induction time were quantified at every 8 days to monitored biodiesel degradation.

4.2.12 Statistical analysis

Experiments were performed in triplicate and values are represent as means ± standard deviation. One-way ANOVA was used to determinate significance differences between means ($p < 0,05$) followed by post hoc Tukey test.

4.3 Result

The fatty acid composition of soybean, canola and macauba biodiese are given in **Table 4.1**. The vulnerability of biodiesel to oxidation is mainly related to the presence of polyunsaturated fatty acid chains in the ester molecules, which react easily with oxygen when exposed to air, because bis-allylic site are highly susceptible to hydrogen abstraction by free radicals (Christensen and McCormick, 2014; Fazal et al., 2022). Therefore, soybean biodiesel is more prone to oxidation due to the high concentration of polyunsaturated fatty acid (56.7%), since its vulnerability to oxidation is mainly related to the presence, number and position of double bonds in the alkyl chain (Kumar, 2017; Varatharajan and Pushparani, 2018).

Table 4.1.Fatty acid methyl ester composition of biodiesel

Fatty acid esters	Composition	Soybean	Canola	Macauba
Palmitic	C16:0	10.5	4.6	22.19
Palmitoleic	C16:1			4.99
Estearic	C18:0	4	2,0	2.4
Oleic	C18:1n9c	28.8	61.0	60.53
Linoleic	C18:2n6c	51.1	18.2	8.98
Linolenic	C18:3n6c	5,6	6,5	
Total				
∑ Saturated (C16:0 + C18:00)		14.5	6.6	24.59
∑ Monounsaturated (C18:1)		28.8	61.0	60.53
∑ Polyunsaturated (C18:2+ C18:3)		56.7	24.7	8.98

4.3.1 Physicochemical characterization biodiesel

The physical-chemical characterization of feedstocks is an important step in biodiesel manufacture because it determinanes the more suitable conversion process

(acid esterification or alkaline transesterification). For instance, water content and acid value are the most crucial parameter. Moreover, higher acid values (5 mg KOH g^{-1}) and water content (200 mg kg^{-1}) negatively affect the conversion yield and quality of biodiesel (Agência Nacional do Petróleo, 2019; Ramos et al., 2019).

Acidity values of oils in this study were 0.244 ± 0,001, 0.249 ± 0.008 and 74.40 ± 0.05 mg KOH g^{-1} for soybean, canola and macauba, respectively. The high acidity value of macauba oil is related to hydrolysis of triglycerides, and consequently formation of free fatty acid (Souza et al., 2016). Because of the high acidity value of macauba oil (74.04 ± 0.50 mg KOH g^{-1}), it was necessary to perform an acid esterification prior to alkaline transesterification, leading to an esterified oil acid value of 4.41 ± 0.007 mg KOH g^{-1}.

The physicochemical parameters of produced biodiesel are presented in **Table 4.2**. Nearly all values are within the requirements of the Brazilian National Agency of Petroleum, Natural Gas and Biofuels (ANP), except for the acidity of macauba biodiesel and oxidation stability of all biodiesel. Particularly, ANP resolution No 798/219 establish a minimum of 12 hours for the stability time and maximum acidity 0.5 mg KOH g^{-1}.

In the study, all biodiesel fell short to meet ANP specification of stability time, which can be attributed to the high content of polyunsaturated fatty acids demonstrating the need for the addition of antioxidants. Specifically, oxidative stability was 4.93, 6.02 and 1.98 h for soybean, canola, and macuaba biodiesel, respectively. Comparable result for soybean biodiesel of 3.77 h and 4.53 h were found by Sousa et al. (2021) and by Nogueira et al. (2020b), respectively. In addition, Moser (2011) found equivalent value for canola biodiesel of 6.4 h. Aguieiras et al. (2014) worked with macauba biodiesel and found similar stability time of 0.95 h.

Table 4. 2. Physicochemical proprieties of ester soybean, canola and macauba methyl esters

			Biodiesel		**ANP**
Parameter	**Unit**	**Soybean**	**Canola**	**Macauba**	**Limit**
Acidity Value	mg KOH g^{-1}	0.17±0.00	0.17±0.00	0.54±0.01	max 0,5
Peroxide Value	meq Kg^{-1}	2.97±0.02	3.31±0.03	1.47±0.01	n.s*
Iodine value	g I2 100 g^{-1}	112.62±0.62	107.59±0.64	83.51±0.75	n.s*
Oxidation Stability (110 °C)	h	4.93±0.05	6.02±0.28	1.98±0.00	min 12

Ester content (wt)	%	97.33	96.89	97.19	96,5
Water content	mg kg^{-1}	141.56±0.00	126.56±0,00	180.50±0.00	max 200
Density	Kg m^{-3}	864.11±1.36	861.51±0.60	867.95±0.27	860-900

*Not specified.

4.3.2 Biomass characterization

The result of the biochemical composition of depigment *Scenedesmus obliquus* is shown in **Table 4.3**. Protein and carbohydrate have the higher participation in the whole biomass (43.56 ± 0.08 and 13.98 ± 0.044 %, respectively). Comparable content in *Scenedesmus obliquus* for neutral carbohydrates were found by Fernandes et al. (2020) (9,7 %) and (Rocha et al., 2019) (12 %). Protein content is also in accordance to the value 48.1 % found by Rocha et al. (2019). Lipid and pigment content were 4.17 and 0.129 %, respectively. Those contents are lower when compared to another studies. For instance, Sharma et al. (2015) found 14 % of lipid content in *Scenedesmus quadricauda,* and Ahmad et al. (2021) found 0.191 % of pigment content, and 0.4 % by Fernandes et al. (2020). The residual pigment in biomass shows that ethanol was not able to extract all pigment content and was not fully selective to pigment also extracting microalgae lipids.

Table 4.3. Physicochemical characterization of depigmented microalgae dry basis (wt%)

Parameter	Values
Protein	45.50 ± 0.080
Total lipids	4.179 ± 0.074
Pigments	0.129 ± 0.005
Chlorophyll *a*	0.059 ± 0.001
Chlorophyll *b*	0.063 ± 0.008
Carotenoid	0.007 ± 0.000
Carbohydrates	13.986 ± 0.044
Ashes	26.070 ± 0.081
Moisture	4.871 ± 0,050

4.3.3 Pyrolysis product yield

Table 4.4 shows the mass fraction from slow pyrolysis from crude soybean, canola and macauba glycerol were similar: solid phase (biochar) variation between 2.7 and 4.5%, liquid phase (aqueous and organic) variation from 64.55 to 72.12%, and gas phase variation from 25.32 to 30.86%. Delgado et al. (2013) studied slow pyrolysis production yield of crude glycerol and found equivalents values for solid (5%), liquid (23%) and gas (72%) phases.

Table 4.4. Mass fraction (w%) of biomasses produced on slow pyrolysis

	Bio-oil			
Mass Fraction	**Soybean**	**Canola**	**Macauba**	***S. obliquus***
Liquid	70.86	72.12	64.55	30.22
Aqueous phase	54.21	55.41	47.20	22.93
Organic phase	16.65	16.70	17.35	7.29
Solid	2.79	2.54	4.57	45.97
Gas	26.33	25.32	30.86	23.8

Campanella et al. (2012) found a 22% for aqueous phase, 22% for bio-oil, 38% for biochar, and 18% for gas phase on slow pyrolysis on *Scenedesmus* sp. Moreover, a study developed by Rocha et al., (2020) reported a product yield of 10.72% for aqueous phase, 13.14% for bio-oil, 46.22% biochar, and 31.33% for gas phase using defatted *Scenedesmus obliquus*, the same strain used in this study. The previous pigment extraction also removed part of lipids from *S. obliquus*, and the high ash content of 26.0 % negatively influence the bio-oil yield found after the pyrolysis (7.29%).

Microalgae pyrolytic bio-oil is a very complex and wide mixture of organic compounds, which can be categorized as: hydrocarbon (benzene, naphthalene fluorene, toluene and xylene) (Yang et al., 2019), oxygenated (alcohols, aldehydes, ketones, phenols, ether, esters, and carboxylic acid), nitrogenated (indole, pyrrole, amine, amide, pyridines and pyrazines) (Zainan et al., 2018).

Carbohydrates in microalgae are present in starch grain, glycolipid, and cell wall constituent, in the form of polysaccharides and oligosaccharides. Carbohydrates are decomposed in pyrolysis by dehydration, glycoside bond cleavage, ring scission and

rearrangement, producing primarily anhydrous sugars and furfurals (Wang et al., 2017).

Triglycerides are ghe main lipids microalgae. During pyrolysis, triglycerides undergo break of the acyl chain from the glycerol back bone via decarboxylation, yielding long chain of fatty acids from fragmentation of glycerides (Wang et al., 2017). Moreover, fatty acid can further undergo decarboxylation, decarbonylation, deoxygenation, generating aldehydes, ketones, acid, alcohols, and olefins. Through cyclization and aromatization processes, olefins could be transformed into aromatic hydrocarbons (Yang et al., 2019).

Protein pyrolysis breaks the peptide bond between the carboxyl and amidogen building block. Therefore, amino acid undergo deamination, direct methylation, decarboxylation, dehydration, decarbonylation, cyclization, dimerization, and homonolysis (Yang et al., 2019). These reactions produce a wide range of nitrogen rich value added compounds, such as amines, pyridines, amides, pyridines, pyrroles, pyrazoles, pyrazines, polyheteroaromatics, nitriles, imidazoles and indoles (Lee et al., 2020; Xiong et al., 2023). Moreover, proteins and carbohydrates may also react via the Maillard reaction, generating a variety of aromatic compounds such phenols, pyridines, pyrroles, and indoles (Yang et al., 2019).

4.3.4 Phenolic content and antioxidant activity

The bio-oil from slow pyrolysis has large amount of phenolic compounds (organic acids, aldehydes, ketones, furans, sugar-based components and phenolics, such as phenol, dimethylphenol, guaiacol and catechol) reported in the literature for having antioxidant activity (Bautista et al., 2017). Total phenolic content and antioxidant activity of bio-oils is shown in **Table 4.5**. Concentrations were significantly different among all samples varied from 15.80 to 158.40 mg GAE g^{-1} extract. Bio-oils from *S. obliquus* and glycerol stand out with the higher phenolic content, 89.90 and 45.31 mg GAE g^{-1}, which is directly correlated to the higher antioxidant activities of 1.25 and 0.48 mM $Trolox^{-1}$, respectively.

Table 4.5.Determination of antioxidant activity, TEAC, DPPH, and phenolic content of bio-oils

	DPPH	TEAC	Total phenol

Antioxidants	IC50 (mg ml^{-1})	(mmol L^{-1} Trolox g^{-1})	(mg GAE g^{-1} extract)
BHT	0.05 ± 0.00 ^{a}	4.72 ± 0.07^{a}	158.40 ± 6.71^{a}
Analytical glycerol	1.47 ± 0.06^{c}	0.48 ± 0.02^{b}	45.31 ± 0.65^{b}
Soybean glycerol	5.57 ± 0,17^{e}	0.11 ± 0.00^{d}	17.97 ± 0.80^{d}
Canola glycerol	5.72 ± 0.16^{e}	0.11 ± 0.00^{d}	15.80 ± 0.92^{d}
Macauba glycerol	4.73 ± 0.16 ^{d}	0.13 ± 0.00^{d}	17.95 ± 0.32^{d}
Scenedesmus bio-oil	0.47 ± 0.00 ^{b}	1.25 ± 0.01^{c}	89.90 ± 1.76^{c}

*Values containing different letters differ from each other by Tukey's test at 5%.

The DPPH antioxidant activity of bio-oils and BHT are shown in **Figure 4.1.** All bio-oil samples experienced the same behavior, increases in the concentration, resulted in higher antioxidant activity. DPPH antioxidant capacity also can be expressed as IC50 parameter, which refers to the amount of antioxidant required to reduce the initial concentration of the antioxidant by 50%. Therefore, among the samples, BHT, *S. obliquus* and glycerol expressed the lower IC50 values, consequently having strong antioxidant activity.

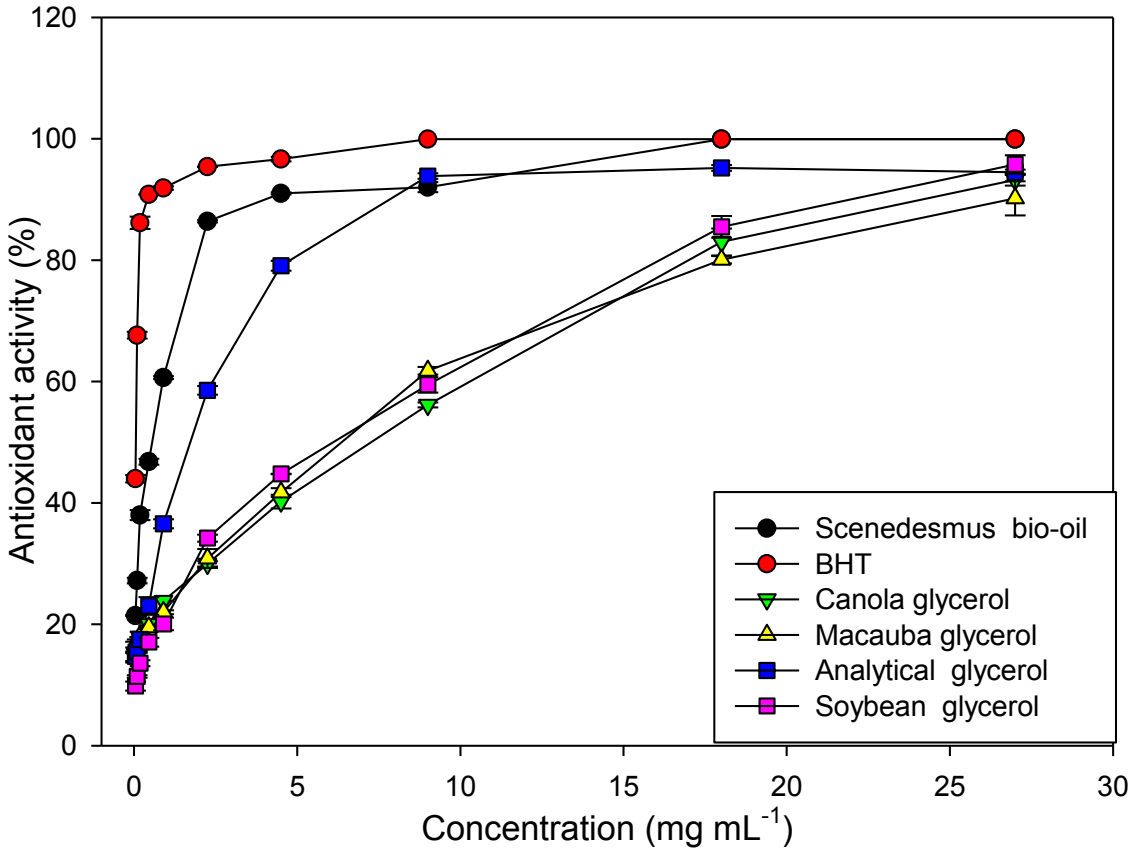

Figure 4. 1. Antioxidant activity of bio-oils and BHT in DPPH assay.

Moreover, pyrolytic compounds are linked to the composition of feedstock used. Therefore, slow pyrolysis of microalgae biomass generates a wide range of products. Due to the high content of protein in *S. obliquus*, the thermochemical processing generates a wide range of nitrogen-rich value added compounds such as amines, pyridines, amides, pyridines, pyrroles, pyrazoles, pyrazines, polyheteroaromatics, nitriles, imidazoles and indoles (Lee et al., 2020; Xiong et al., 2023). Pyrolytic compound variation is results of the intrinsic microalgae characteristics such as species, culture condition, which are directly related to the biochemical composition, consisting mainly of proteins, carbohydrates and lipids, each one contributing in a different way to the quality and product yield (Yang et al., 2019).

4.3.5 GC-MS analysis of bio-oils

GC-MS analysis was carried out to identify the organic compound in the bio-oil. The pyrolysis of residues results in diverse chemical compounds (classified in 7 groups, Table 4.6) with varying relative content (%). The striking difference between the bio-oils were higher concentration of aromatic compounds, and hydrocarbons. Thus, a correlation between higher phenolic content result in higher antioxidant activity can be verified in Table 4.5. Therefore, among the bio-oil samples *S. obliquus* and analytic glycerol stand out as potential antioxidants.

Some studies investigated the composition of bio-oils from microalgae using GC-MS and reported the presence of a wide variety of compounds, highlighting aromatic amines and phenolic compounds. Despite the high content of protein in the defatted biomass, GC-MS analysis showed that bio-oil from *S. obliquus* is poor in nitrogenous compounds. This result is in close agreement with data from previous study on the composition of bio-oils from microalgae of the Scenedesmus genus (Rocha et al., 2020). Also, this find indicates that the pyrolysis was efficient to promote the breakdown of proteins and deamination of the corresponding products. It might also be an advantage of such bio-oil as antioxidants for biodiesel, since high contents of N-compounds in bio-oils may result in undesirable emissions of toxic nitrogenous gases.

Table 4.6. Relative content % of major organic compounds identified by GC-MS analysis of natural extracts after derivatization by sylilation

Compound group	Analytic glycerol	Bio-oil			
		Soybean	Canola	Macauba	*Scenedesmus obliquus*
Aromatic	9.41	0.77	n.i*	0.53	11.27
Hydrocarbons	25.57	46.85	15.32	33.43	2.28
Carboxylic acids	8.93	9.45	47.73	25.04	27.14
Esters	5.89	21.21	26.51	25.58	4.65
Alcohols	9.14	5.31	2.00	7.06	8.97
Others	3.87	7.77	1.17	2.92	4.57

*not identified.

4.3.6 Bio-oils performances on soybean, canola and macauba biodiesel via Rancitmat test

Figure 4.2 illustrates the oxidative stability of control and additive samples of soybean biodiesel as function of the bio-oil type and concentration. The mean value of control soybean biodiesel was 4.93 ± 0.05 h. Only BHT, analytic glycerol and *S. obliquus* bio-oil showed a measurable positive impact on the biodiesel stability time. Moreover, *S. obliquus* bio-oil improvement on oxidative stability is found as dosage dependent, where higher dosages such as 4000 ppm had an improvement of 116.85% in the induction period (10.68 h).

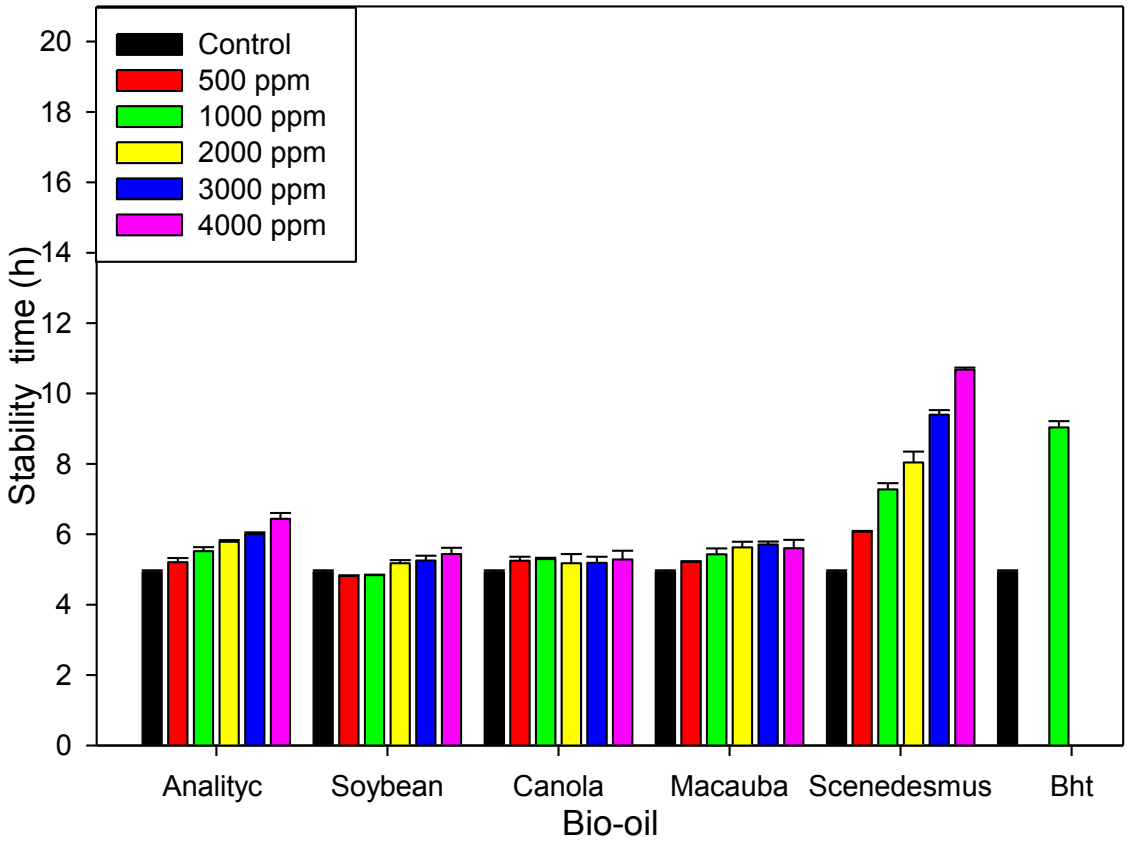

Figure 4.2. Stability time of soybean biodiesel additive with bio-oils.

The mean value of not doped canola biodiesel (**Figure 4.3**) was 6,02 ± 0,28 h. Similar trend of soybean biodiesel is observed, in which BHT, analytic, and *S. obliquus* bio-oil showed a noticeable effect on the stability time, as dosage depended. In addition, *S. obliquus* bio-oil had greater positive impact on canola biodiesel, where specific concentrations of 3000 and 4000 ppm resulted on an improvement of 133.26% (14.04 ± 0.01 h) and 194.51% (17.73 ± 0.27 h) on the stability time, respectively, Therefore, *S. obliquus* bio-oil concentrations above 2000 ppm are able to fulfil the requirement of ANP resolution No 798/219 of minimum stability time 12 hours.

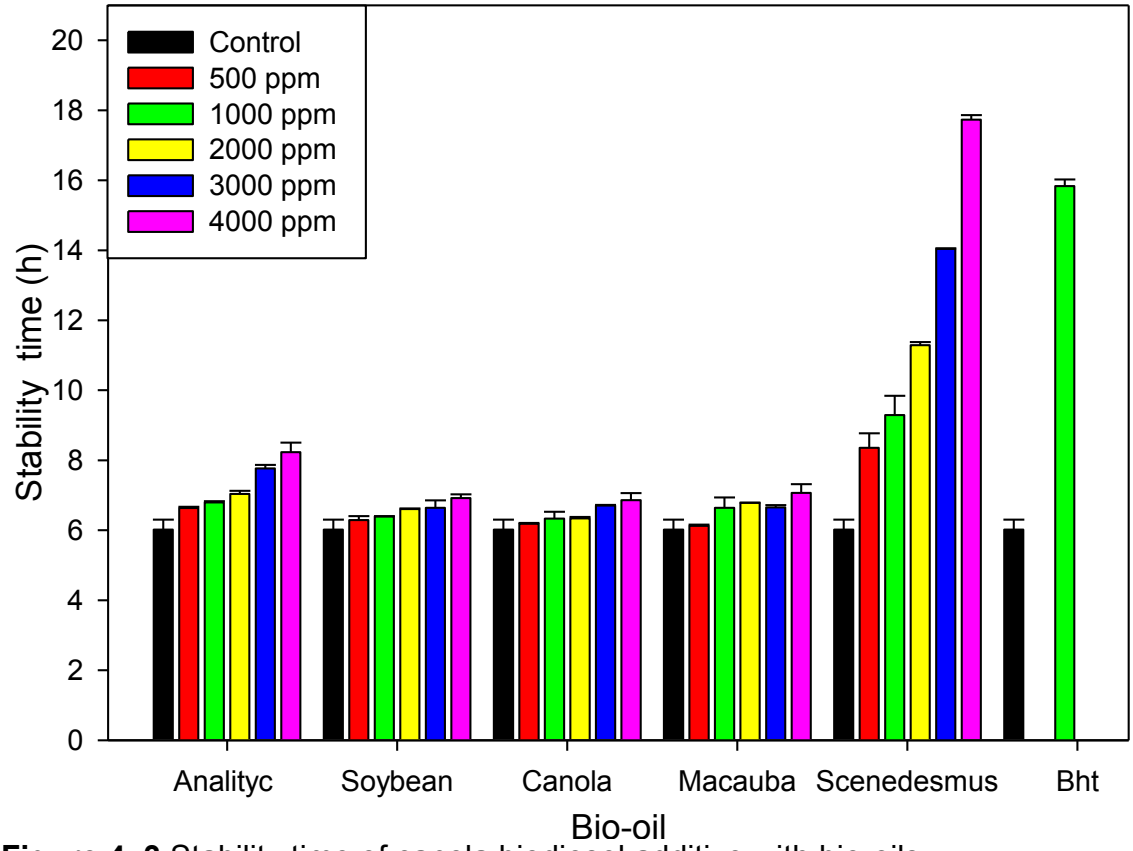

Figure 4. 3. Stability time of canola biodiesel additive with bio-oils.

As shown on **Figure 4.4**, stability of dopped and neat macauba biodiesel depends on the bio-oil dosage. The mean value of non-additivated macauba biodiesel was 1.98 ± 0.00 h. As previous result on soybean and canola oil, *S. obliquus* bio-oil stand out as the best impact on macauba biodiesel stability time. A specific concentration of 4000 ppm produces an improvement of 563.56% (13.21 h) and accomplishes the ANP resolution No 798/219 of minimum stability time 12 hours. Finally, it was confirmed that *S. obliquus* bio-oil improvement on stability time is verified in all biodiesel matrices investigated.

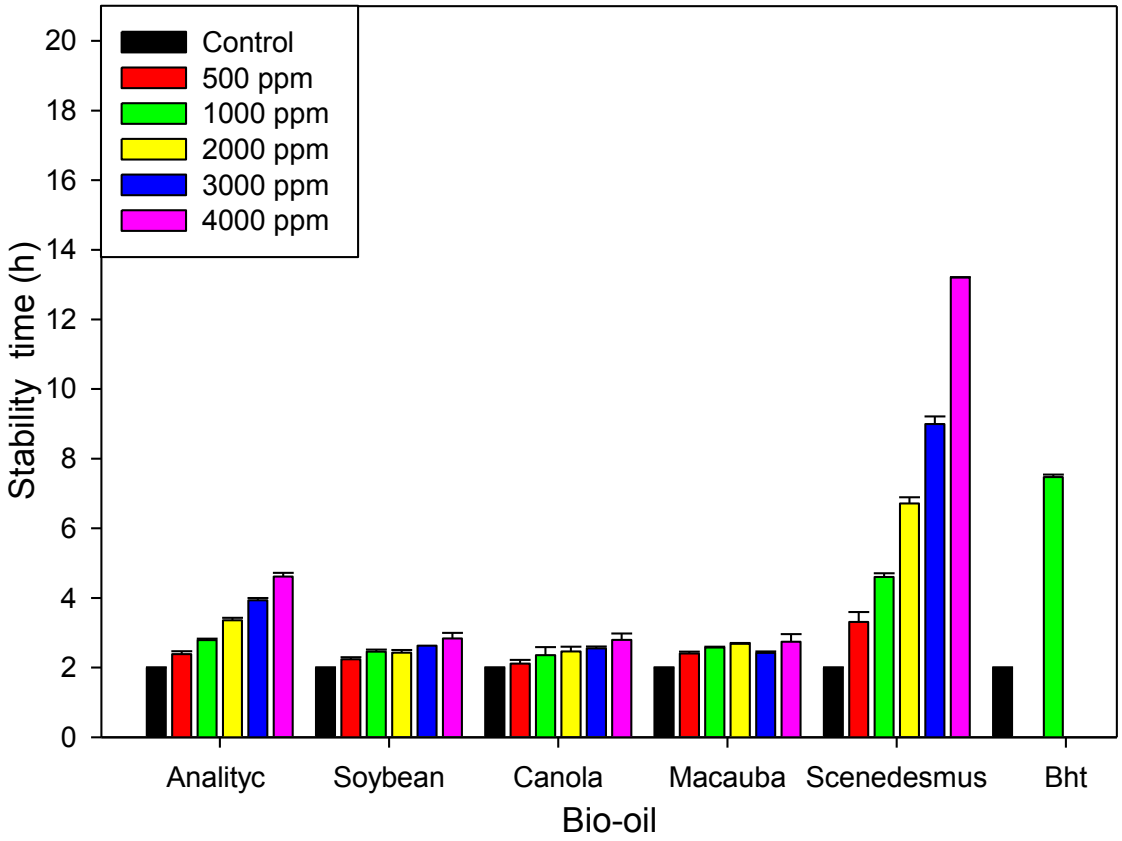

Figure 4.4. Stability time of macauba biodiesel additive with bio-oils.

A restricted number of studies had been reported evaluating the effect of boi-oil, mainly form woody biomasses, on the oxidation stability of biodiesel. Garcia-Perez et al. (2010) observed that canola biodiesel dopped with 10 to 50% in mass of bio-oil, produced from pyrolysis of woody fraction of mallee eucalyptus and pine, resulted in an increment of the stability time of 10.2 to 28.1 h for mallee pine, and 10.2 to 26 h for mallee eucalyptus. Another study, reported that an addition of 4% by weight of pure bio-oil directly to biodiesel increased oxidative stability by 475% (García et al., 2016). In addition, Gil-Lalaguna et al. (2017) evaluated the effect bio-oil produced form pyrolysis of pinewood where an addition of 2% directly in sunflower biodiesel produced an improvement of 135% in the stability time.

4.3.7 Long term storage stability test

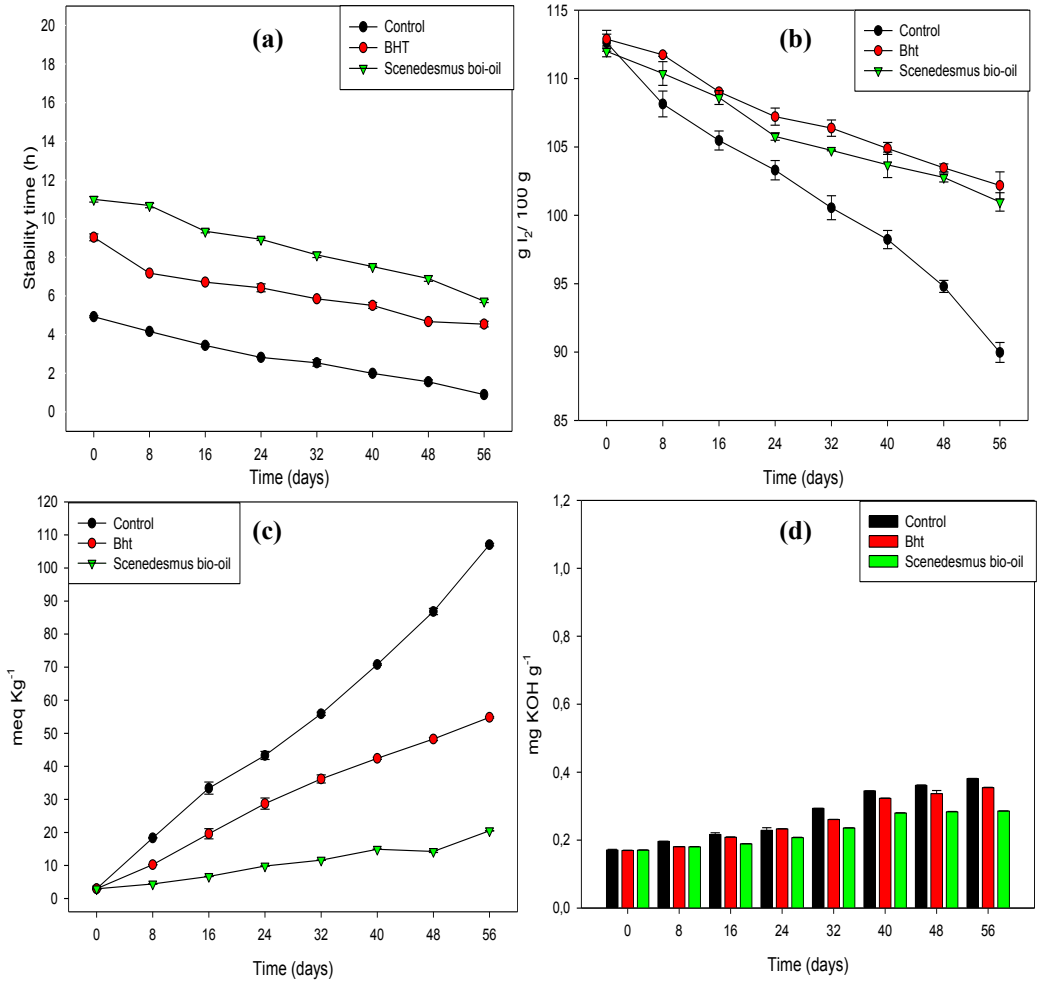

Figure 4. 5. Physicochemical parameters of soybean biodiesel at 43°C during 56 day of storage time. A) stability time, b) Iodine value, c) peroxide value and d) acid value

During storage test, as expect, soybean biodiesel stability time decreased (**Figure 4.5a**), attributed to the high level of polyunsaturated fatty acids (56.7%). All samples experience a decreased in the stability through the storage test. Neat soybean biodiesel was prone to oxidation, demonstrated on the sharply decrease stability time 81.73% (4.93 ± 0.04 to 0.90 ± 0.00 h). Conversely, the biodiesel samples with BHT and *S. obliquus* bio-oil showed a better resistance to oxidation. Particularly initial and eddying stability time of doped samples were: 9.04 ± 0.17 to 4.55 ± 0.14 h (decrease of 49.70%) for BHT, and 11.00 ± 0.14 to 5.74 ± 0.07 h (decrease of 47.86%) for *S. obliquus* bio-oil. However, dopped biodiesel show great oxidation stability results, they did not accomplish the ANP requirement of resolution No 798/219 of minimum stability time of 12 h.

Iodine value indicates the total degree of unsaturation and the tendency of biodiesel to oxidation, polymerization, deposit formation and is based on the reactivity of alkyl double bonds (J. S. Rodrigues et al., 2020). Even though, iodine value only

measured the total unsaturation, thus does not consider its position and type of the double bonds conjugated versus unconjugated or cis versus trans orientations, for instance (R. Kumar et al., 2016; Moser, 2011). The iodine value in this study was 112.62 ± 0.62 gI^2 /100 g for neat biodiesel (**Figure 4.5b**), comparable value for neat soybean biodiesel were found by Sousa et al. (2021) of 103.35 gI^2 /100 g, Sousa et al. (2014) 110.9 gI^2 /100 g, and Lapuerta et al. (2012) of 113.6 gI^2 /100 g. As expected, neat biodiesel undergoes a more noticeable decline in the iodide value over the storage time, due to oxidation and polymerization reactions. Particularly, initial and eddying iodine value of samples were: 112.62 ± 0.62 to 89.97 ± 0.73 gI^2 /100 g (decrease of 20.10%) for neat biodiesel, 112.88 ± 0.64 to102.19 ± 0.99 gI^2 /100 g for BHT (decrease 9.47%), and 111.99 ± 0.39 to 100.98 ± 0.67 (decrease of 9.83%) for *S. obliquus* bio-oil.

ANP resolution No 798/219 does not establish a limit for peroxide value, it is a very important parameter for monitoring biodiesel on storage time. This parameter indicates the initial stage of oxidation by measuring primary oxidation products (hydroperoxide and conjugated dines) (Varatharajan and Pushparani, 2018). In addition, peroxide value reach a plateau and abruptly decreased, due to the transformation of peroxides in to aldehydes an ketones, secondary oxidation products (Kumar, 2017). Peroxide values in all samples were low (2.97 ± 0.02 meq kg^{-1}, **Figure 4.5c**). However, during the storage time of 56 days, all samples experienced an increase on the peroxide value. Particularly, neat biodiesel (control) peroxide value drastically increased and reached 107.08 ± 0.40 meq kg^{-1} on day 56. Particularly, initial, and eddying iodine value of doped samples were: 2.97 ± 0.15 to 54.80 ± 0.22 meq Kg^{-1} for BHT, and 2.96 ± 0.01 to 20.50 ± 0.05 meq kg^{-1}. Therefore, BHT and *S. obliquus* bio-oil showed a greater resistance to initial stage of oxidation, which is correlated to the higher antioxidant activity.

During storage biodiesel undergo a series of interconnect chemical reaction, which directly impact on the physicochemical proprieties, reflecting in the fuel quality. Upon biodiesel storage, monitoring acid value is very important, because variation over the time may be related to water presences. Water on biodiesel leads oxidation of fatty acid methyl ester via hydrolysis, resulting in the dissociation products alcohol and free fatty acid, which subsequently increase acid value (Kumar, 2017; Yaakob et al., 2014). Acidity value of soybean biodiesel during storage is shown on **Figure 4.5d.** Specifically, initial, and eddying acid value of samples were: 0.171 to 0.381 mg KOH

g^{-1} (increased 122.98%) for neat biodiesel, 0.170 to 0.35 mg KOH g^{-1} for BHT (increase of 109.00%), and 0.170 to 0.286 mg KOH g^{-1} (67.56%) for *S. obliquus* bio-oil.

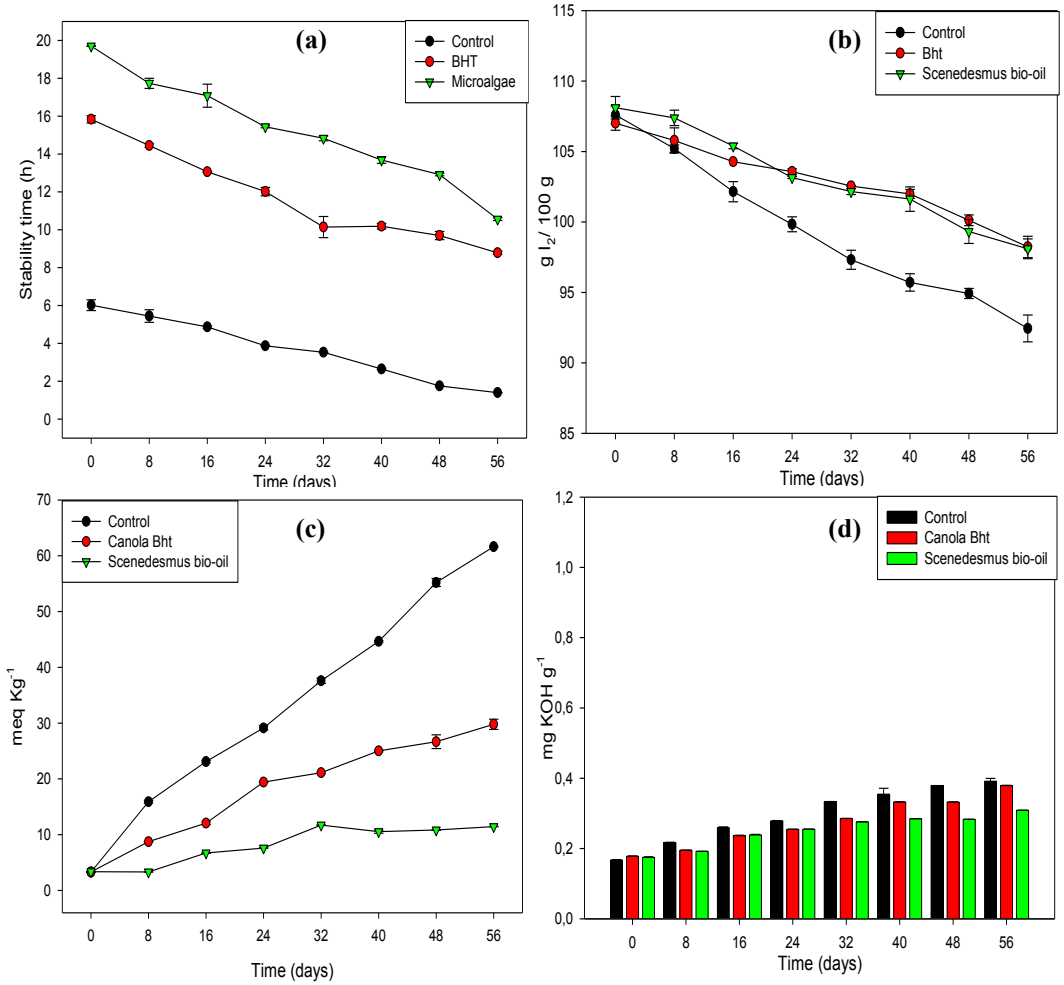

Figure 4.6. Physicochemical parameters of canola biodiesel at 43°C during 56 day of storage time. A) stability time, b) Iodine value, c) peroxide value and d) acid value

Storage time on neat canola biodiesel, were more susceptible to oxidation, over the 56 days of storage, as shown on **Figure 4.6a**. All samples experienced a decrease in stability through the storage test. Greater reduction stability time were observer on neat biodiesel 6.04 ± 0.28 to 1.40 ± 0.14, a decreased of 76.74% confirming the necessity of an additive. Moreover, dopped samples presented a better resistance to oxidation, as expected. Particularly initial and eddying stability were: 15.84 ± 0.19 to 8.79 ± 0.07 h for BHT (decreased 44.52%), and 19.70 ± 0.01 to 10.56 ± 0.07 h (decrease of 46.40%) for *S. obliquus* bio-oil. Therefore, *S. obliquus* bio-oil showed better performance than BHT, showing great oxidation stability, been able to satisfy, until day 48, ANP requirement of resolution No 798/219 of minimum stability time of 12 h.

Iodine value of canola biodiesel is shown on **Figure 4.6b**. As expected, all samples undergo a decline in the iodide value over of storage test, due to oxidation. Therefore, canola biodiesel was prone to oxidation, because of its chemical composition, 68% monounsaturated and 24% polyunsaturated fatty acid. Furthermore, neat canola biodiesel experienced a significant variation in iodine value (107.59 ± 0.64 to 92.44 ± 0.95 gI^2/100g), declined of 14.08%, On the other hand, doped samples experienced a lower iodine variation. Particularly, initial, and eddying iodine vales of doped samples were: 107.01 ± 0.50 to 98.23 ± 0.76 gI^2/100g for BHT (decreased of 8.20%), 108.11 ± 0.80 to 98.10 ± 0.70 gI^2/100g for *S. obliquus* bio-oil.

Peroxide concentration on canola biodiesel samples is displayed on **Figure 4.6c.** At the beginning of the storage experiment peroxide value were low around 3.30 meq Kg^{-1}. Therefore, there was not significant concentration of primary oxidation products (hydroperoxide and conjugated dines). During storage canola biodiesel free from antioxidants were more susceptible to peroxide formation, presented a linear increase, 3.31 ± 0.03 to 61.63 ± 0.13 meq Kg^{-1}. Particularly initial and eddying peroxide values of doped samples were: 3.32 ± 0.05 to 29.78 ± 0.92 meq Kg^{-1} for BHT, and 3.37 ± 0.01 to 11.45 ± 0.03 meq Kg^{-1} for *S. obliquus* bio-oil. These results are directly related to stability time, dopped canola biodiesel *S. obliquus* bio-oil shows greater oxidation resistance over 56 days, been able to maintain a stability time over 12 h for 48 days, therefore, satisfying ANP resolution No 798/219.

Acidity value of canola biodiesel is shown on **Figure 4.6d**. Initial, and eddying acid value of samples were: 0.16 to 0.39 mg KOH g^{-1} for neat biodiesel (increase of 134.23%), 0.17 to 0.37 mg KOH g^{-1} for BHT (increase of 113.26 and 0.17 to 0.30 mg KOH g^{-1} for *S. obliquus* bio-oil. Therefore, all biodiesel samples, neat and dopped, were able to maintain acid value within 0.5 mg KOH g^{-1} establish on ANP resolution No 798/219.

In other study Moser (2011) evaluated the acid value, iodine, and storage time on canola methyl ester over a period d 12 month at different temperatures (-15, 20 and 40 °C). Similar outcomes were found on neat canola biodiesel, acid value increased to 0.26 mg KOH g^{-1} at 60 days of experiment, a drastically reduction of the stability time from 6.4 to 0.8 h, and an iodide decreasing from 107 to 104 gI^2/100g.

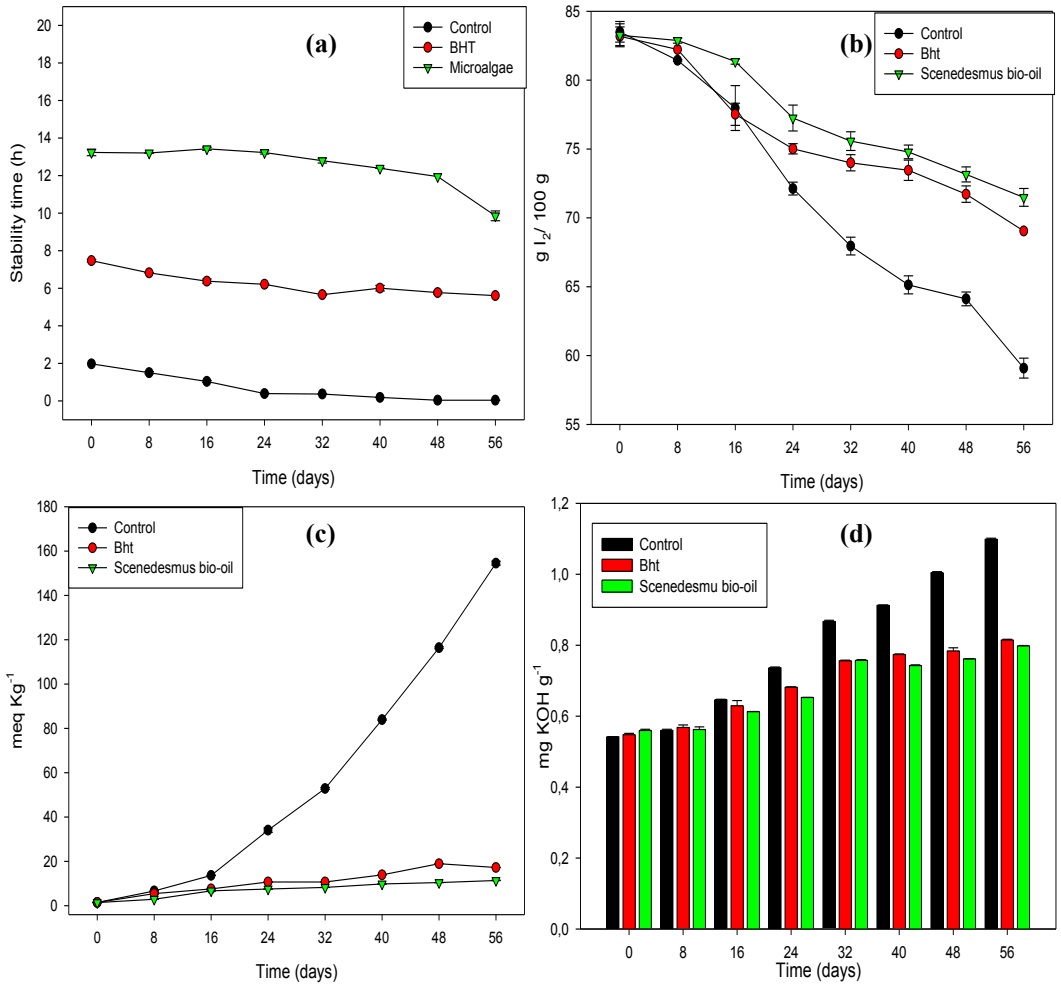

Figure 4.7. Physicochemical parameters of macauba biodiesel at 43°C during 56 day of storage time. A) stability time, b) Iodine value, c) peroxide value and d) acid value.

Biodiesel processing quality and quantity is strongly affected by high degree of free fatty acid content on the feedstocks (Ramos et al., 2019). Macauba pup oil as reported early on **Table 4.3.** As shown in **Figure 4.7**, the physicochemical parameter evaluated on storage macauba biodiesel. Stability time of macauba biodiesel is presented in **Figure 4.7a**. Stability time of all samples decreased in function on the storage time. A more prominent reduction was record on neat macauba biodiesel 1.98 ± 0.00 to 0.04 ± 0.00 h (decrease of 98.10%), correlating to lower acid, peroxide and iodine value having the worst performance over the storage time, been therefore very predisposed to oxidation. Particularly, initial, and eddying stability times of samples were: 7.47 ± 0.07 to 5.61 ± 0.00 h (decreased of 24.90%) for BHT, 13.24 ± 0.17 to 9.86 ± 0.26 h (decrease of 25.54%) for *S. obliquus* bio-oil.

Neat macauba biodiesel undergo a significant negative effect on the peroxide value, showed an abruptly increase from 1,48 to 154,51 meq Kg^{-1}. Among dopped

samples, *S. obliquus* bio-oil demonstrated greater resistance to oxidation, prevention the formation peroxide and hydroperoxide, initial degradation product, confirmed by the slightly variation 1.45 ± 0.01 to 11.42 ± 0.06 meq Kg^{-1} along the storage time. Similar value was displayed by BHT additive, specifically 1.48 ± 0.02 to 17.26 ± 0.00 meq Kg^{-}(**Figure 4.7c**).

The iodine value of macauba biodiesel is shown on **Figure 4.7b.** In this study, the initial iodine value macauba biodiesel prior to alkaline transesterification were 83.51 gI^2 /100 g, consistent value to the fatty acid composition presented on **Table 4.3**. Neat macauba biodiesel, were more prone to oxidation, experienced a great reduction since day 24, specifically variation 29.24% (83.51± to 59.09). Furthermore, dopped antioxidant presented more slightly variation, standing out *S. obliquus* bio-oil. Particularly, decreasing iodine value were: 14.13% (83.26 ± 0.83 to 71.49 ± 0.65 h) for *S. obliquus* bio-oil and 16.99% (83.51 ± 0.68 to 59.09 ± 0.65h) for BHT.

Macauba oil used in the study presented an extremely high acidity, acid esterification was used to transform free fatty acid into ester, lowering acidity value and increase transesterification yield. Macauba biodiesel acidity shorth after transesterification was 0,54 ± 0,014 mg KOH g^{-1}, which is marginally above ANP regulation on 0,5 mg KOH g^{-1}. Furthermore, acid value of macauba biodiesel upon storage is displayed on **Figure 4.7d**. A Similar trend as previously result obtained on soybean and canola biodiesel, acid value on neat macauba, presented a susceptibility to oxidation, confirmed by significantly increased on the acid value 102.71% (0.54 ± 0.00 to 1.09 ± 0,00 mg KOH g^{-1}). In contrast, *S. obliquus* bio-oil displayed the lower increased 42.59% (0.56 ± 0.01 to 0.79 ± 0.00 mg KOH g^{-1}), followed by BHT (positive control) increased of 48.69% (0.54 to 0.81 mg KOH g^{-1}).

4.4 Conclusion

This study assessed the antioxidant effect of organic phase of bio-oil produced from slow pyrolysis of analytic glycerol, crude soybean, canola, macauba glycerol, and *Scenedesmus obliquus* depigmented biomass. Those bio-oils were investigated as an alternative source of antioxidants on soybean, canola and macauba biodiesel.

The antioxidant potential of the bio-oils (in mmol L^{-1} Trolox g^{-1}) was confirmed by the antioxidant activity in a decreasing manner: 1.25 ± 0.01 for *S. obliquus*, 0.48 ± 0.02 for glycerol, 0.13 ± 0,00 for macauba, 0.11 ± 0,0 for soybean, and 0.11 ± 0,0 for

canola. Antioxidant activity was also corroborated by its positive effect on IC50, expressed in mg mL^{-1}, as 1.47 ± 0.06 for *S. obliquus*, 1.47 ± 0.06 for analytic glycerol bio-oil, 4.73 ± 0.16 for macauba, 5.57 ± 0.17 for soybean, and 5.72 ± 0.16 for canola glycerol bio-oil.

The screening test demonstrated that antioxidant performance was dosage and matrix dependent. At 4000 ppm, higher stability times were presented by *S. obliquus* bio-oil with 116.85% of increasing (13.21 h) for soybean, 194.52% of increasing (17.73 h) for canola, and 563.57% of increasing (13.21 h) for macauba . Consequently, the addition of bio-oils satisfied the requirement of ANP resolution No 798/219 of minimum stability time of 12 hours, but on canola and macauba biodiesel. During the long storage test, neat biodiesel were prone to oxidation, as evidenced by the decreasing in the stability time at the 56-day of. Specifically, 81.73, 76.74 and 98.11% decreased for soybean, canola anda macauba biodiesel respectively. *S. obliquus* bio-oil was proven as a promising alternative for synthetic (BHT) and natural antioxidants, due to protective effect on delaying lipid oxidation, confirmed on lower iodine value, practically immutable low acid value, peroxide value and stability time decreasing over the time.

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5. General conclusions

In this study, the neat biodiesel did not attend the ANP specification of stability time, which can be attributed to the high level of polyunsaturated fatty acids. It clearly demonstrates the need for the addition of some chemical species, antioxidants, that can prevent or delay the oxidation of biofuel. Specifically, neat biodiesel stability time were 1.99 ± 0.01h for macauba, 4.93 ± 0,05 h for soybean, and 6.02 ± 0.28 h for canola. Likewise, most published articles tested a restricted number of synthetic antioxidants. However, there is a great diversity of antioxidants that have not yet been studied. Therefore, this study demonstrated the oxidative stability of soybean, canola and macauba biodiesel can be improved by the addition of natural extracts, stand out turmeric and *scenedesmus obliquus*, and bio-oil from slow pyrolysis of crude glycerol from biodiesel processing and from the depigmented microalgae *Scenedesmus obliquus*.

Antioxidant screening concentration on accelerated oxidation test (Rancimat) demonstrated that natural extracts and bio-oil antioxidants were dose and matrix dependent. Among the natural extract at the highest concentration (3000 ppm) turmeric and *S. obliquus* stood out for higher stability times. Specifically, stability time increases with turmeric were 35, 51.8, 338.4% on soybean, canola, and macauba biodiesel, respectively. *S. obliquus*, the increases were 36.0, 46.7 and 122.0% on soybean, canola, and macauba biodiesel, respectively. On the other hand, depigmented S. obliquus bio-oil at 4000 ppm produced an increased in the stability time of 116.9, 194.5 and 563.6% on soybean, canola, and macauba biodiesel, respectively.

During the 56 days of storage test, oxidation process on neat and dopped biodiesel samples was monitored using stability time, acid, iodine, and peroxide value. As expected, neat biodiesel were more susceptible to oxidation, evidenced in the lower stability times of 0.90 ± 0.00 h for soybean, 1.40 ± 0.01 h for canola and 0.04 ± 0.00 h for macauba. The addition of natural extract from turmeric and *S. obliquus* were able to maintain biodiesel quality along the storage test, except for stability time of ANP resolution n. º 798/219 of minimum 12 hours. However, *S. obliquus* bio-oil is a promising alternative to synthetic (BHT) and natural antioxidants, due to protective effect on delaying lipid oxidation, confirmed on lower iodine value, practically immutable low acid value, peroxide value and stability time decreasing over the time.

Therefore, *S. obliquus* bio-oil were able to maintain stability time above 12 h until the 48th day on canola (12.92 ± 0.06 h) and macauba (11.95 ± 0.00 h) biodiesel, consequently fulfilling the ANP resolution No 798/219.

6. Appendix

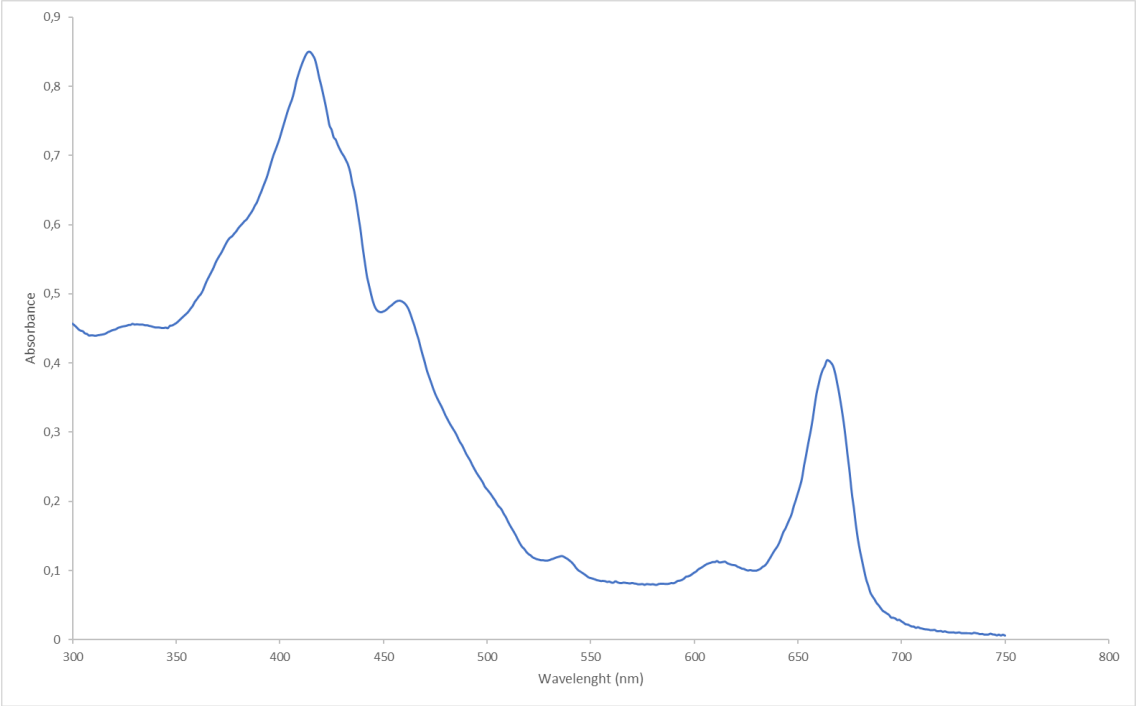


Figure A 1. UV-visible spectrum of ethanolic *S. obliquus* extract.

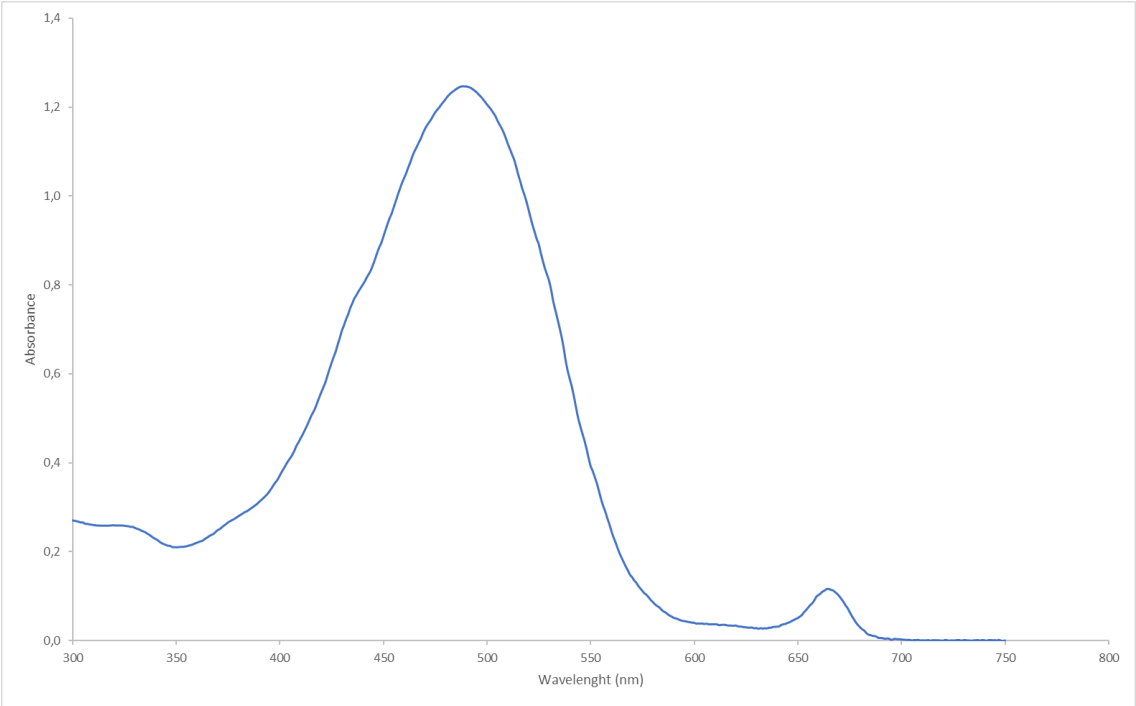


Figure A 2.UV-visible spectrum of ethanolic *H pluvialis* extract.

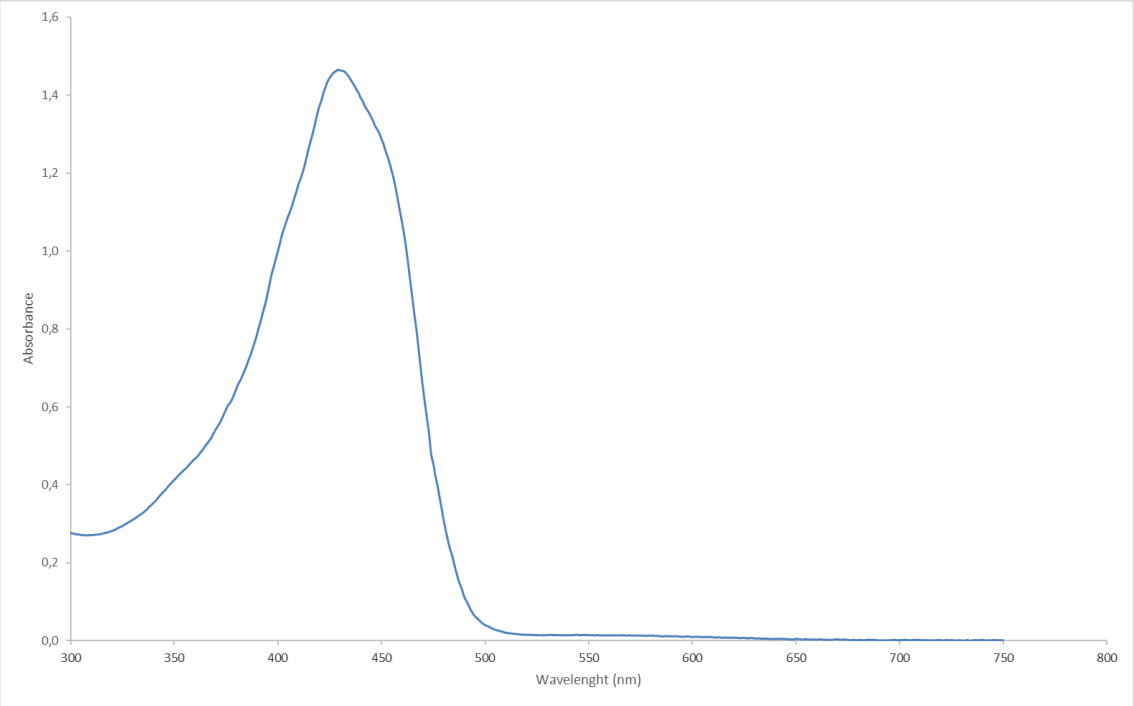


Figure A 3. UV-visible spectrum of ethanolic turmeric extract.

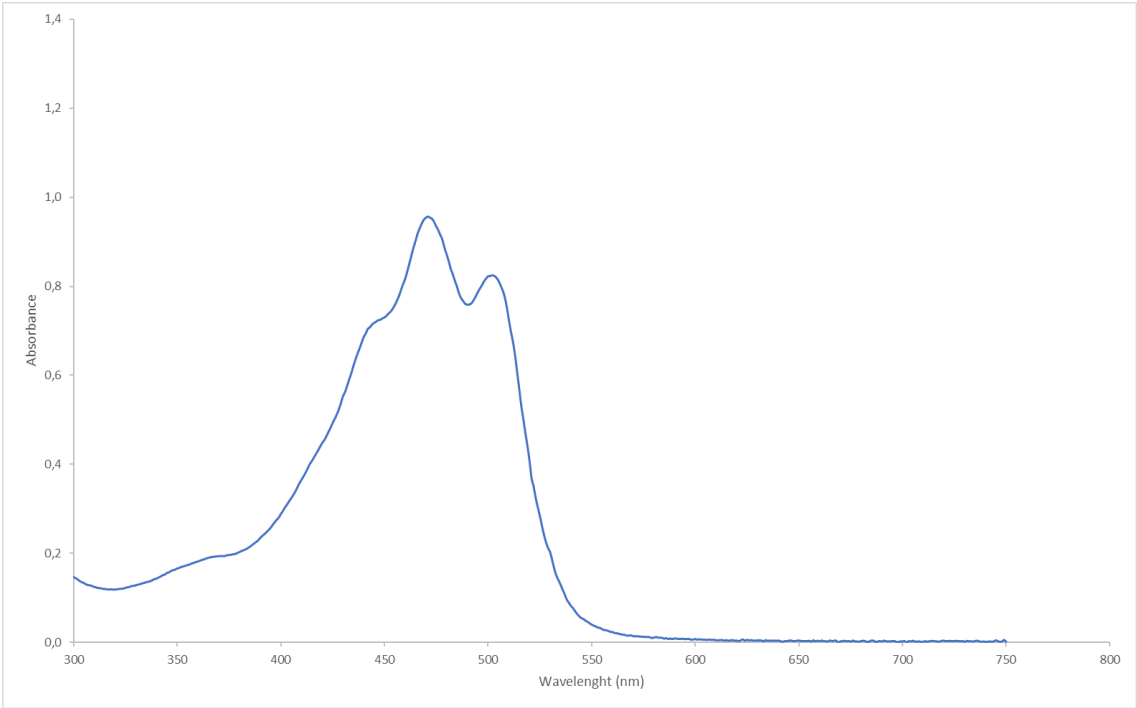


Figure A 4. UV-visible spectrum of ethanolic annatto extract.

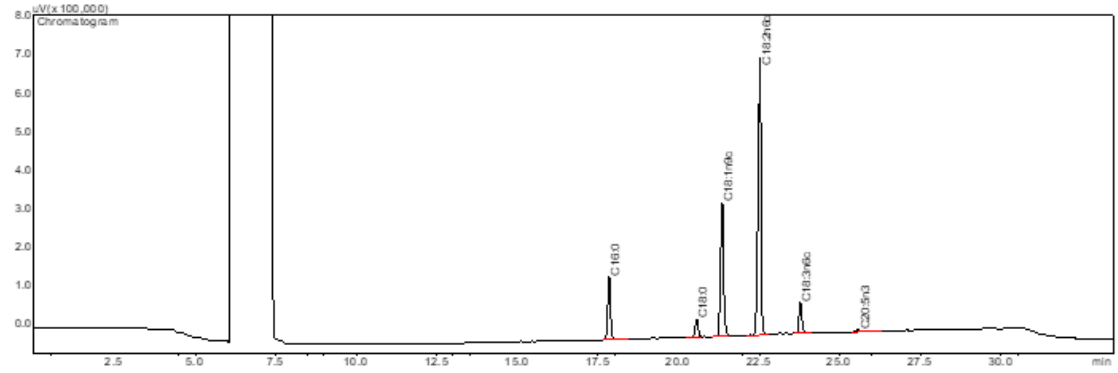


Figure A 5. Chromatogram soybean biodiesel.

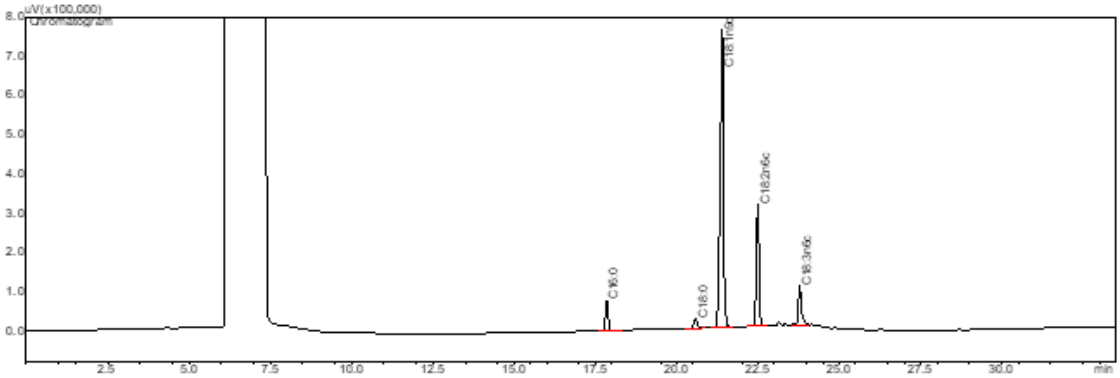


Figure A 6. Chromatogram canola biodiesel

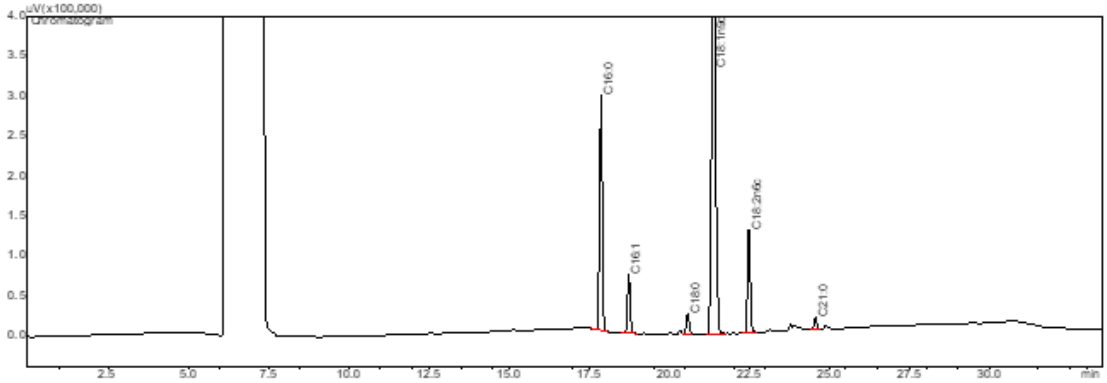


Figure A 7. Chromatogram Macauba biodiesel.

Natural Extracts

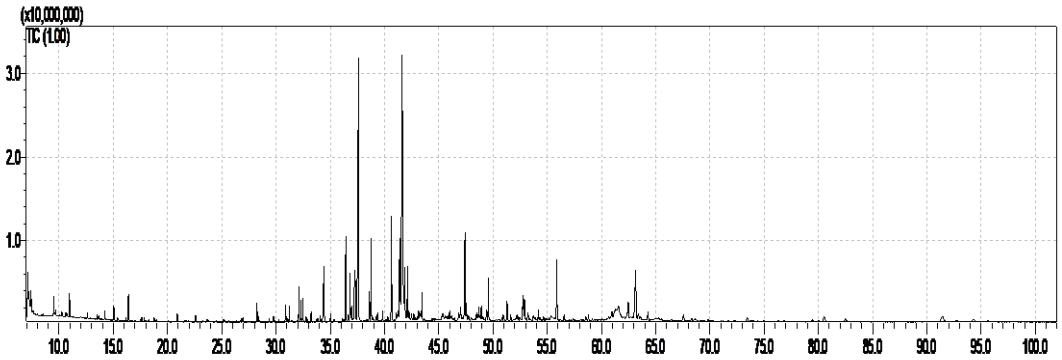


Figure A 8. GC-MS Chromatogram *S. obliquus* extract.

Table A. 1.Chemical constituents identified in *Scenedesmus obliquus* extract. Relative percentage as determined by GC-MS after derivatization by silylation.

Rt (min)	Relative percentage (%)	Compounds
	0.7	**Aromatic compounds**
13.525	0.12	Benzoic Acid TMS
28.351	0.33	Butylated Hydroxytoluene TMS
48.497	0.25	Diisooctyl phthalate
	5.75	**Aliphatic hydrocarbons**
28.234	0.74	Tetradecane
32.118	1.22	Neophytadiene
43.149	0.22	1-Nonadecene
47.416	3.57	3-Eicosene, (E)-
	46.84	**Aliphatic carboxylic acids**
7.227	0.55	a-Hydroxyisobutyric acid 2TMS
9.671	0.18	Oxalic acid, 2TMS
14.213	0.26	Octanoic acid TMS
16.167	0.13	Butanedioic acid 2TMS
17.631	0.10	Nonanoic acid TMS
20.901	0.24	Decanoic acid TMS
26.961	0.14	Dodecanoic acid TMS
31.225	0.47	Azelaic acid 2TMS
32.465	0.73	Myristic acid TMS
35.024	0.25	Pentadecanoic acid TMS
37.237	1.54	Palmitelaidic acid TMS
37.615	16.09	Palmitic acid TMS
39.834	0.32	Heptadecanoic acid TMS
41.415	4.33	9,12-Octadecadienoic acid (Z,Z)- TMS
41.633	16.96	9-Octadecenoic acid, (E)- TMS
41.716	1.63	Oleic acid (Z) TMS
42.130	1.94	Stearic acid TMS
43.457	0.98	a-Linolenic acid TMS
	10.17	**Aliphatic esters**

33.244	0.38	Phytol acetate
33.854	0.09	9-Hexadecenoic acid, methyl ester, (Z)-
34.423	1.78	Hexadecanoic acid, methyl ester
36.133	0.23	Hexadecanoic acid, ethyl ester
38.600	0.98	9,12-Octadecadienoic acid, methyl ester, (E,E)-
38.770	2.98	6-Octadecenoic acid, methyl ester, (Z)-
38.880	0.12	9-Octadecenoic acid, methyl ester, (E)-
39.358	0.24	Methyl stearate
40.307	0.16	Ethyl Oleate
45.405	0.13	Hexanedioic acid, bis(2-ethylhexyl) ester
47.592	0.14	Carbonic acid, 2-ethylhexyl octyl ester
49.586	1.40	1-Monopalmitin 2TMS
51.297	0.71	1-Heneicosyl formate
52.786	0.83	1-Monooleoylglycerol 2TMS
	9.16	**Alcohols**
15.047	0.50	Glycerol 3TMS
32.772	0.21	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
34.351	1.46	6,10,14-trymethyl-2-pentadecanol
40.654	3.87	Phytol TMS
46.997	0.49	9-Octadecen-1-ol, (Z)-
55.884	2.13	1-Hexacosanol TMS
62.466	0.50	Ergost-7-en-3b-ol TMS
	2.32	**Others**
32.285	0.76	2-Pentadecanone, 6,10,14-trimethyl-
37.290	1.56	1,3-dioxolane, 2-pentadecyl

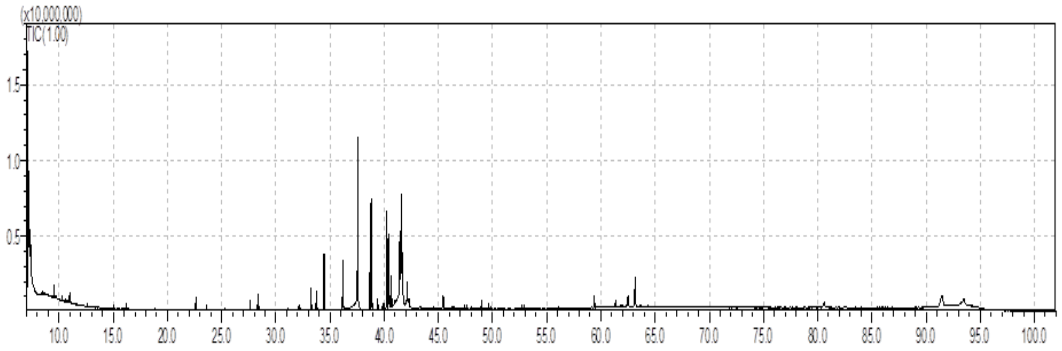


Figure A 9.GC-MS Chromatogram *H. pluvialis* extract.

Table A.2 Chemical constituents identified in bio-oil from *Haematococcus pluvialis*. Relative percentage as determined by GC-MS after derivatization by silylation.

Rt (min)	Relative percentage (%)	Compounds
	1.69	**Aromatic compounds**
28.390	1.69	Butylated Hydroxytoluene TMS
	2.20	**Aliphatic hydrocarbons**
27.636	0.52	8-Heptadecene
28.260	0.30	Tetradecane
32.145	0.23	Neophytadiene
33.746	1.15	Hexadecane
	56.46	**Aliphatic carboxylic acid**
7.136	15.44	Lactic Acid 2TMS
16.186	0.27	Butanedioic acid 2TMS
37.583	12.26	Palmitic Acid TMS
40.234	7.01	Linoelaidic acid
41.451	6.09	9,12-Octadecadienoic acid (Z,Z)-TMS
41.610	12.05	a-Linolenic acid TMS
41.716	3.35	9-Octadecenoic acid, (E)- TMS
	28.58	**Aliphatic esters**
34.451	3.57	Hexadecanoic acid, methyl ester
36.182	3.11	Hexadecanoic acid, ethyl ester
38.634	2.44	9,12-Octadecadienoic acid, methyl ester, (E,E)-
38.800	7.49	9-Octadecenoic acid (Z)-, methyl ester
38.918	0.32	10-Octadecenoic acid, methyl ester
39.399	0.59	Methyl stearate
39.955	0.38	(6Z,9Z,12Z,15Z)-Methyl octadeca-6,9,12,15-tetraenoate
38.634	2.44	9,12-Octadecadienoic acid, methyl ester, (E,E)-
40.389	6.04	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-
40.494	0.72	(E)-9-Octadecenoic acid ethyl ester
45.453	0.72	Hexanedioic acid, bis(2-ethylhexyl) ester
48.976	0.45	2-Palmitoylglycerol, 2TMS derivative
49.616	0.31	1-Monopalmitin, 2TMS derivative
	2.09	**Alcohols**

40.671	2.09	Phytol TMS

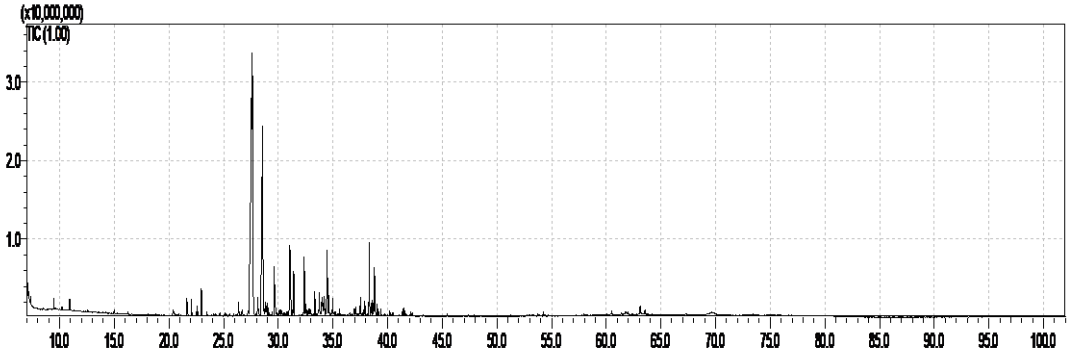


Figure A 10.GC-MS Chromatogram turmeric extract.

Table A. 3. Chemical constituents identified in turmeric extract. Relative percentage as determined by GC-MS after derivatization by silylation.

Rt (min)	Relative percentage (%)	Compounds
	30.95	**Aromatic compounds**
21.639	0.69	Benzene, 1-(1,5-dimethyl-4-hexenyl)-4-methyl-
22.593	0.46	Butylated Hydroxytoluene
23.453	0.13	Vanillin TMS
27.602	29.12	AR-Turmerone
34.967	0.55	4-Coumaric acid 2TMS
	1.7	**Aliphatic hydrocarbons**
22.051	0.57	1,3-Cyclohexadiene, 5-(1,5-dimethyl-4-hexenyl)-2-methyl-, [S-(R*,S*)]-
22.484	0.14	b-Bisabolene
22.974	0.99	Cyclohexene, 3-(1,5-dimethyl-4-hexenyl)-6-methylene-, [S-(R*,S*)]-
	1.3	**Aliphatic carboxylic acids**
37.513	0.60	Palmitic Acid TMS
41.389	0.26	9,12-Octadecadienoic acid (Z,Z)- TMS
41.518	0.31	9-Octadecenoic acid, (E)- TMS
42.113	0.13	Stearic acid TMS
	4.22	**Aliphatic esters**
38.622	0.61	9,12-Octadecadienoic acid, methyl ester, (E,E)-
38.787	3.29	9-Octadecenoic acid (Z)-, methyl ester
39.379	0.23	Methyl stearate
45.428	0.09	Hexanedioic acid, dioctyl ester
	0.41	**Alcohols**

63.099	0.41	b-Sitosterol TMS
	26.51	**Anothers ketones**
27.672	9.47	Tumerone
28.568	15.57	Curlone
29.709	0.81	(6R,7R)-Bisabolone
33.992	0.66	(S)-3-Methyl-6-((S)-6-methyl-4-oxohept-5-en-2-yl)cyclohex-2-enone

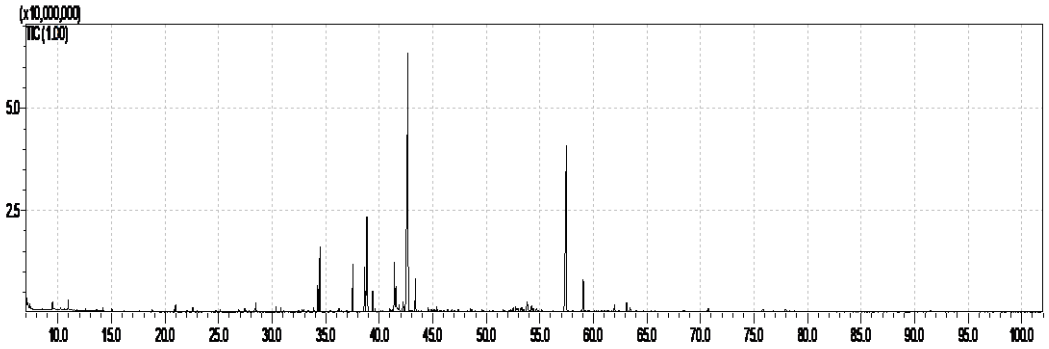


Figure A 11.GC-MS Chromatogram annatto extract.

Table A. 4. Chemical constituents identified in annatto extract. Relative percentage as determined by GC-MS after derivatization by silylation.

Rt (min)	Relative percentage (%)	Compounds
	1.67	**Aromatic compounds**
13.508	0.05	Benzoic Acid TMS
22.588	0.51	Butylated Hydroxytoluene
32.944	0.09	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester
48.516	0.20	Diisooctyl phthalate
53.307	0.51	Phthalic acid, bis(7-methyloctyl) ester
54.375	0.31	1,2-Benzenedicarboxylic acid, dinonyl ester
	3.03	**Aliphatic hydrocarbons**
20.975	0.41	(1R,1aR,2aS,6R,6aS,7aS)-1,6,6a-Trimethyldecahydro-1,2a-methanocyclopropa[b]naphthalene
34.252	1.54	5,9,13-Pentadecatrien-2-one, 6,10,14-trimethyl-, (E,E)-
53.822	1.08	Supraene
	9.61	**Aliphatic carboxylic acids**
7.196	0.19	a-Hydroxyisobutyric acid 2TMS
14.186	0.15	Octanoic acid TMS
20.887	0.19	Decanoic acid TMS

32.464	0.09	Myristic acid TMS
37.533	2.72	Palmitic Acid TMS
41.423	3.39	9,12-Octadecadienoic acid (Z,Z)-TMS
41.545	1.88	9-Octadecenoic acid, (E)- TMS
42.221	0.69	Stearic acid TMS
45.352	0.18	Dehydroabietic acid TMS
46.375	0.13	Arachidic acid TMS
	19.61	**Aliphatic esters**
18.733	0.11	Decanoic acid, ethyl ester
33.860	0.25	9-Hexadecenoic acid, methyl ester, (Z)-
34.462	4.29	Hexadecanoic acid, methyl ester
36.139	0.10	Hexadecanoic acid, ethyl ester
38.640	2.79	9,12-Octadecadienoic acid, methyl ester, (E,E)-
38.838	7.56	9-Octadecenoic acid (Z)-, methyl ester
38.910	0.49	11-Octadecenoic acid, methyl ester, (Z)-
39.386	1.11	Methyl stearate
41.843	0.31	(2E,6E,10E)-3,7,11,15-Tetramethylhexadeca-2,6,10,14-tetraen-1-yl formate
43.378	2.11	2,6,10,14-Hexadecatetraen-1-ol, 3,7,11,15-tetramethyl-, acetate, (E,E,E)-
49.596	0.12	1-Monopalmitin 2TMS
70.730	0.37	(2E,6E)-3,7,11-Trimethyldodeca-2,6,10-trien-1-yl decanoate
	37.44	**Alcohols**
15.019	0.16	Glycerol 3TMS
42.687	36.10	Farnesol TMS
61.971	0.47	Stigmasterol TMS
63.097	0.71	b-Sitosterol

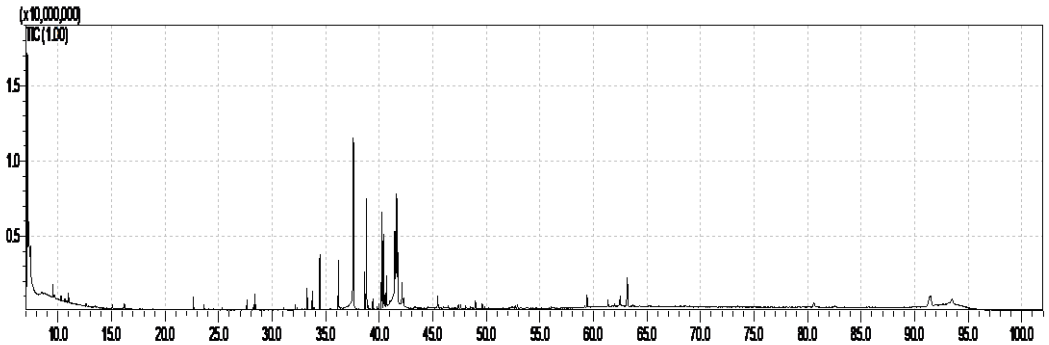


Figure A 12.GC-MS Chromatogram coffee husk extract.

Table A. 5. Chemical constituents identified in coffee husk extract. Relative percentage as determined by GC-MS after derivatization by silylation.

Rt (min)	Relative percentage (%)	Compounds
	7.43	**Aromatic compounds**
13.525	1.20	Benzoic acid TMS
15.412	0.20	Benzeneacetic acid TMS
22.610	0.82	Butylated Hydroxytoluene
22.778	0.14	Salicylic acid 2TMS
26.325	0.35	4-Hydroxybenzoic acid 2TMS
30.374	0.11	Vanillic Acid 2TMS
32.058	2.95	Protocatechoic acid 3TMS
48.510	0.36	Diisooctyl phthalate
32.967	0.28	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester
54.203	0.42	Phthalic acid, bis(7-methyloctyl) ester
54.687	0.43	1,2-Benzenedicarboxylic acid, dinonyl ester
55.162	0.17	Phthalic acid, nonyl pentadecyl ester
	0.64	**Aliphatic hydrocarbons**
28.248	0.09	Heptadecane
33.717	0.09	Nonane, 3-methyl-5-propyl-
36.270	0.13	Hexadecane, 2,6,10,14-tetramethyl-
47.489	0.18	Heneicosane
55.026	0.15	Heptadecane, 2,6,10,15-tetramethyl-
	34.29	**Aliphatic carboxylic acids**
7.135	2.64	Lactic Acid 2TMS
7.233	0.35	2-Hydroxybutyric acid 2TMS
7.615	0.34	Glycolic acid 2TMS
9.675	0.28	Oxalic acid 2TMS
10.034	0.10	Hydracrylic acid 2TMS
10.815	0.28	2-Hydroxy-3-methylbutyric acid 2TMS
14.221	0.66	Octanoic acid TMS
16.177	0.91	Butanedioic acid 2TMS
17.646	0.13	Nonanoic acid, TMS
19.334	0.98	Pentanedioic acid 2TMS
20.913	0.49	Decanoic acid TMS
26.992	0.16	Dodecanoic acid TMS
31.245	0.72	Azelaic Acid, 2TMS derivative
32.496	0.31	Myristic acid TMS
36.982	0.43	Palmitelaidic acid TMS

37.577	11.82	Palmitic Acid TMS
41.421	4.42	9,12-Octadecadienoic acid (Z,Z)- TMS
41.560	5.69	9-Octadecenoic acid, (E)- TMS
42.134	2.23	Stearic acid TMS
46.374	0.49	Arachidic acid TMS
69.917	0.86	Ursolic acid 2TMS
	30.51	**Aliphatic esters**
18.760	0.37	Decanoic acid, ethyl ester
33.870	0.55	9-Hexadecenoic acid (Z)-, methyl ester
34.471	8.21	Hexadecanoic acid, methyl ester
36.151	0.26	Hexadecanoic acid, ethyl ester
38.638	4.37	9,12-Octadecadienoic acid, methyl ester, (E,E)-
38.846	14.16	6-Octadecenoic acid, methyl ester, (Z)-
38.926	0.77	9-Octadecenoic acid, methyl ester, (E)-
39.385	1.20	Methyl stearate
40.319	0.17	(E)-9-Octadecenoic acid ethyl ester
43.916	0.13	Hexadecanoic acid, 15-methyl-, methyl ester
49.589	0.32	1-Monopalmiti, 2TMS
	3.45	**Alcohols**
15.052	0.25	Glycerol 3TMS
61.395	0.61	Campesterol TMS
61.952	0.66	Stigmasterol TMS
63.092	1.93	*b-Sitosterol* TMS
	6.76	**7,06**
32.743	6.76	Caffeine

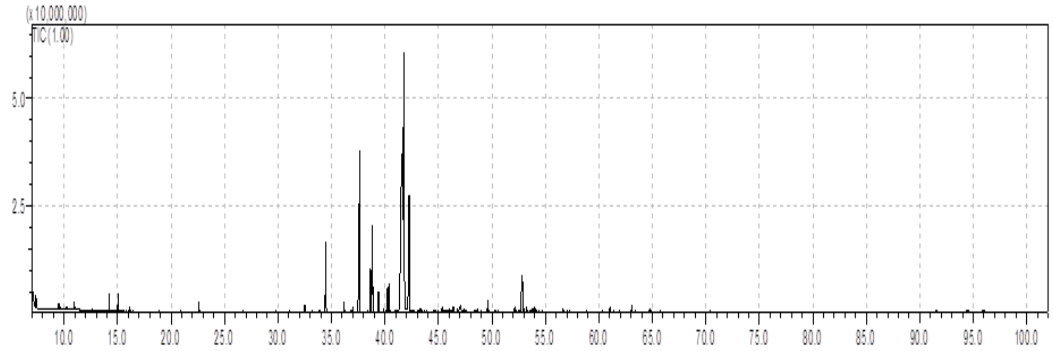


Figure A 13.GC-MS Chromatogram coffee C. *H. pluvialis* extract.

Table A. 6. Chemical constituents identified in coffee C. *H. pluvialis* extract. Relative percentage as determined by GC-MS after derivatization by silylation

Rt (min)	Relative percentage (%)	Compounds
	0.68	**Aromatic compounds**
22.602	0.49	Butylated Hydroxytoluene
45.354	0.19	Dehydroabietic acid TMS
	0.59	**Aliphatic hydrocarbons**
46.942	0.22	1,E-8,Z-10-Hexadecatriene
53.821	0.37	Squalene
	62.78	**Aliphatic carboxylic acids**
7.365	0.53	Hexanoic acid TMS
14.200	0.67	Octanoic acid TMS
16.155	0.17	Butanedioic acid 2TMS
39.868	0.15	Heptadecanoic acid TMS
40.205	1.12	9,12-Octadecadienoic acid (Z,Z)-
41.577	23.15	Linoelaidic acid TMS
41.797	28.63	9-Octadecenoic acid, (E)- TMS
42.258	7.88	Stearic acid TMS
46.384	0.21	Arachidic acid TMS
54.012	0.27	Lignoceric acid TMS
	31.09	**Aliphatic esters**
34.465	3.82	Hexadecanoic acid, methyl ester
36.151	0.45	Hexadecanoic acid, ethyl ester
36.977	0.19	Palmitelaidic acid TMS
37.646	14.39	Palmitic Acid TMS
38.648	2.26	9,12-Octadecadienoic acid, methyl ester, (E,E)-
38.839	5.34	9-Octadecenoic acid (Z)-, methyl ester
38.923	0.24	10-Octadecenoic acid, methyl ester
39.391	0.88	Methyl stearate
40.349	1.27	(E)-9-Octadecenoic acid ethyl ester
49.597	0.49	1-Monopalmitin, 2TMS derivative
52.837	1.52	1-Monooleoylglycerol, 2TMS derivative
53.238	0.24	Glycerol monostearate, 2TMS derivative
	1.06	**Alcohols**
15.040	0.71	Glycerol 3TMS
63.081	0.35	*b-Sitosterol* TMS

Bio-oils

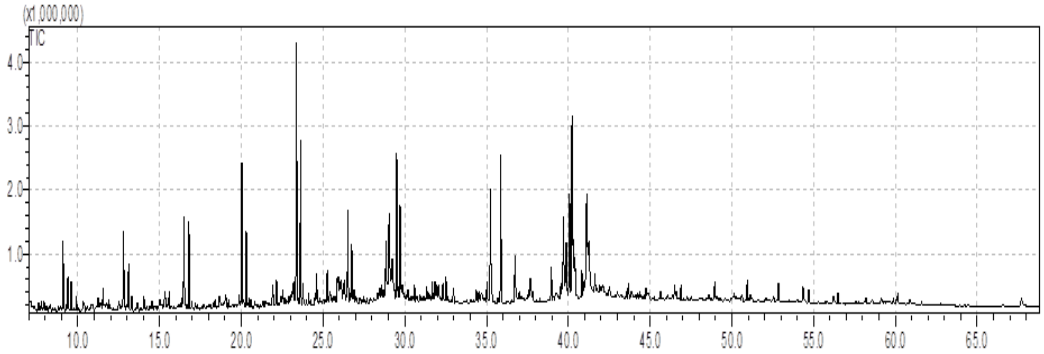


Figure A 14.GC-MS Chromatogram soybean bio-oil.

Table A. 7. Chemical constituents identified in bio-oil soybean. Relative percentage as determined by GC-MS after derivatization by silylation.

Rt (min)	Relative percentage (%)	Compounds
	0.77	**Aromatic compounds**
11.540	0.40	Benzene, pentyl-
11.911	0.17	Benzene, 1-methyl-4-butyl
19.225	0.20	1-Methyl-2-n-hexylbenzene
	46.85	**Aliphatic hydrocarbons**
9.097	1.68	1-Undecene
9.401	0.72	Undecane
9.591	0.65	2-Undecene, (E)-
9.915	0.31	2-Undecene, (Z)-
10.325	0.42	5-Dodecyne
12.798	1.94	Cyclopropane, nonyl-
13.112	0.97	Dodecano
13.306	0.30	2-Dodecene, (E)-
14.045	0.26	6-Dodecyne
16.380	0.24	6-Tridecene, (E)-
16.496	2.19	1-Tridecene
16.794	1.94	Tridecane
18.398	0.16	Cyclopentane, hexyl-
19.869	0.17	7-Tetradecene, (E)-
20.033	3.20	1-Tetradecene
20.305	1.73	Tetradecano
23.377	6.25	1-Pentadecene
23.627	3.76	Pentadecane
23.774	0.48	7-Hexadecene, (Z)-
24.514	0.29	Cyclohexene, 3-(2-methylpropyl)-
25.255	0.81	Cyclohexane, (4-methylpentyl)-
25.933	0.60	Cyclododecene, (Z)-
26.292	0.47	9-Octadecene, (E)-
26.519	1.99	3-Octadecene, (E)

26.749	1.30	Hexadecane
28.849	1.83	1,13-Tetradecadiene
29.232	2.13	8-Heptadecene, (E)-
29.504	3.49	1-Heptadecene
29.712	2.20	Heptadecane
29.845	0.35	5-Octadecene, (E)-
30.195	0.20	9-Octadecene, (E)-
31.674	0.55	1,13-Tetradecadiene
32.325	0.36	9-Eicosene
32.517	0.54	Octadecane
44.745	0.19	Nonadecane
46.891	0.34	Eicosane
48.956	0.40	Heneicosane
50.942	0.49	Docosane
52.856	0.42	Pentacosane
54.703	0.31	2-methyloctacosane
56.488	0.22	Triacontane
	9.45	**Aliphatic carboxylic acids**
35.325	0.27	9-Hexadecenoic acid, (Z)- TMS
36.749	1.24	Pentadecanoic acid TMS
38.962	0.76	Hexadecanoic acid TMS
41.125	4.51	Oleic Acid
41.264	2.25	9,12-Octadecadienoic acid (Z,Z)-
41.625	0.42	Octadecanoic acid
	21.21	**Aliphatic esters**
35.870	3.52	Hexadecanoic acid, methyl ester
39.696	2.71	Z-(13,14-Epoxy)tetradec-11-en-1-ol acetate
40.070	2.83	9,12-Octadecadienoic acid, methyl ester
40.220	5.36	9-Octadecenoic acid, methyl ester, (E)-
40.407	1.03	11,14-Eicosadienoic acid, methyl ester (E)
40.824	0.61	Methyl stearate
	5.31	**Alcohols**
24.123	0.22	n-Tridecan-1-ol
29.044	4.29	9-Octadecen-1-ol, (Z)-
30.595	0.35	1-Dodecanol, 3,7,11-trimethyl-
35.016	0.45	1-Heneicosanol
	7.77	**Others**
28.691	0.17	Oleyl alcohol, trifluoroacetate
31.851	0.61	cis-9-Hexadecenal
32.971	0.32	Heptadecanal
34.535	0.24	Oxirane, hexadecyl-
35.248	3.13	2-Heptadecanone
37.675	0.98	9-Octadecenal

40.308	1.97	2-Nonadecanona
54.347	0.35	13-Docosenamide, (Z)-

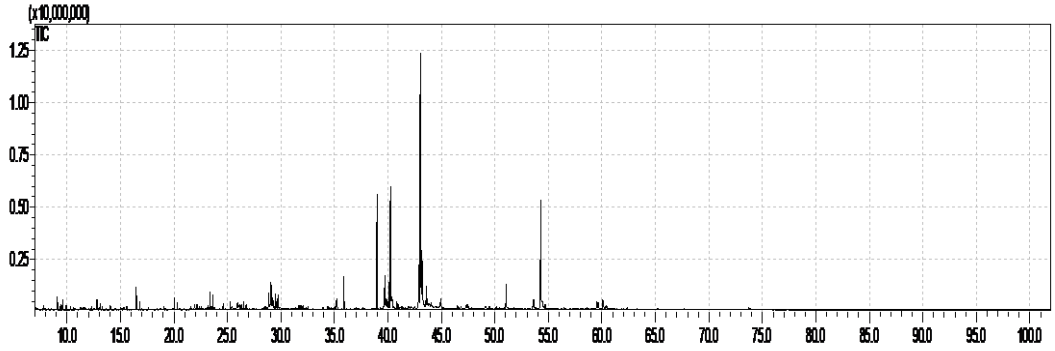


Figure A 15.GC-MS Chromatogram canola bio-oil.

Table A. 8. Chemical constituents identified in bio-oil Canola. Relative percentage as determined by GC-MS after derivatization by silylation.

Rt (min)	Relative percentage (%)	Compounds
	-	**Aromatic compounds**
		-
	15.32	**Aliphatic hydrocarbons**
9.097	0.93	1-Undecene
9.402	0.35	Undecane
9.593	0.70	2-Undecene, (E)-
9.917	0.32	2-Undecene, (Z)-
12.799	0.85	3-Tetradecene, (Z)-
13.114	0.35	Dodecane
16.796	0.60	Tridecane
20.030	0.83	1-Tridecene
20.307	0.42	Tetradecane
23.370	1.18	1-Pentadecene
23.623	1.04	Pentadecane
25.255	0.60	Cyclohexane, (4-methylpentyl)-
26.519	0.50	3-Hexadecene
26.750	0.33	Hexadecane
28.853	1.26	1,13-Tetradecadiene
29.058	2.43	8-heptadecene
29.237	0.64	9-Octadecene, (E)-
29.503	1.04	3-Eicosene, (E)-
29.714	0.95	Triacontane, 11,20-didecyl-
	47.73	**Aliphatic carboxylic acids**
38.979	9.15	Hexadecanoic acid TMS
42.872	3.40	9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester

43.030	28.03	trans-9-Octadecenoic acid, trimethylsilyl ester
43.169	4.99	Oleic acid, trimethylsilyl ester
43.580	1.52	Octadecanoic acid, trimethylsilyl ester
44.926	0.64	9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester
	26.51	**Aliphatic esters**
35.872	2.47	Hexadecanoic acid, methyl ester
40.073	1.98	9,12-Octadecadienoic acid, methyl ester
40.234	9.93	9-Octadecenoic acid, methyl ester, (E)-
40.827	0.42	Methyl stearate
51.047	1.81	Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester
54.274	9.90	1-Monooleoylglycerol trimethylsilyl ether
	2.00	**Alcohols**
16.457	2.00	Glycerol 3TMS
	1.17	**Others**
35.202	1.17	Hexadecanenitrile

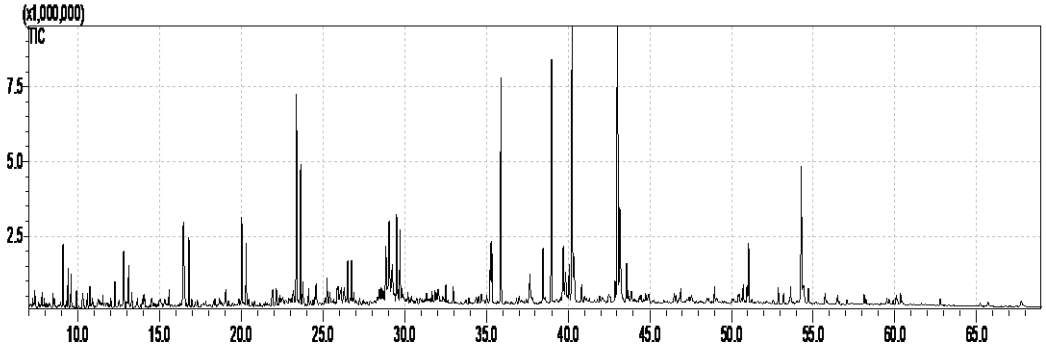


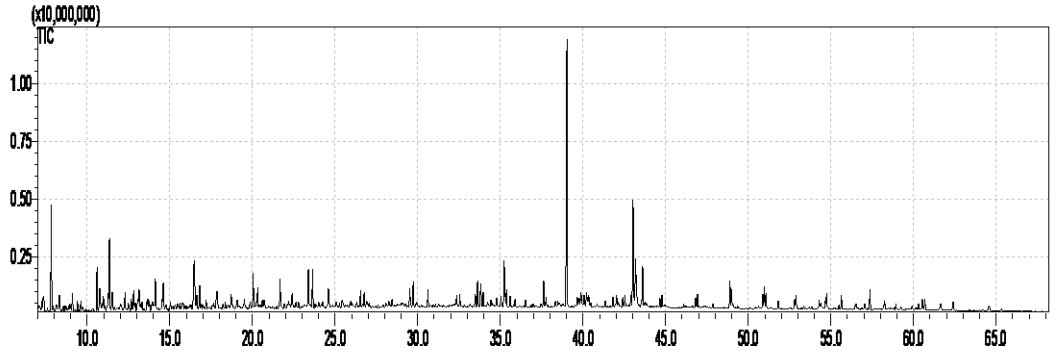
Figure A 16. GC-MS Chromatogram macauba bio-oil.

Table A. 9.Chemical constituents identified in bio-oil Macauba. Relative percentage as determined by GC-MS after derivatization by silylation.

Rt (min)	Relative percentage (%)	Compounds
	0.53	**Aromatic compounds**
7.817	0.23	Phenol TMS
10.588	0.30	*O-cresol* TMS

	33.43	**Aliphatic hydrocarbons**
9.103	1.43	1-Undeceno
9.407	0.88	Undecane
9.595	0.73	2-Undecene, (E)-
9.921	0.37	Cyclopropane, 1-ethyl-2-heptyl-
12.803	1.58	1-Dodecene
13.116	0.84	Tridecane
13.309	0.26	2-Dodecene, (E)-
16.799	1.63	Tridecane
20.036	2.07	1-Tetradecene
20.310	1.42	Tetradecano
21.933	0.20	Cyclopentane, decyl-
22.153	0.36	Cyclopentene, 1-octyl-
23.388	5.37	1-Pentadecene
23.637	3.43	Pentadecane
23.780	0.48	5-Octadecene, (E)-
24.127	0.35	9-Octadecene, (E)-
25.258	0.68	Cyclohexane, undecyl-
26.524	0.97	1-Pentadecene
26.754	0.97	Hexadecane
28.574	0.27	9-Octadecyne
28.859	1.75	1,13-Tetradecadiene
29.245	1.45	8-heptadecene
29.512	2.06	1-Heptadecene
29.720	1.75	2-methyloctacosane
32.524	0.39	Nonadecane
46.900	0.23	Heneicosane
48.966	0.35	Docosane
50.955	0.43	Tetracosane
52.867	0.38	Pentacosane
54.712	0.35	Tetratetracontane
	25.04	**Aliphatic carboxylic acids**
8.489	0.23	Hexanoic acid TMS
38.463	1.31	cis-9-Hexadecenoic acid TMS
38.993	6.74	Hexadecanoic acid TMS
42.874	0.52	9,12-Octadecadienoic acid (Z,Z) TMS
43.033	11.70	trans-9-Octadecenoic acid, trimethylsilyl ester
43.170	3.57	Oleic acid, trimethylsilyl ester
43.587	0.97	Octadecanoic acid, trimethylsilyl ester
	25.58	**Aliphatic esters**
29.853	0.17	Dichloroacetic acid, 4-hexadecyl ester
35.334	1.52	9-Hexadecenoic acid, methyl ester, (Z)-
35.894	6.18	Hexadecanoic acid, methyl ester

39.709	1.18	Z-(13,14-Epoxy)tetradec-11-en-1-ol acetate
40.078	0.89	9,12-Octadecadienoic acid, methyl ester
40.255	9.47	9-Octadecenoic acid, methyl ester, (E)-
40.367	1.80	11-Octadecenoic acid, methyl ester
40.833	0.39	Methyl stearate
50.723	0.41	1-Monooleoylglycerol TMS
54.278	3.57	1-Monooleoylglycerol trimethylsilyl ether
	7.06	**Alcohols**
16.460	2.75	Glycerol 3TMS
29.055	3.53	9-Octadecen-1-ol, (Z)-
25.936	0.78	E-7-Tetradecenol
	2.92	**Others**
32.978	0.38	Heptadecanal
35.258	1.92	2-Heptadecanone
37.664	0.62	9-Octadecenal, (Z)-



***F*igure A 17.**GC-MS Chromatogram *S. obliquus* depigment bio-oil.

Table A. 10. Chemical constituents identified in *S. obliquus* depigment bio-oil. Relative percentage as determined by GC-MS after derivatization by silylation.

Rt (min)	Relative percentage (%)	Compounds
	11.27	**Aromatic compounds**
7.822	4.40	Phenol TMS
10.592	1.75	O-cresol TMS
10.977	0.36	*p-cresol* TMS
11.345	3.06	*m-cresol* TMS

14.119	1.26	2,3-Dimethylphenol TMS
14.520	0.44	2,6-Dimethylphenol TMS
	20.28	**Aliphatic hydrocarbons**
9.103	0.71	1-Dodecene
9.410	0.39	Undecane
12.806	0.96	Cyclopropane, nonyl-
16.630	0.61	Cyclopentane, 3-hexyl-1,1-dimethyl-
16.803	0.85	Tridecane
19.511	0.21	Dodecane, 2,6,10-trimethyl-
20.042	1.44	1-Tetradecene
20.315	0.61	Tetradecane
22.404	0.79	Nonane, 3-methyl-5-propyl-
23.384	1.45	1-Pentadecene
23.636	1.58	Pentadecane
26.534	0.66	1-heptadecene
29.518	0.85	9-Eicosene, (E)-
29.729	1.15	Octadecane
32.541	0.47	Nonadecane
33.811	1.54	2-Hexadecene, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]-
34.770	0.39	3-Eicosyne
37.767	0.41	Nonadecane, 9-methyl
40.214	0.78	Heneicosane
42.538	0.52	Docosane
44.777	0.49	Tetracosane
46.922	0.59	Pentacosane
48.991	0.70	Hexacosane
50.979	0.97	Octacosane, 2-Methyl
52.892	0.56	Nonacosane
54.739	0.60	Dotriacontane
	27.14	**Aliphatic carboxylic acids**
15.606	0.23	Octanoic acid TMS
33.959	0.58	Tetradecanoic acid TMS
35.590	0.44	*n-Pentadecanoic* acid TMS
38.325	0.08	*cis-9-Hexadecenoic* acid TMS
39.029	14.85	Hexadecanoic acid TMS
41.341	0.24	Heptadecanoic acid TMS
43.026	5.35	*trans-9-Octadecenoic* acid TMS
43.184	2.94	11-trans-Octadecenoic acid TMS
43.608	1.75	Octadecanoic acid TMS
51.814	0.31	Docosanoic acid TMS
62.394	0.37	Tetracosanoic acid, trimethylsilyl ester
	4.65	**Aliphatic esters**
33.619	1.15	Phytol, acetate
35.889	0.31	Hexadecanoic acid, methyl ester

37.622	1.23	Hexadecanoic acid, ethyl ester
39.655	0.30	Octadecanoic acid, 2-propenyl ester
41.814	0.42	Ethyl Oleate
42.406	0.37	Dichloroacetic acid, heptadecyl ester
51.076	0.64	Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester
54.289	0.23	1-Monooleoylglycerol trimethylsilyl ether
	8.97	**Alcohols**
16.465	3.00	Glycerol 3TMS
30.616	0.70	1-Dodecanol, 3,7,11-trimethyl-
32.350	0.37	n-pentadecanol
35.042	0.34	n-Hexadecanol
40.066	0.59	n-Nonadecanol
44.654	0.35	n-Heneicosanol
46.813	0.40	n-Docosanol
48.891	1.15	n-Tetracosanol
50.886	0.66	n-Heptacosanol
52.810	0.40	1-Octacosanol
55.637	0.59	22-Methyltetracosanol TMS
60.689	0.42	Silane, trimethyl(octacosyloxy)-
	4.57	**Others**
35.231	3.15	Heptadecanenitrile
35.371	0.82	Cyclohexane, 2-chloro-4-methyl-1-(1-methylethyl)-, [1S-(1.alpha.,2.beta.,4.beta.)]-
42.034	0.38	Hexadecanamide
58.252	0.22	Hexadecane, 1-iodo-

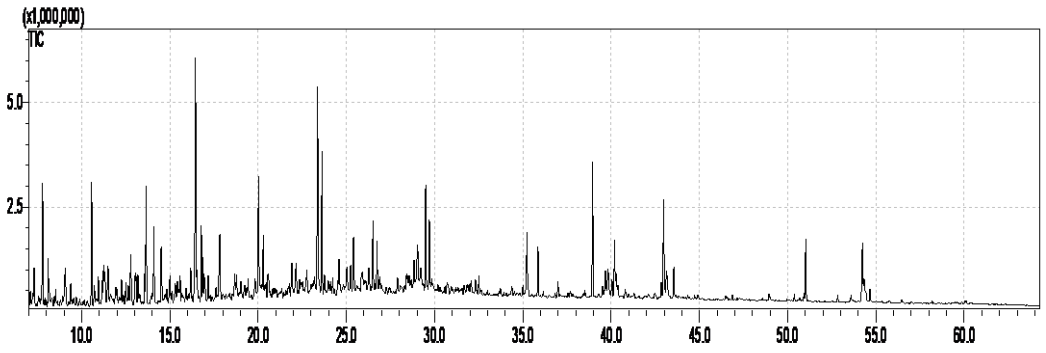


Figure A 18. GC-MS Chromatogram of analytic glycerol bio-oil.

Table A. 11.Chemical constituents identified in analytic glycerol bio-oil. Relative percentage as determined by GC-MS after derivatization by silylation.

Rt (min)	Relative percentage (%)	Compounds
	9.41	**Aromatic compounds**
7.789	3.17	Phenol TMS
10.567	2.78	*O*-cresol TMS
10.950	0.57	*p*-Cresol TMS
13.570	0.72	2,6-Dimethylphenol TMS
14.094	1.66	2,3-Dimethylphenol TMS
17.614	0.18	Pyrocatechol 2 TMS
36.985	0.33	Diamyl phthalate
	25.57	**Aliphatic hydrocarbons**
9.076	1.07	1-Undecene
12.780	1.73	3-Tetradecene, (Z)-
13.095	0.46	Dodecane
16.782	1.84	Tridecane
20.021	2.79	1-Tetradecene
20.293	1.11	Tetradecane
23.367	5.14	1-Pentadecene
23.616	3.32	Pentadecane
23.759	0.36	5-Octadecene, (E)-
24.110	0.19	9-Octadecene, (E)-
25.241	0.61	Cyclohexane, undecyl-
26.507	1.52	9-Eicosene, E
26.738	1.11	Hexadecane
28.832	0.63	1,13-Tetradecadiene
29.491	2.46	1-Heptadecene
29.699	1.58	Hexacosane
32.313	0.24	1-Heptadecene
32.504	0.41	Nonadecane
	8.93	**Aliphatic carboxylic acids**
8.464	0.17	Hexanoic acid TMS
38.951	3.36	Hexadecanoic acid TMS
42.839	0.62	9,12-Octadecadienoic acid (Z,Z)-TMS
42.970	2.87	trans-9-Octadecenoic acid TMS
43.130	1.17	Cis-9-Octadecenoic acid TMS
43.554	0.74	Octadecanoic acid TMS
	5.89	**Aliphatic esters**
35.850	1.21	Hexadecanoic acid, methyl ester
40.043	0.40	9,12-Octadecadienoic acid, methyl ester
40.195	1.56	9-Octadecenoic acid, methyl ester, (E)-

51.023	1.50	Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester
54.235	1.22	1-Monooleoylglycerol trimethylsilyl ether
	9.14	**Alcohols**
16.447	7.57	Glycerol 3TMS
29.027	1.10	9-Octadecen-1-ol, (Z)-
29.219	0.28	1-Tetradecanol
35.000	0.19	1-Heneicosanol
	3.87	**Others**
7.309	0.68	2-Cyclopenten-1-one, 2,3-dimethyl-
35.231	1.57	2-Heptadecanone
39.677	0.51	Ethanone, 1-cyclododecyl-
40.295	0.72	2-Pentadecanone
54.327	0.39	13-Docosenamide, (Z)-