Photosynthetic Pigments from Marine Algae: Isolation, Characterization, Biological Properties, and Life Cycle Assessment

Shorog Mohammed AlOtaiby

Submitted in accordance with the requirements for the degree of

Doctor of Philosophy

The University of Leeds

School of Chemistry

April, 2024

The candidate confirms that the work submitted is his/her own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others. Details of the jointly authored publications and the contributions of each author are outlined on the next page.

Details of jointly authored publications and the contributions of the candidate and the other authors to the work are included below:

Chapter 4:

Alotaiby, S., Zhao, X., Boesch, C., & Sergeeva, N. N. (2023). Sustainable approach towards isolation of photosynthetic pigments from Spirulina and the assessment of their prooxidant and antioxidant properties. *Food Chemistry*, 137653.

Details of authorship contributions

Shorog Mohammed Alotaiby: designed the question for literature search, conducted the experiments, collected and analyzed data, drafted and edited the manuscript, and replied to the comments from reviewers.

Xiao Zhao: contributed by conducting cell culture and cytotoxicity assays, Intracellular reactive oxygen species (ROS) assay of isolated pigments, proofreading and editing manuscript.

Natalia Sergeeva and Christine Boesch: provided supervision and feedback on the experiment methodologies and contributed to the proofreading and editing of the manuscript.

List of accepted conference abstracts

Shorog Mohammed Alotaiby, Natalia Sergeeva and Christine Bösch (2021). Sustainable method for extraction, isolation, and purification of bioactive pigments from Spirulina. Plant Based Foods Congress – Leeds, UK.

Shorog Mohammed Alotaiby, Natalia Sergeeva and Christine Bösch (2023). “Extraction, isolation, purification, and characterization of bioactive pigments from spirulina and evaluation of their pro and antioxidant activity, 23rd World Congress on Nutrition and Food Chemistry - London, UK.

Shorog Mohammed Alotaiby, Natalia Sergeeva and Christine Bösch (2024). “Extraction, isolation, purification, and characterization of bioactive pigments from spirulina and evaluation of their pro and antioxidant activity, Saudis Innovation – Coventry, UK.

Acknowledgements

I would like to extend my deepest appreciation to my primary supervisor, Dr. Natalia Sergeeva, for her essential assistance, motivation, guidance, and forbearance over the duration of my doctoral studies. The provision of moral support and ongoing supervision by her was vital in facilitating the effective completion of my PhD.

I am extremely grateful to my co-supervisor, Christine Bösch, for her invaluable support and guidance throughout the duration of my research until the completion of the thesis.

I am highly thankful to “Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia” for granting me the PhD scholarship and financial support during my studies.

I would also like to extend my thanks to the technical staff in the School of Chemistry, Martin Huscroft, Jeanine Williams, Mohammed Asaf, and Dr. Mark Howard, for their great support.

Further, I would like to thank all my colleagues who made my PhD journey wonderful and enjoyable.

Finally, I am very grateful to my father, mother, husband, son, daughters, sisters, and brothers for their unwavering support throughout my studies. It would have been difficult for me to finish my studies without their wonderful understanding and encouragement throughout the last few years.

Thank you.

Dedication

Who made my dreams a reality.

To myself

Who planted the seeds of faith, love, giving and truth in my heart.

To My Father “Mohammad”

Who was the reason for my success, and thanks to her prayers, God made my dreams come true.

To My Mother “Ratha “

Who supported me with his time, money, love, forgiveness, and patience and gave me beautiful moments in difficult days.

To My husband “Awad”

Who shared with me the journey of research and spoke about me with pride in front of his teacher and his friends, and who asks me daily what I accomplished in the PhD project.

To My son “Abdul Malik”

Who encouraged me a lot to reach my goals and who showered me with love, kindness, and mercy and who were drawing their dreams in my footsteps.

To My daughters “Sondes and Hoor”

Abstract

Marine algae's photosynthetic system is distinguished by the existence of phycobiliprotein pigments, along with chlorophyll and carotenoid pigments. Photosynthetic pigments have recently garnered interest for their biological activity. The aim of this study was to optimize the extraction, purification and evaluation of the antioxidant and pro-oxidant activities of photosynthetic pigments. Primarily, photosynthetic pigments were extracted and characterized from four marine algal sources, utilizing environmentally friendly procedures and solvents, followed by comparative data for pigment extracts, including total pigment content and antioxidant activity. Secondly, a novel and sustainable procedure was suggested for extracting and analyzing photosynthetic pigments from spirulina, followed by a new workflow to purify photosynthetic pigments and convert them into more stable pigments without the need for pretreatment using flash chromatography. As the precise effects of individual photosynthetic pigments on antioxidant and prooxidant characteristics have not been completely understood. a comparison of the antioxidant and pro-oxidant properties of isolated extracts and individual pigments was carried out. Finally, the sustainability of the approaches for extracting pigments from marine algae was evaluated using a gate-to-gate life cycle assessment (LCA). The results of the proposed method for purifying chlorophyll pigments showed a high yield of individual chlorophyll derivatives, pheophytin a and pheophorbide a with high purity. The results also showed that all the purified photosynthetic pigments possessed antioxidant and free radical activity, with chlorophyll derivatives showing a much stronger ability compared to other pigments. The LCA results showed that water may be a suitable solvent for extracting the three photosynthetic pigments green, orange, and blue from the same batch of Spirulina through a synergy between mechanical processes such as ultrasound and non-mechanical processes such as freezing and thawing. Furthermore, based on this life cycle analysis of pigment production, electricity had the greatest environmental impact in most categories of extraction procedures. This study could constitute a starting point for expanding the extraction of marine algae pigments and their use as antioxidants in various applications.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Chl *a*	Chlorophyll *a*
Chl *b*	Chlorophyll *b*
Chl *c*	Chlorophyll *c*
PBP	Phycobiliproteins
C-PC	Phycocyanin
R-PC	Phycoerythrin
A-PC	Allophycocyanin
FTE	Freezing - thawing extraction
UAE	Ultrasound-assisted extraction
LNE	liquid nitrogen extraction
SLE	Solid-liquid extraction
MAE	Microwave-assisted extraction
SFE	Supercritical Fluid Extraction
ROS	Reactive oxygen species
TLC	Thin layer chromatography
HPLC	High-performance liquid chromatography
CCC	Counter-current chromatography
NMR	Nuclear magnetic resonance
^{1}H NMR	Proton NMR
^{13}C NMR	Carbon-13 (C13) NMR
UV-Vis	Ultraviolet-visible spectroscopy
FT-IR	Fourier transmission infrared spectroscopy
MS	Accurate mass spectroscopy
PS	Photosensitizer
PDT	Photodynamic therapy
THF	Tetrahydrofuran
DMF	Dimethyl formamide
LUMO	Lowest unoccupied molecular orbital

HOMO	Highest occupied molecular orbital
DPBF	1,3-Diphenylisobenzofuran
DPPH	2,2-Diphenyl-1- picrylhydrazyl
ABTS	2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid
TPA	12-O-tetradecanoylphorbol-13-acetate
FRAP	Ferric reducing antioxidant power
TEAC	Trolox equivalent antioxidant capacity
ORAC	Oxygen radical antioxidant capacity
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
TBHQ	Tert-butylhydroquinone
PG	Propyl gallate
FDC	Food-drug-cosmetic

Chapter 1 Introduction and Thesis Outline

Highlights

- There is an increasing trend in consumer demand for naturally derived pigments.
- Microalgae exhibit a wide range of pigments and demonstrate rapid growth with minimal resource utilization.
- To facilitate commercialization, there is a need to enhance the pigment production process.
- The integration of sustainability has become a key aspect in the advancement of production systems with the objective of conserving energy and material resources for future use.
- This requires insight into methods for extracting, isolating, and purifying algal pigments and evaluating their biological activity. Additionally, evaluate the sustainability of pigment extraction on a lab scale, as described in this thesis.

1.1. General introduction

A colorant is a chemical that adds color to matter. The most common types of colorants are dyes and pigments. The primary distinction between the two is that dyes are normally soluble, but pigments are insoluble and must be suspended in a medium or binder. This is because dyes and pigments have different particle sizes, which impact how they function. Dyes are commonly used in textiles, paper, leather, and wood [1]. Natural pigments or synthetic dyes that have been authorized as safe for human consumption are frequently used to color cosmetics, food, and pharmaceuticals. Pigments, on the other hand, are commonly used to color rubber, plastic, and resin items [1].

Color requirements for cosmetics, food, and pharmaceutical additives are changing as the world's population expands. Although the synthesis, fabrication, and usage of artificial colors have expanded in response to human preferences over the last decade, a widespread use of synthetic colorants has been linked to several negative consequences [2]. Carcinogenic mutations and immunosuppression are among the most serious side effects [3]. This has prompted an increased interest in natural products as sources of metabolites with significant biological activity. Among natural products, pigments are the most common metabolites in nature. Because of the structural diversity and broad biological activities, natural pigments are extensively used in food, pharmaceutical, and cosmetic industries; their inclusion can also improve and contribute to overall quality, shelf-life (act as antioxidant reagents) and improved stability, product assuranceand visual appeal [4].

Natural pigments are classified into four categories based on their origin: (1) plant-derived pigments; (2) marine-derived pigments (including algae and cyanobacteria); (3) animal-derived pigments (including insect and invertebrate pigments); and (4) pigments derived from microorganisms (including fungal and bacterial) [1]. Natural pigments cover a wide range of colors such as green, orange, yellow, brown, purple, red, and blue [5]. In the search for natural pigments, plants were commonly used to produce pigments, which can be obtained from any part of the plant, such as leaves, fruits, twigs, seeds, flowers, bark root, etc. [6]. The two biggest disadvantages of plant pigment

production are linked to the need for wide arable cultivation land and the reduction of CO_2 emissions [7]. Presently, algae and cyanobacteria are gaining attention for their distinctive pigment content. The great thing about algae and cyanobacteria is that they grow 10 times more rapidly than terrestrial plants, and less than a tenth of the land is needed to produce an equivalent amount of biomass. It grows on non-productive and non-arable land, so it doesn't compete with other crops for land. Because it does not require fresh water, and it can be fertilized more efficiently than land crops. Additionally, the intensive water usage, wasteful fertilizer runoff, and downstream eutrophication associated with modern agriculture can be minimized [7]. Also, algae and cyanobacteria can be found in both seawater and freshwater, and they have more efficient photosynthetic mechanisms than land plants that produce high biomass yields. This may be due to the reduced complexity of the cell structure and, in the meantime, the consumption of carbon dioxide and nutrients happens easier as they grow in aquatic environments [8].

Algae pigments are classified chemically as chlorophylls, carotenoids, phycobilins, and flavonoids [4]. In natural sources, each type performs a vital role, for example, chlorophylls (green) and carotenoids (yellow and orange) are principally responsible for photosynthetic activity in green and brown algae. In addition to chlorophyll and carotenoids, phycobilin (red and blue) plays a function in photosynthetic processes in red algae and cyanobacteria [9]. These three pigments (chlorophylls, carotenoids, and phycobilins) are called photosynthetic pigments. While flavonoids are involved in protective processes [10], apart from other natural pigments, there is a lack of studies directed towards the biological features and mode of action of the individual photosynthetic pigments found in edible algae. This provides a strong motivation for further investigation of these compounds.

1.2. Rationale and overall research aim

Natural pigments serve the dual purpose of providing color and presenting possible health advantages as intriguing bioactive chemicals. Photosynthetic pigments are a class of bioactive compounds that occur naturally in various sources. These pigments possess the ability to serve as colorants and are

utilized in the production of dietary supplements, cosmetics, and pharmaceuticals. Numerous studies have been conducted to analyse the composition of photosynthetic pigments in various natural sources. Nevertheless, the existing body of research on the biological properties of isolated photosynthetic pigments derived from algae remains limited. This is likely because of a restricted access to the individual pigments as they are known for their sensitivity to light, heat, and pH, as well as their limited solubility in water [11].

Therefore, the first aim of this thesis was to extract, characterize and purify the main photosynthetic pigments from different edible algae sources such as: 1) green algae; 2) red algae; 3) brown algae, and 4) blue-green algae. Second is to investigate their individual antioxidant and prooxidant activities using chemical and biological models. Finally, the project will use the life cycle assessment to evaluate the sustainability of the developed methodologies to produce pigments from algae.

1.3. Problem and Challenge Statement

Despite the inherent benefits associated with natural pigments and the extensive research conducted on the successful extraction of pigments from their natural sources, there are certain drawbacks involving the natural source itself. Furthermore, the acquisition of a satisfactory quantity and quality of biological pigments to analyse the biological activity is limited. In addition, the understanding of the structure-activity relationship through the generation of structural analogues is a considerable challenge, as the synthetic methodologies are intricate for many natural pigments. These drawbacks prompted industrial companies and researchers to reduce programs for the isolation, purification, and application of pigments [11].

1.4. Research goals

- A methodology to use water as a sole solvent to recover all types of the photosynthetic pigments albeit significant variations in their stability and solubility.

- A methodology that prioritizes both purity and high yields allows for subsequent biological experiments.

- The antioxidant properties of the algae have been the subject of numerous studies. However, the reported studies were conducted using the unpurified extracts as the primary research material rather than individual components. To investigate and evaluate the real contribution of individual pure pigments on the antioxidant properties of these extract as this question remains unclear.

- Evaluation of the antioxidant and prooxidant properties of the photosynthetic pigments as these characteristics can change in response to light.
- In the context of employing green solvents and technologies, is it necessary to conduct a life cycle assessment of pigment production to determine whether the developed extraction process is indeed sustainable.

1.5. Thesis Objectives

- To design and optimize extraction conditions for photosynthetic pigments and analysis of extracts from selected algal sources
- To purify of photosynthetic pigments using flash chromatography and characterization of isolated pigments
- To assess antioxidant and radical scavenging activities of extracts and pure isolated pigments
- To assess the prooxidant properties of pure, isolated pigments
- To carry out the life cycle assessment of photosynthesis pigments extracted from selected algal sources

1.6. Proposed Solution

The present thesis proposes that improving the procedure of extracting and separating photosynthetic pigments from algae is a viable and efficient approach to obtain pure pigments in a sustainable manner. **Figure 1.1** provides an overview of the challenges and possible solutions during the process of extracting and separating photosynthetic pigments from the algae. The process steps are represented in the purple boxes, while the associated significant restrictions are outlined in the orange boxes. The green boxes provide the advancements being made to overcome these limitations to isolate individual pigments.

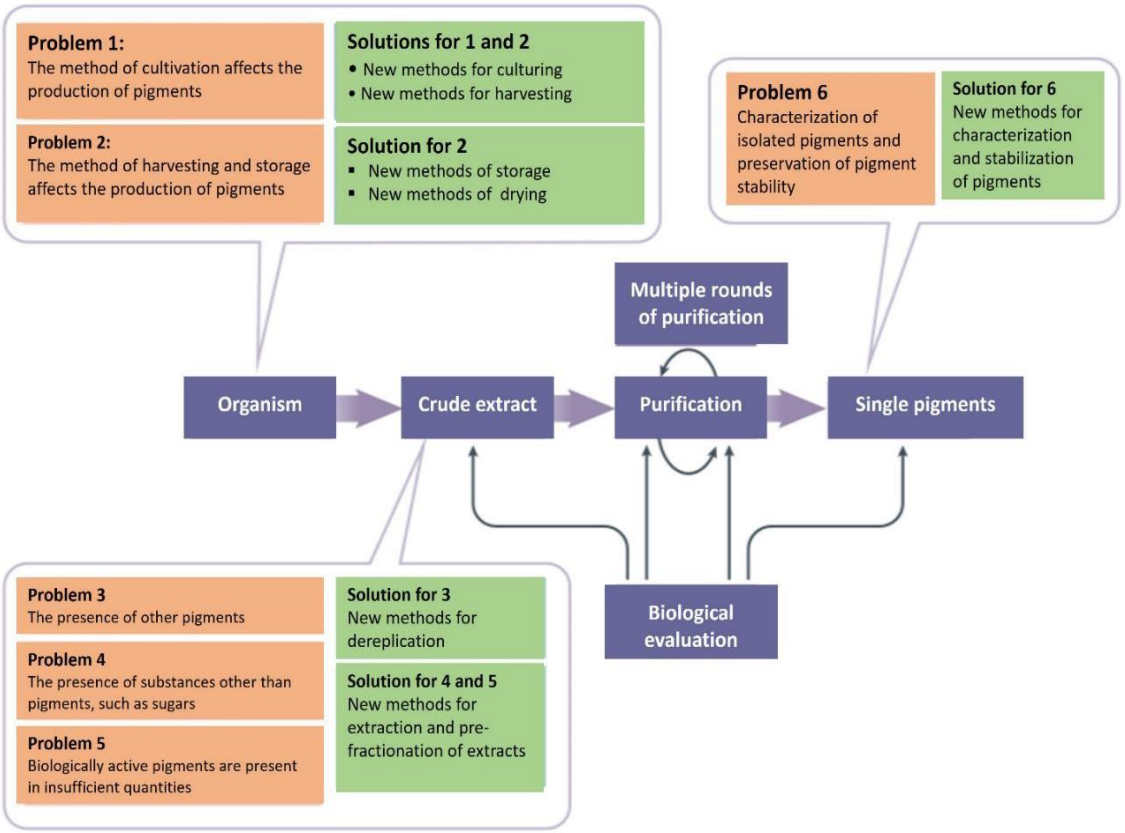


Figure 1.1 An outline of the challenges and potential solutions for the extraction of photosynthetic pigments from algae.

The initial step involves the extraction of photosynthetic pigments from algae, where the selection of the extraction technique is a determining factor. Once

a crude extract with a significant biological activity is identified, the next stage is the purification process, which typically entails numerous iterations focused on the isolation of the individual pigments that may possess biological activity [12]. The production of photosynthetic pigments in algae is primary influenced by the factors such as cultivation methods and geographic location. Additionally, the processes of harvesting and drying algae also have an impact on the content of photosynthetic pigments. These constrains are being addressed through developing new methods of cultivation, harvesting, and drying [13].

Typical limitations to accurately evaluate the bioactivity of the crude extracts is the complex matrix of natural compounds that are not pigments and along with a too low concentration of photosynthetic pigments. The development of more efficient extraction and pre-fractionation methods for extracts can address these issues [14]. Finally, when individually bioactive photosynthetic pigments are identified, significant time and effort are usually required to determine their biological activity. The creation of techniques for quick in vitro and in vivo screening can address this issue [15].

1.7. Thesis outline

This thesis focuses on the methods for the isolation, characterization, and purification of photosynthetic pigments from marine algae. This work evaluates the biological properties of the isolated pigments in terms of antioxidant and pro-oxidant properties. Finally, this thesis evaluates the sustainability of the dye production process. The outline of this thesis is highlighted in **Figure 1.2**.

Chapter 2 provides an overview of basic biological information, including the distribution of pigments across the most common microalgae groups, the roles of pigments in algae, and their biosynthetic pathways. This chapter also presents the biological activities and potential applications of pigments derived from marine algae. Additionally, it explains the limitations of producing dyes from marine algae and the rationale for choosing different analytical techniques described in the subsequent chapters. **Chapter 3** focuses on the content of non-protein and protein-based pigments in four types of marine algae: red, brown, green, and blue-green, using different extraction methods and

different solvents. This chapter also evaluates the relative efficiency of the pigment extraction, identifies, and estimates the pigments in the extracts by UV-vis spectrophotometry and HPLC-DAD methods. This chapter also evaluates and compares the antioxidant and prooxidant activities of algae extraction. **Chapter 4** describes the development of the methodology to isolate both non-protein and protein-based pigments from the same batch of Spirulina using water as a sole solvent assissted by ultrasonication. It also reports the methodology based on flash chromatography to purify chlorophyll and convert it directly into its stable products. This chapter also evaluates and compares the antioxidant and prooxidant activities of purified pigments from Spirulina. **Chapter 5** provides the life cycle assessment for the pigment production and compares the extraction efficiency and sustainability of the developed extraction methods. **Chapter 6** outlines a general summary and includes the conclusion, recommendations, and future work.

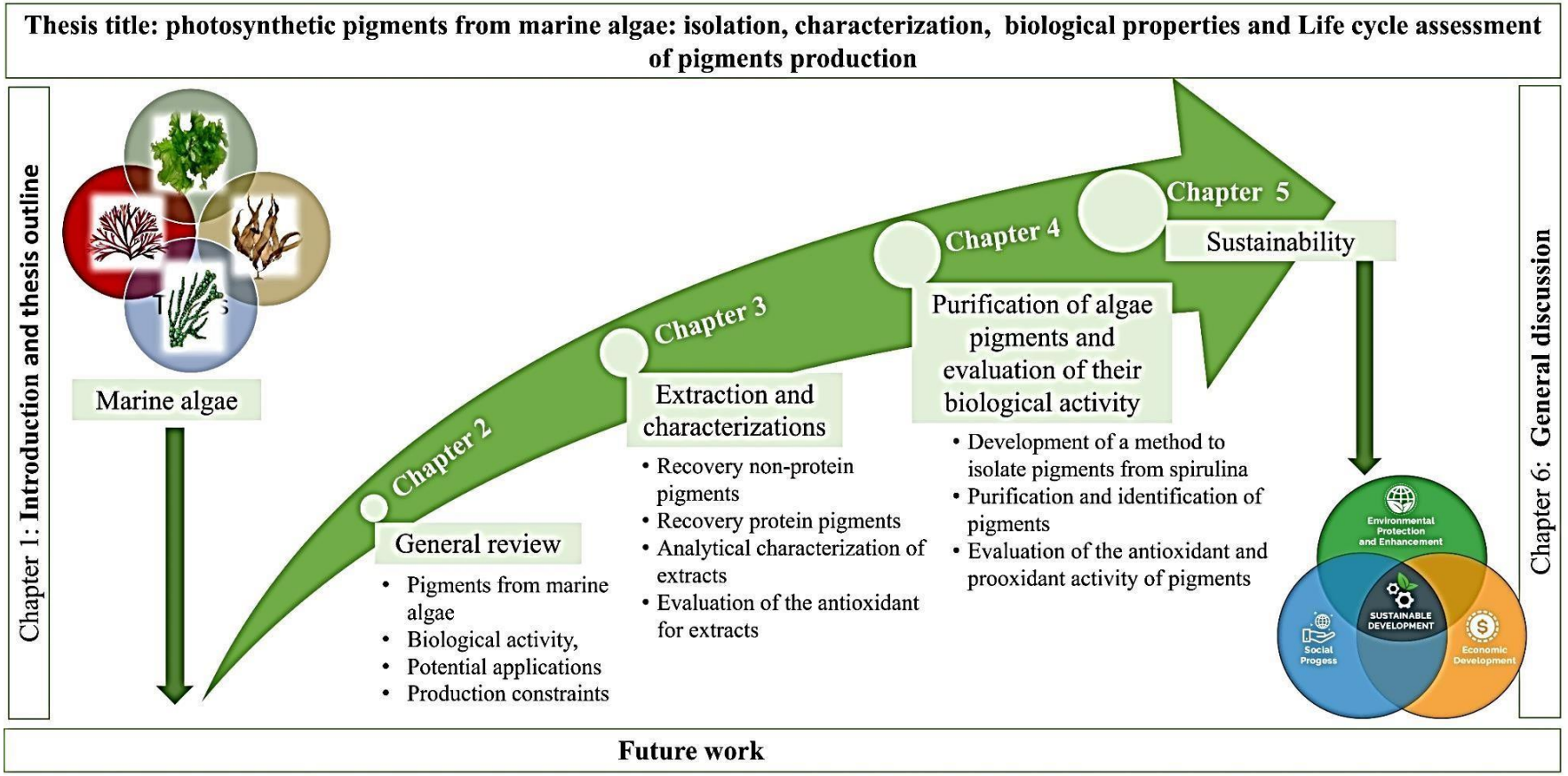


Figure 1.2 Schematic overview of this thesis.

1.8. Reference

1. Pasdaran, A., et al., *A review of the chemistry and biological activities of natural colorants, dyes, and pigments: challenges, and opportunities for food, cosmetics, and pharmaceutical application.* Chemistry & Biodiversity, 2023. **20**(8): p. e202300561.

2. Reza, M., et al., *Study of a common azo food dye in mice model: Toxicity reports and its relation to carcinogenicity. Food Sci. Nutr. 7 (2), 667–677*. 2019.

3. Al-Shabib, N.A., et al., *Synthetic food additive dye "Tartrazine" triggers amorphous aggregation in cationic myoglobin.* International Journal of Biological Macromolecules, 2017. **98**: p. 277-286.

4. Atanasov, A.G., et al., *Natural products in drug discovery: Advances and opportunities.* Nature reviews Drug discovery, 2021. **20**(3): p. 200-216.

5. Laleh, G., et al., *The effect of light, temperature, pH and species on stability of anthocyanin pigments in four Berberis species.* Pakistan Journal of Nutrition, 2006. **5**(1): p. 90-92.

6. GOGOI, M.N. and M. Kalita, *DYEING OF SILK WITH NATURAL DYES.(PART-I).* Colourage, 1999. **46**(1): p. 23-26.

7. Parry, M.L., et al., *Climate change 2007-impacts, adaptation and vulnerability: Working group II contribution to the fourth assessment report of the IPCC*. Vol. 4. 2007: Cambridge University Press.

8. Morocho-Jácome, A.L., et al., *(Bio) Technological aspects of microalgae pigments for cosmetics.* Applied Microbiology and Biotechnology, 2020. **104**(22): p. 9513-9522.

9. Pradhan, B., et al., *Bioactive metabolites from marine algae as potent pharmacophores against oxidative stress-associated human diseases: A comprehensive review.* Molecules, 2020. **26**(1): p. 37.

10. Lotito, S.B. and B. Frei, *The increase in human plasma antioxidant capacity after apple consumption is due to the metabolic effect of*

fructose on urate, not apple-derived antioxidant flavonoids. Free Radic Biol Med, 2004. **37**(2): p. 251-8.

11. Di Salvo, E., et al., *Natural Pigments Production and Their Application in Food, Health and Other Industries.* Nutrients, 2023. **15**(8): p. 1923.

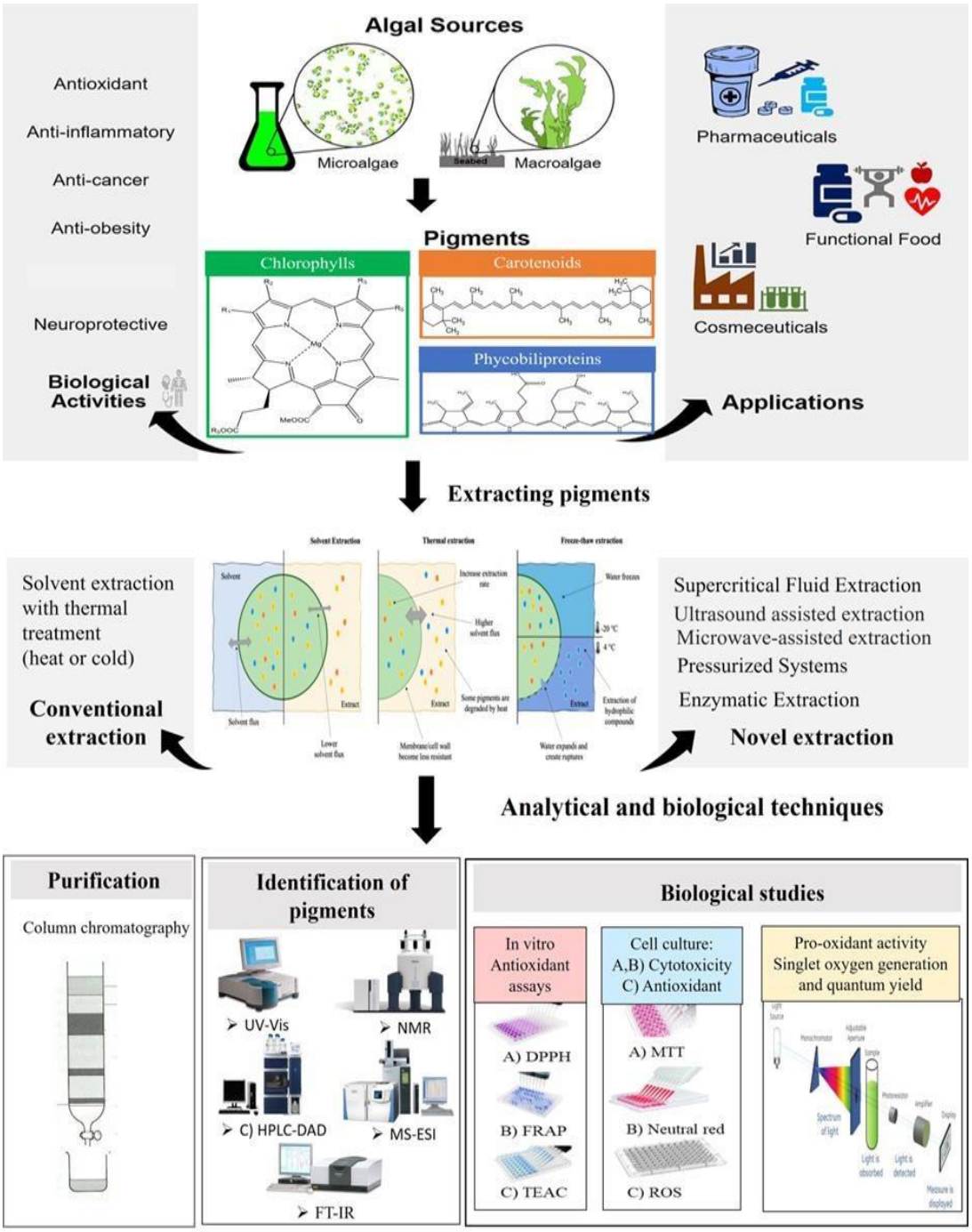
12. Pagels, F., et al., *Extraction of pigments from microalgae and cyanobacteria—A review on current methodologies.* Applied Sciences, 2021. **11**(11): p. 5187.

13. Scoglio, S., *Microcystins in water and in microalgae: Do microcystins as microalgae contaminants warrant the current public alarm?* Toxicology reports, 2018. **5**: p. 785-792.

14. Osório, C., et al., *Pigments content (chlorophylls, fucoxanthin and phycobiliproteins) of different commercial dried algae.* Separations, 2020. **7**(2): p. 33.

15. Pérez-Gálvez, A., I. Viera, and M. Roca, *Carotenoids and chlorophylls as antioxidants.* Antioxidants, 2020. **9**(6): p. 505.

Chapter 2 Photosynthetic Pigments Derived from Marine Algae: Biological Activity, Potential Applications, Extraction Methods, and Analytical Techniques.



Abstract

There is an increasing interest in colorants that are derived from natural sources in the most common applications called the Big Five: energy, technology, industry, medicine, and the environment. Marine algae are an exciting source of pigments, whereby both biotic (such as organisms) and abiotic (such as temperature, pH, salinity, and light intensity) factors affect their production. Marine algae produce a range of pigments, such as chlorophyll (green), carotenoids (red, orange, yellow), and phycobiliproteins (red, blue), which have been reported to exhibit various biological activities, including antioxidant properties. Additionally, these compounds have demonstrated anticancer and antimicrobial effects. Efficient, successful, and environmentally friendly recovery methods for pigment extraction include novel solid-liquid and liquid-liquid extraction techniques combining supercritical, high-pressure, microwave, ultrasonic, and enzymatic approaches. Nevertheless, these extraction techniques often yield low concentrations, making the production of algae-derived pigments economically unattractive. Hence, the improvement of extraction procedures depends on a thorough understanding of the chemical composition, their biosynthesis, and functional role in biological pathways. In this chapter, the distribution of commercially interesting pigments across the most common marine algae groups is highlighted, along with their biological properties and possible applications. This overview also covers the roles of these pigments, their chemical compositions, and the processes of biosynthesis. This overview also discusses the most recent developments in the extraction techniques and methodologies applied in this field.

2.1 Introduction

Algae have been identified as a promising natural producer of bioactive pigments. A wide variety of natural pigments are found in algae; the nature of these pigments and the quantity of them differ between species, which is what gives algae their distinct colors. The pigments found in algae are categorized into chlorophylls, carotenoids, and phycobiliproteins, which are the three primary types isolated from algae up until now [1]. These pigments are proven to exert antioxidant, anti-carcinogenic, anti-inflammatory, anti-obesity, anti-angiogenic, and neuroprotective properties [2, 3].

These colorants have been considered environmentally friendly and safe, thus, current research in this field is focusing on replacing synthetically produced dyes with natural dyes to develop a reliable food, cosmetic, and pharmaceutical industry [3]. Despite the significance of algal pigments and the benefits they offer, the process of producing pigments is fraught with difficulties and obstacles, some of which were discussed in **Chapter 1, Section 6**. Due to the issues and obstacles, current research aims at devising approaches for efficient extraction, separation, and purification of pigments, and relevant analytical and biological methodologies.

Extensive literature exists regarding the pigment composition of specific microalgae that have been cultivated under well-defined conditions. This body of knowledge encompasses the identification and characterization of pigments found in various groups of microalgae [4-6], as well as the investigation of the functional roles played by these pigment groups within the organisms [7]. Additionally, research has been conducted on specific segments of pigment biosynthesis pathways [8] and various techniques for extracting these pigments from microalgae [9-11]. Nevertheless, the available knowledge is fragmented and lacks a comprehensive synthesis that incorporates all pertinent details.

Thus, the aim of this review was to identify the biochemical constraints and opportunities for extracting pigments from marine algae and to give an

overview of all relevant basic chemical, biological, and technical information. An adequate understanding of these opportunities and constrains is important for the design of an economically feasible algae-based pigment extraction process.

2.1.1 Distribution of pigments groups within marine algae classes

In coastal aquatic environments, the interaction between solar radiation with sediments and their microscopic components leads to a deviation of the spectral distribution of light from that of the original input light at the surface. The relative pigment composition in marine algae is shaped by these varying spectral ratios [12]. The major pigments present in marine algae are listed in **Table 2.1**.

In fact, two classes of marine algae have been identified: 1) Eukaryotes (macro), including three families that are divided according to their prominent coloration: brown (Phaeophyta), red (Rhodophyta), and green (Chlorophyta); 2) Prokaryotes (micro), including the genus Cyanophyta, a blue-green algae [13].

The color and categorization of marine algae are mostly determined by the abundance and distribution of their pigment content. *Chlorophyta* also known as green algae, is characterized by a notable presence of chlorophyll *a* and *b*, alongside lesser quantities of β-carotene and xanthophyll. The brown seaweeds, belonging to the *Ochrophyta* and *Phaeophyceae*, exhibit a wide range of hues, including yellow, olive green, and brown. These seaweeds are known for their significant accumulation of xanthophyll and fucoxanthin while containing relatively low levels of other xanthophylls and β-carotene, as well as chlorophyll *a*. *Rhodophyta*, also known as red seaweeds, are characterized by their elevated concentrations of phycoerythrin and phycocyanin while exhibiting relatively low quantities of chlorophyll *a*, β-carotene, and xanthophyll [14, 15]. In contrast, *Cyanophyceae,* also known as cyanobacteria, exhibit elevated concentrations of chlorophyll *a* and phycocyanin, along with varying concentrations of β-carotene and lutein [16].

Table 2.1.Distribution of main pigments within marine algae classes

Pigment group	Pigments	Cyanobacteria	Red algae	Green algae	Brown algae	References
Chlorophylls and derivatives	Chlorophyll *a*	+	+	+	+	[17]
	Chlorophyll *b*	-	-	+	-	[15, 18, 19]
	Chlorophyll *c*	-	-	-	-	
	Chlorophyll *d*	-	+	-	*	
	Pheophorbide *a*	*	*	*	*	
	Pyropheophytin *a*	*	*	*	*	
	Pheophytin *a*	+	*	+	*	
Carotenes	α-carotene	*	+	*	-	[17]
	β-carotene	+	+	+	+	[15, 18, 19]
	γ-carotenes	-	-	+	-	
Xanthophylls	Lutein	+	+	+	+	[17]
	Violaxanthin	-	+	+	+	[15, 18, 19]
	Neoxanthin	-	-	+	-	
	Astaxanthin	-	-	+	-	
	Fucoxanthin	-	-	-	+	
	Diatoxanthin	-	-	-	+	
	Taraxanthin	-	+	-	-	
	Zeaxanthin	+	+	+	+	
	Canthaxanthin	*	-	+	-	
Phycobiliproteins	phycocyanin,	+	+	-	-	[15, 17-19]
	phycoerythrin,	+	+	-	-	
	allophycocyanin,	+	+	-	-	
Example of algae		 *Arthrospira platensis*	 *Palmaria palmata*	 *Ulva lactuca*	 *Laminaria japonica*	(https://www.algae-base.org)

+: present, -: absent, *: Sometimes not detected.

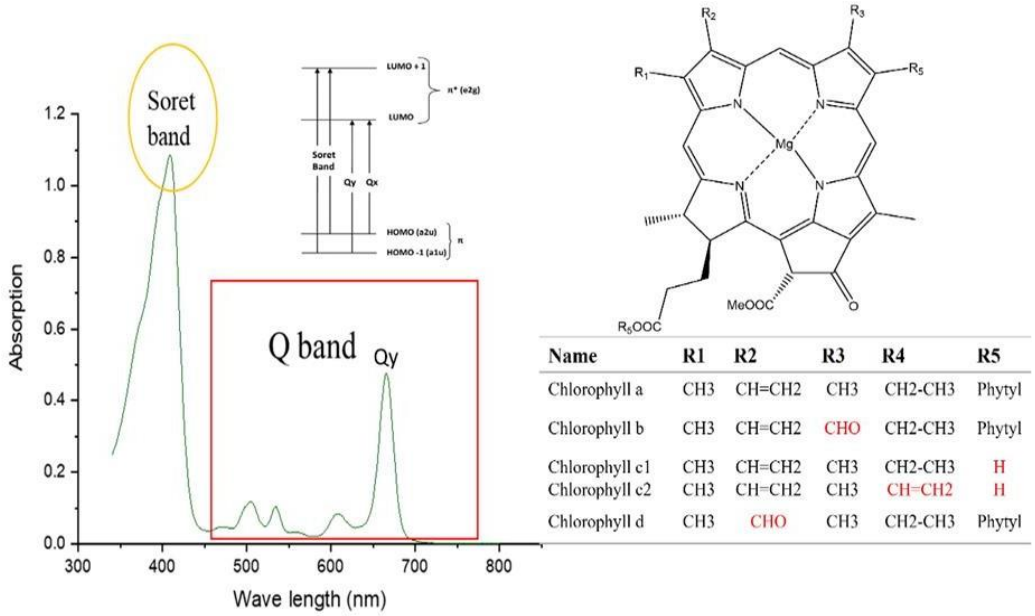
2.1.2 Photosynthetic pigments derived from marine algae

2.1.2.1 Chlorophylls

Chlorophylls are a class of cyclic tetrapyrrolic pigments with shared structural and functional characteristics. These pigments demonstrate their highest light absorption capacity within the blue and red regions of the visible spectrum [20]. These pigments are the primary photosynthetic chromophores present in algae, plants, and cyanobacteria. Chlorophylls are lipid-soluble pigments that exhibit a greenish color. They consist of a porphyrin ring with a centrally located magnesium ion and typically possess a lengthy hydrophobic chain (phytyl) [2]. The molecular structure, which may differ due to one or more side-chain substitutions, affects the light absorption properties of various chlorophylls. Algae have four basic types of chlorophyll molecules, which are denoted as Chl *a*, Chl *b*, Chl *c*, and Chl *d* [21]. Their presence depends on the type of algae [2, 20].

Chlorophyll *a* (Chl *a*), which has a blue-green color, is abundant in the marine environment and is always found in all algae, which is responsible for the process of photosynthesis [18]. It efficiently visible light exhibiting two absorption bands: Soret at 430 nm and Qy at 660 nm (measured in acetone) [22]. Chlorophyll *c* (Chl *c*) is an accessory pigment of blue-green color working in conjunction with Chl *a*. Its structure features the double bond at ring D instead of the single bond in Chl *a*, and the phytyl chain is missing. Comparing the absorption spectra of Chl *a* and *c*, the latter is dominated by a large Soret band (at 450 nm in acetone) and a small Qy band at 630 nm [23]. There are two types of Chl *c* in marine algae: Chl *c1* and Chl *c2*. Their structures differ only in the configuration of the R4 side chain (in Chl *c1*, it is CH_2CH_3, and in Chl *c2*, it is CH=CH2) [20], as shown in **Figure 2.1**. Chlorophyll *b* and chlorophyll *d* pigments have a yellow-green coloration. They are also accessory pigments, playing a crucial role in the process of photosynthesis by exerting their influence indirectly [5]. Chlorophyll *a*, chlorophyll *b*, and chlorophyll *d* share a fundamental structural framework. They differ only slightly in the configuration

of their respective side chains, R2 and R3. In chlorophyll *a* and chlorophyll *b*, the R2 group is $CH=CH_2$, but in chlorophyll *d*, it is HC=O. Methyl is the R3 group in chl *a* and *d*, whereas chlorophyll *b* has aldehyde group (HC=O) [20], as shown in **Figure 2.1**. These differences result in the variation in their absorption properties, in the case of chlorophyll *b* the maximum absorption occurs at 450 nm and 642 nm (in acetone) [5].



Name	R1	R2	R3	R4	R5
Chlorophyll a	CH3	CH=CH2	CH3	CH2-CH3	Phytyl
Chlorophyll b	CH3	CH=CH2	CHO	CH2-CH3	Phytyl
Chlorophyll c1	CH3	CH=CH2	CH3	CH2-CH3	H
Chlorophyll c2	CH3	CH=CH2	CH3	CH=CH2	H
Chlorophyll d	CH3	CHO	CH3	CH2-CH3	Phytyl

Figure 2.1 Chemical composition of chlorophyll: Chl a, Chl b, Chl d and Chl c, in addition to the classical absorption spectra for all types of chlorophyll.

Chlorophyll *d* has distinctive spectrum characteristics due to the absorption of far-red light, with the Qy band observed at 697 nm and the Soret band observed at 458 nm (in acetone) [24]. The presence of the Soret band in all types of chlorophylls as a condensed band can be attributed to the π-π* electronic transitions of the tetrapyrrolic macrocycle. In contrast, the Q bands usually consist of four bands of varying intensities, with their intensity being around 10 to 20 times lower than that of the Soret band. The occurrence of Q bands can be attributed to weak n-π* transitions [25] (**Figure 2.1**).

2.1.2.2 Chlorophylls derivatives:

Recently, the biomedical applications of chlorophyll derivatives have expanded greatly due to their antibacterial, antioxidant, anti-inflammatory [26], and anti-mutagenic properties. These derivatives are known as decomposition products due to the instability of chlorophylls when exposed to high temperatures, excessive light, acidic or enzymatic conditions [27]. The primary degradation reactions of chlorophyll, which produce more stable derivatives, are outlined in **Figure 2.2**.

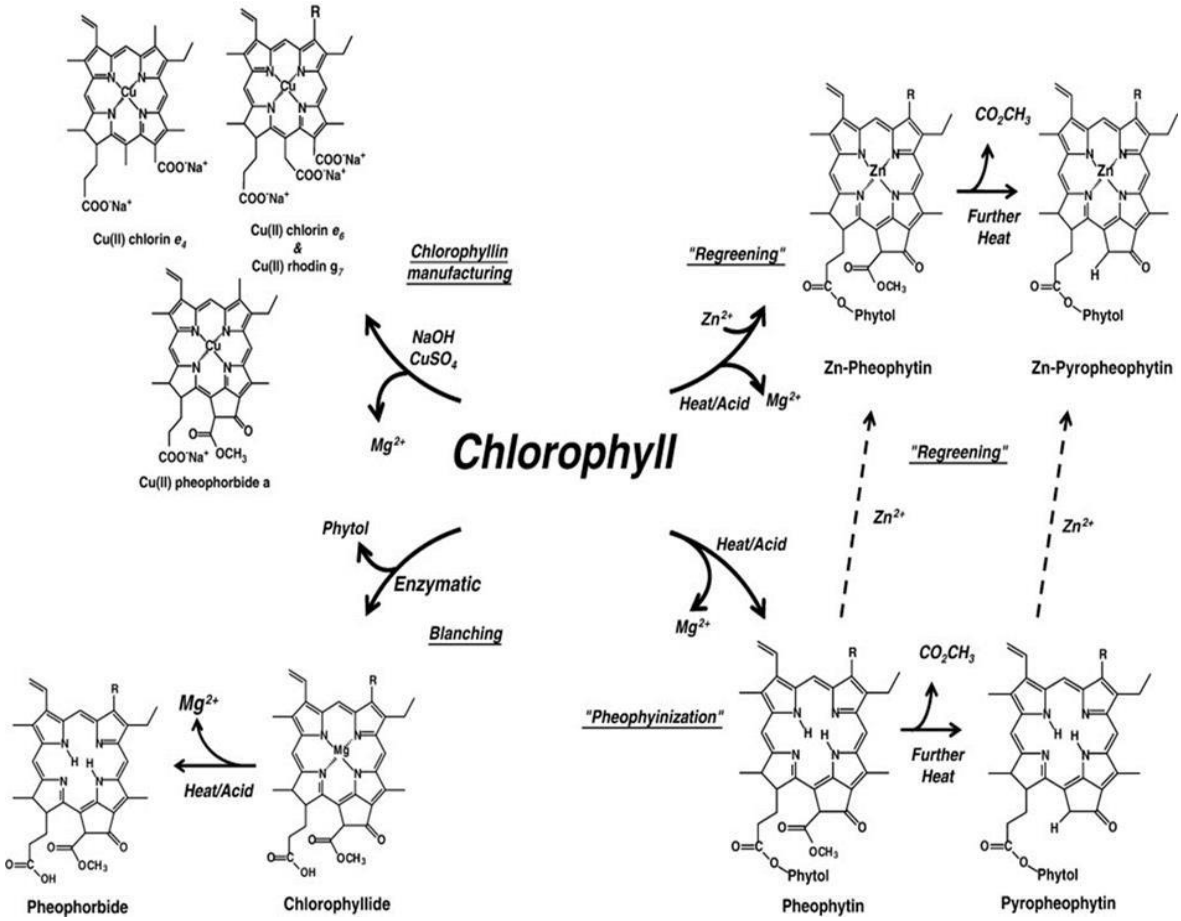


Figure 2.2 The primary degradation pathways of chlorophyll leading to more stable derivatives [27]

When natural chlorophylls undergo heat or acid treatment, they undergo a process known as pheophytinization [27]. Natural chlorophyll is changed into pheophytins and pyropheophytins, which do not contain magnesium (**Figure 2.2**). When the chlorophyllase enzyme is present during the extraction process, it catalyzes the formation of chlorophyllides. These chlorophyllides can then undergo decomposition into pheophorbide pigment through heat treatment or acidification. These derivatives exhibit significant light absorption within the red wavelength range [26].

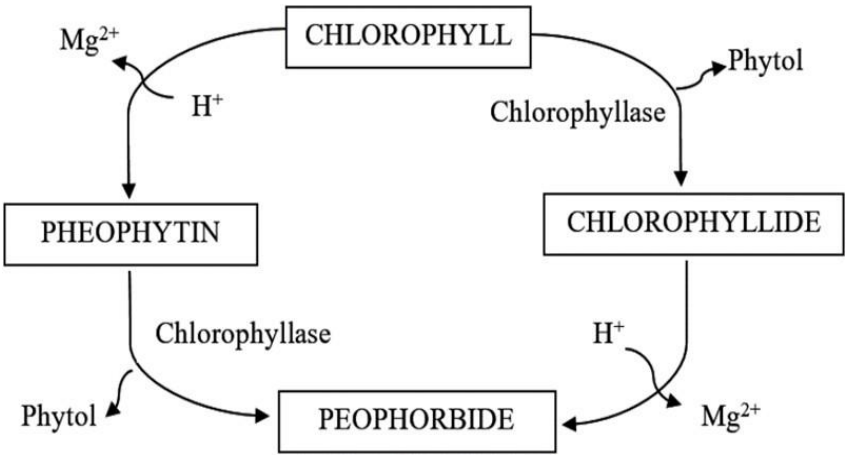


Figure 2.3 Pathways to chlorophyll degradation [27].

2.1.2.3 Carotenoids

Carotenoids are the most common class of pigments observed in natural products [7]. These pigments belong to a class of lipophilic linear polyenes consisting of isoprene units. They usually appear yellow, orange, or red [28]. These pigments play an important role in the process of photosynthesis, as they help protect chlorophyll from photooxidation [29].

Carotenoids can be classified into two distinct categories:

- Carotenes are unsaturated hydrocarbons that include α-carotene, β-carotene, and δ-carotene (**Figure 2.4**) [30].

- Xanthophylls are unsaturated hydrocarbons bound to one or more oxygen-containing functional groups and include lutein, violaxanthin, neoxanthin, astaxanthin, fucoxanthin, diatoxanthin, zeaxanthin, and taraxanthin (**Figure 2.4**) [30].

Spectroscopically, carotenoids and xanthophylls absorb the blue part of the spectrum (400–480 nm), and their spectra can present three bands in the case of carotenoids and xanthophylls containing OH group or one broad peak if a carbonyl group is present (**Figure 2.4)**. Algae usually contain several carotenoids, such as β-carotene, lutein, astaxanthin, zeaxanthin, violaxanthin, canthaxanthin, and fucoxanthin [31, 32].

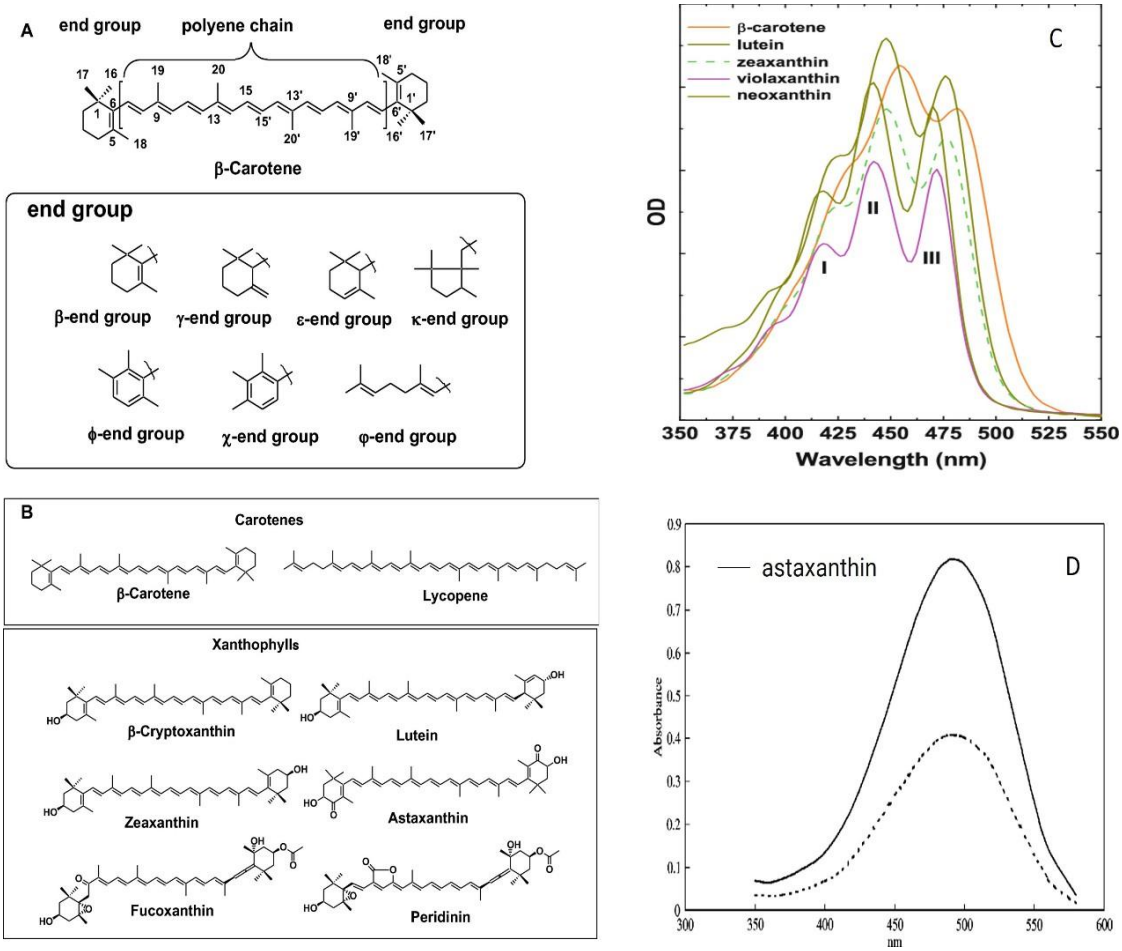


Figure 2.4 A) Basic structure of carotenoids and their end groups. B) Typical structures of carotenes and xanthophylls [30]. C) UV-Vis absorption spectra of carotenes (β-carotene) and the xanthophylls bearing OH-group, such as

lutein, violaxanthin, neoxanthin, zeaxanthin, and taraxanthin. D) UV-Vis absorption spectra of xanthophylls bearing a carboxyl group, such as astaxanthin[33]

2.1.2.4 Phycobiliproteins

Phycobiliproteins (PBP) are a family of water-soluble pigment proteins. These pigments are made up of open tetrapyrrole chains. These chains are linked to certain proteins through a sulfur bond of thioether inside the protein. PBPs are found on the outside of thylakoid membranes in the form of phycobilisome PBPs, which are big groups that help algae and cyanobacteria absorb light and transfer energy to chlorophylls [34] (**Figure 2.5**).

Phycobiliproteins mainly consist of three classes of PBP, which are classified according to the nature of their light absorption into: phycocyanin (PC, $\lambda_{A\,max}$ = 600-620 nm; $\lambda_{F\,max}$: 640-645 nm), phycoerythrin (PE, $\lambda_{A\,max}$ = 540-565 nm; $\lambda_{F\,max}$ = 580-585 nm), and allophycocyanin (APC, $\lambda_{A\,max}$ = 650-653 nm; $\lambda_{F\,max}$ = 655-660 nm) [35] as shown in **Figure 2.5**. PBP is recognized by its bright colors, with PE is pink, PC is dark blue, and APC is light blue [36].

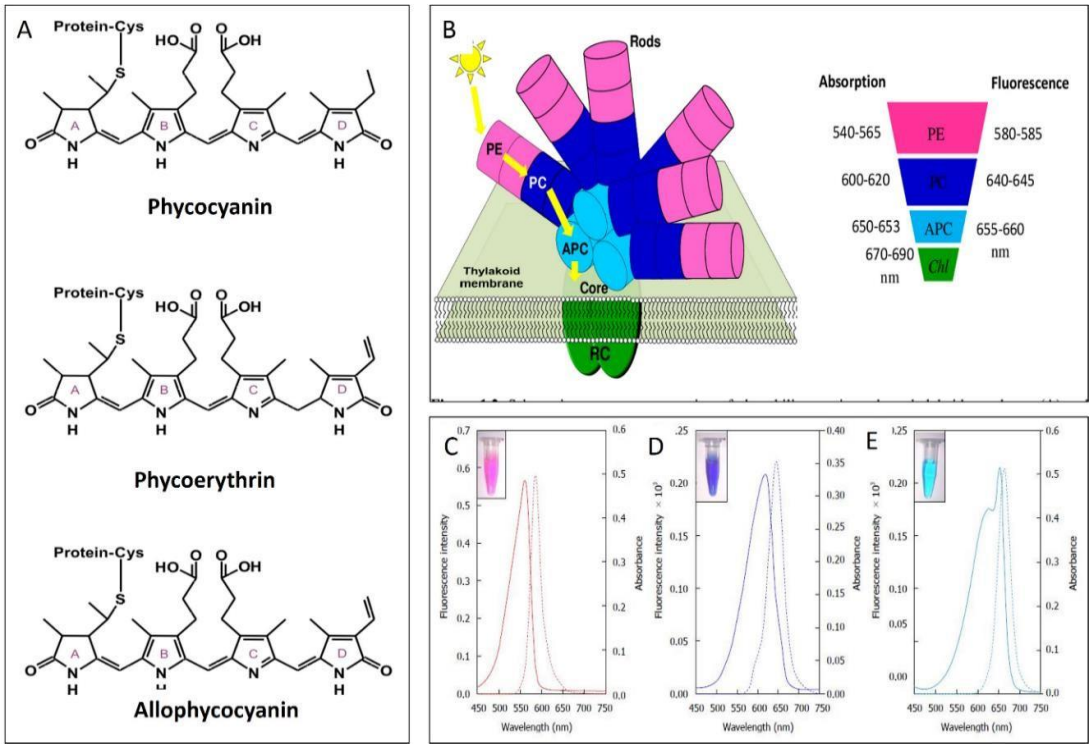


Figure 2.5 The general structure of phycobiliproteins' chromophores [37]. UV-visible absorbance (solid line) and fluorescence (dotted line) spectra, and appearance (inset) of A: Phycoerythrin, B: Phycocyanin, and C: Allophycocyanin [36].

2.1.3 Biosynthesis of pigments in marine algae

Cyanobacteria are a group of prokaryotic organisms that engage in the process of oxygenic photosynthesis. Cyanobacteria exhibit promise as a viable host for sustainable production of terpenoids, such as carotenoids and sterols, which are a broad class of natural products. This potential stems from their capacity to utilize solar energy in the form of photons to convert carbon dioxide into biomass. These terpenoids hold significant potential as advanced biofuels and high-value added chemicals [38]. Additionally, cyanobacteria exhibit a remarkable capacity for synthesizing tetrapyrroles, demonstrating versatility in their ability to create end products that encompass the entire spectrum of the

tetrapyrrole biosynthetic pathway, including hemes, chlorophylls, and phycobilins [39].

In addition to light, microalgae rely on many substances, such as water, carbon dioxide, inorganic nitrogen (ammonia or nitrate), and phosphate, to sustain their photoautotrophic development [8]. **Figure 2.6** provides a schematic representation of the routes involved in the synthesis of pigments from these components. It should be noted that many microalgae groups possess only a subset of these routes (**see Section 2**). The elements nitrogen and carbon are assimilated into the molecular structure of protoporphyrin IX, denoted by the purple-green arrow. This compound is composed of a sizable aromatic ring known as a tetrapyrrole [40]. The ring is susceptible to oxidation processes, leading to the production of biliverdin which later produces phycobilins. The biosynthetic pathway of phycobilins refers to the series of biochemical reactions involved in the synthesis of these pigments [34]. The process of bilin synthesis commences with the enzymatic cleavage of heme b, also known as bilin b, by hemeoxygenase (HO). This enzymatic reaction results in the production of biliverdin IXα, which serves as the pivotal molecule within the many routes of bilin biosynthesis. Biliverdin IXα undergoes further reduction by the action of either NAD(P)H-dependent or ferredoxin-dependent bilin reductases (FDBR). The FDBR reductase, like PebA synthase and PebB synthase, facilitates the transfer of two electrons to its substrate in a two-step catalytic process, resulting in the production of phycoerythrobilin. The enzymes PcyA and PebS facilitate the transfer of four electrons from ferredoxin to two separate double bonds of biliverdin, resulting in the production of phycocyanobilin and phycoerythrobilin, respectively, in a single enzymatic reaction. Phycocyanobilin and phycoerythrobilin undergo additional reduction processes to form phycoviobilin and phycourobilin, respectively, facilitated by the PecE/F enzymes of Rpc G reductase [34], as shown in **Figure 2.7**.

Within an alternative metabolic pathway for oxidation of the tetrapyrrole ring, carbon is directed towards geranyl geranyl-PP (shown by the green-orange arrow), which can subsequently undergo reduction to yield phytyl-PP. During

the process of phosphate release, phytyl-PP becomes linked to a derivative of protoporphyrin IX, which forms a coordination complex with a magnesium ion. Consequently, the chlorophylls are formed (shown by the green arrows) [8]. As indicated in **Section 3.1**, chlorophyll *c* does not possess the phytyl tail. The attachment and subsequent release of the tail remain ambiguous in the given context [40].

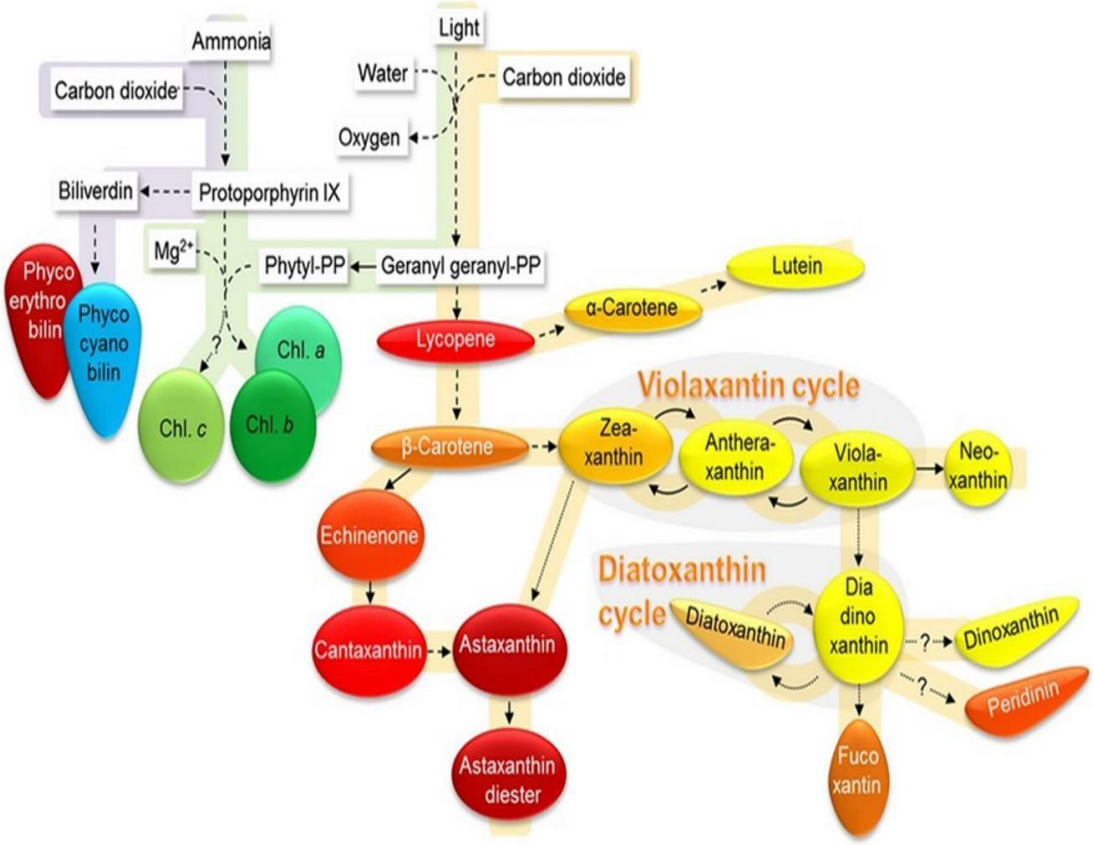


Figure 2.6 A simplified depiction of the pigment biosynthesis pathways in cyanobacteria and microalgae [8]. The solid arrows depict individual conversion stages, dashed arrows represent lumped reactions [41], and dotted arrows indicate hypothesized pathways [42]. The arrows with a question mark symbolize pathways that have yet to be addressed. The classification of pigment colors is delineated according to the descriptions provided in [4]. It is important to acknowledge that the violaxanthin cycle is commonly known as the xanthophyll cycle.

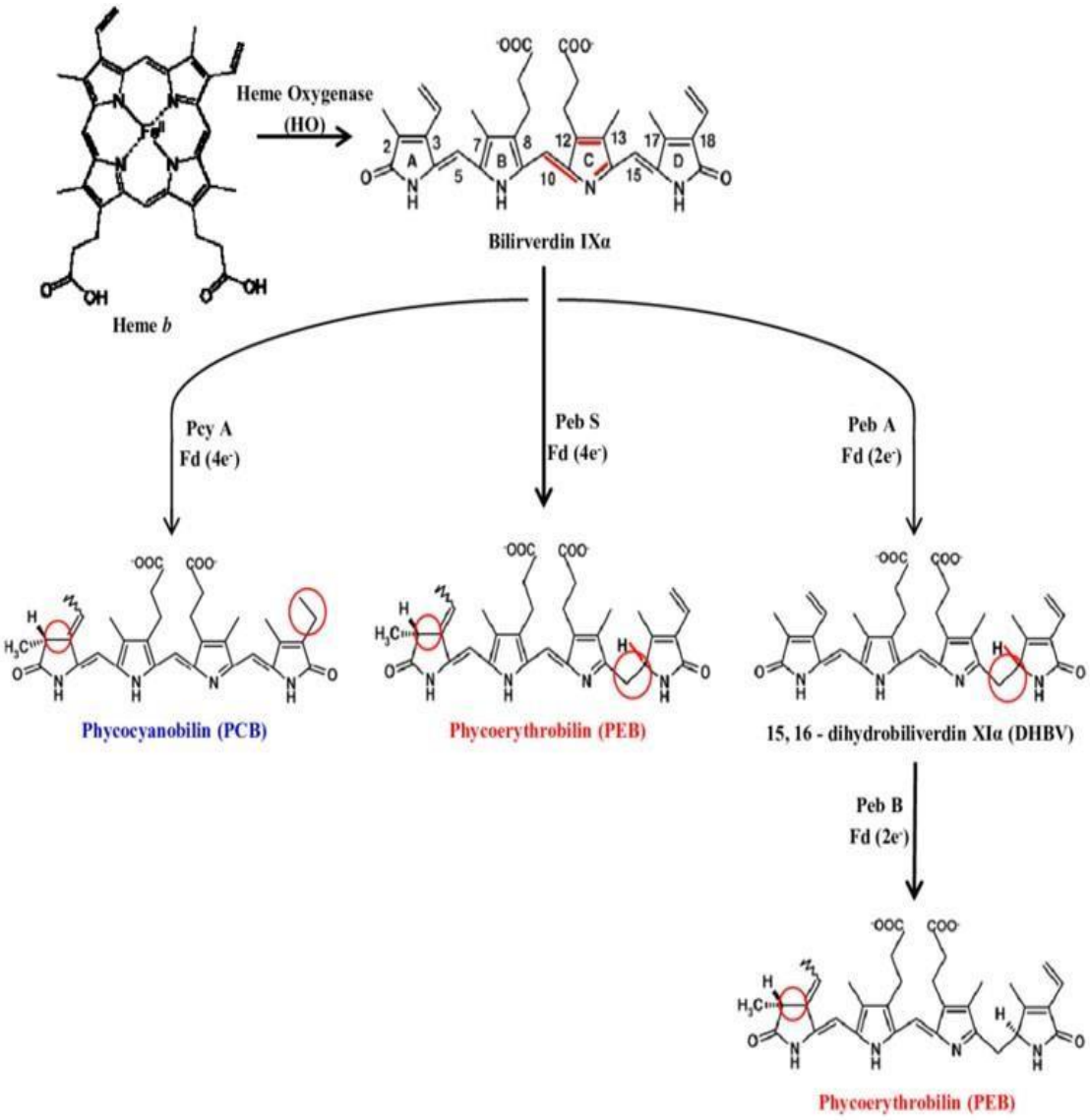


Figure 2.7 The biosynthetic pathway of phycobilins [34].

In addition to being a precursor for chlorophylls, geranyl geranyl-PP also functions as a precursor for carotenoids, as indicated by the orange arrows. The formation of lycopene, a carotenoid compound, involves the condensation of two molecules of geranyl-geranyl-PP, followed by an isomerisation reaction and the introduction of four double bonds. Following the synthesis of lycopene, the biosynthetic pathway undergoes a division into two distinct branches. One

of the branches leads to the yellow pigment known as lutein. Lutein has the potential to undergo conversion into loroxanthin in certain species, such as the *Chlorophyte Chlamydomonas reinhardtii* (Dangeard) [43]. The other branch leads to the orange β-carotene, which represents another point of divergence. The conversion of β-carotene to red astaxanthin involves a series of chemical processes, including two oxidation reactions and two hydroxylation reactions. The carotenoid with hydrophobic properties has the potential to undergo esterification, either once or twice, with a fatty acid. This process results in the formation of a more lipophilic compound known as astaxanthin mono- or diester. The main esters identified in *Haematococcus pluvialis* include those composed of fatty acids C18:1 and C20:0. The orange zeaxanthin is derived from β-carotene through an alternative pathway. Zeaxanthin is a constituent of the violaxanthin cycle, as depicted in **Figure 2.6**. The violaxanthin cycle is alternatively referred to as the xanthophyll cycle; violaxanthin and diatoxanthin cycles, both using xanthophylls, are referred to as distinct mechanisms to avoid any potential mistakes [42].

The utilization of the violaxanthin cycle rather than the xanthophyll cycle will be employed in this context. Zeaxanthin undergoes two successive epoxidation processes, resulting in the formation of antheraxanthin and subsequently violaxanthin. Both antheraxanthin and violaxanthin have a yellow coloration. The reactions are triggered under conditions of light that are below saturation levels, whereas the opposite reactions are triggered under situations of light that exceed saturation levels. The isomerization of violaxanthin can result in the formation of neoxanthin. The metabolic pathways discussed earlier have been the subject of extensive research, in contrast to the processes associated with diatoxanthin, dinoxanthin, and fucoxanthin (shown by the dotted lines in **Figure 2.6**. Previous work conducted by Lohr and Wilhelm (1999) [44] has reported that the conversion of violaxanthin to diadinoxanthin occurs when exposed to extended periods of sub saturated light. This conversion likely necessitates multiple sequential conversion steps. Additionally, the findings of their study indicate that diadinoxanthin undergoes further metabolic processes leading to the formation of fucoxanthin, likely including many

sequential conversion stages. Furthermore, as per the research conducted by Lohr and Wilhelm (1999) [44], it has been observed that excessive levels of light intensity led to the process of de-epoxidation of diadinoxanthin, resulting in the formation of diatoxanthin. The xanthophyll undergoes a process of epoxidation once more when exposed to light intensities that are below saturation levels. The process by which diadinoxanthin and diatoxanthin are converted into one other is referred to as the diatoxanthin cycle [44] (**Figure 2.6**).

2.2 Biological activities and potential application of pigments derived from marine algae.

Algae has always held a unique place in the culture of East Asia (Japan and China). This ancient tradition and daily consumption of algae have allowed for many epidemiological studies demonstrating its health benefits [45]. The results of epidemiological studies have shown that the decrease in cases of breast and prostate cancer in Japan and China compared to North America and Europe is related to nutritional factors [46].

According to these studies, the importance of marine algae as sources of biologically active substances has been realized. Therefore, a new trend has emerged to isolate and identify bioactive compounds from marine algae. Among the identified functional components of marine algae, natural pigments have been reported to have many health benefits, such as antioxidant, anticancer, anti-inflammatory, anti-obesity, anti-angiogenic, and neuroprotective activities [2]. **Table 2.2** summarizes the main biological functions of algae-derived pigments.

Table 2.2 Table Marine algae-derived natural pigments and potential biological activities.

Natural pigments	Biological activities	Sources	References
Chlorophyll *a*	Antioxidant Antimutagenic	*Enteromorpha prolifera, Fucus vesiculosus* *Cyanobacteria* *Porphyra tenera*	[47] [48] [49]
Pheophorbide *a*	Antioxidant	*Enteromorpha prolifera* *Cyanobacteria*	[50] [51]
Pyropheophytin *a*	Antioxidant	*Eisenia bicyclis*	[52]
Pheophytin *a*	Antioxidant Antimutagenic Neuroprotective Anti-inflammatory	*Porphyra tenera* *Sargassum fulvellum* *Enteromorpha prolifera* *Cyanobacteria*	[53, 54] [55] [56] [57] [58]
Lutein	Antimutagenic	*Porphyra tenera*	[49]
β-Carotene	Antimutagenic	*Porphyra tenera*	[49] [48]
Siphonaxanthin	Anticancer Anti-angiogenic	*Codium fragile* *Codium fragile*	[2] [59]
Fucoxanthin	Antioxidant Anticancer Anti-inflammatory Anti-obesity Anti-angiogenic Neuroprotective Prevent osteoporosis. Photoprotective	*Hijikia fusiformis,* *Undaria pinnatifida, Fucus serratus,* *Padina tetrastromatic* *Undaria pinnatifida* *Myagropsis myagroides* *Undaria pinnatifida* *Undaria pinnatifida* *Hijikia fusiformis* *Laminaria japonica* *Laminaria japonica*	[60] [61] [62] [63] [64] [65] [66]
phycoerythrin	Antioxidant	*Porphyra sp*	[67]
phycocyanin	Antioxidant Antiviral Anti-inflammatory	*Cyanobacteria* *Cyanobacteria* *Cyanobacteria*	[68, 69] [70] [71]

2.2.1 Antioxidant activity

Antioxidants are molecules that inhibit or postpone substrate oxidation. Antioxidants can mop up free radicals and decrease oxidation in processed food and medicines during storage [51]. It was reported [72] that antioxidants could limit the impacts of free radicals and reactive oxygen species (ROS) in the human body, which in turn delays the onset of chronic illnesses. Consequently, a multitude of synthetic commercial antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), and propyl gallate (PG), have been extensively utilized within the food and pharmaceutical sectors to inhibit oxidation [73]. Due to the potential risks linked to the utilization of these synthetic antioxidants, the search for natural antioxidants as viable substitutes is of utmost importance in the food-drug-cosmetic(FDC) sector [2].

Recent years have seen a surge in research and development efforts by the FDC industries for antioxidants derived from marine algae and plants. Algae are one of the marine resources with the highest concentration of natural antioxidants. Different kinds of natural products, such as fucoxanthin, phycoerythrobilin, chlorophyll *a*, and their derivatives, have been found to be powerful antioxidants, suggesting that marine algae may have useful medicinal properties [74]. The antioxidant activity of natural pigments is contingent upon their structural characteristics, including the presence of a porphyrin ring, a phythyl chain, and an extended system of conjugated double bonds. Multiple studies have documented the antioxidant effects of Chl *a* and its derivatives found in marine algae [48].

Some chlorophyll derivatives, like pheophorbide *a*, that do not have the central Mg(II) ion and phytyl chain, worked better as antioxidants than chlorophyll itself. In a recent study [50], it was proposed that the robust antioxidant activity exhibited by *Enteromorpha prolifera* can be attributed to chlorophyll *a* derivatives, specifically pheophorbide *a*, rather than polyphenols. According to another study [52], it was found that pyropheophytin *a*, derived from the brown seaweed *Eisenia bicyclis*, exhibited higher antioxidant activity compared to

synthetic antioxidants such as BHT and α-tocopherol. Furthermore, Chl *b* derivatives showed higher antioxidant activity than Chl *a* derivatives. This suggests that the presence of an aldehyde group makes these compounds more antioxidant [51, 57].

Also, fucoxanthin, which comes from brown seaweeds, has better antioxidant and reduction properties than commercially available alternatives [75, 76]. It was observed [67] that phyoerythrobilin, obtained from *Porphyra sp*., demonstrated significant antioxidant activity. Many studies have also shown the antioxidant activity of phycocyanin isolated from *Spirulina* [77, 78]. These studies indicate that among the diverse naturally occurring chemicals found in marine algae, natural pigments demonstrate potential as effective and non-toxic options in the quest for substances with antioxidant activity.

2.2.2 Anticancer activity

Over the past few years, cancer has emerged as a significant and pressing issue in human health. Hence, natural compounds with anticancer properties, such as chemo-preventive medicines, have gathered significant attention in the field of cancer therapy. The development of cancer cells in the human body can be triggered by the presence of free radicals. Consequently, compounds with radical scavenging properties, such as natural pigments, have the potential to indirectly mitigate the formation of cancer in the human body [2, 26].

Several research articles have been published on the association between carotenoids and their potential protective effects against several types of cancer, including breast cancer and lung cancer [79, 80, 81]. Furthermore, a recent study conducted in Japan reported that fucoxanthin exhibits an apoptosis-inducing impact on human leukaemia [82]. Previously, Hosokawa *et al* [62] and Kotake *et al* [83] reported that fucoxanthin had antiproliferative effects and induced apoptosis in human colon and prostate cancer cells.

Extensive research has been conducted on the anticancer properties of chlorophyll and its derivatives, particularly focusing on their ability to counteract the mutagenic effects of various dietary and environmental substances. Moreover, the effectiveness of natural and commercially available chlorophyll derivatives as photosensitizers has facilitated their application in the treatment of cancer through photodynamic therapy [27]. According to the literature reviewed in this domain, there may be potential to develop new anti-cancer products in the pharmaceutical industry as well as in the food industry as new chemo-preventive agents for the treatment of cancer from natural pigments derived from marine algae.

2.2.3 Anti-inflammatory activity

The primary approach to managing inflammatory illnesses evolves around the regulation of macrophage activity, as these cells are responsible for generating pro-inflammatory cytokines and mediators. Therefore, the suppression of the synthesis of these inflammatory mediators represents a promising therapeutic approach for the management of inflammatory disorders [84] [85]. The anti-inflammatory properties of metabolites obtained from algae have been documented in previous studies [37, 56, 71, 77].

Nevertheless, limited research has been dedicated to investigating the possible anti-inflammatory properties exhibited by pigments derived from seaweed. An instance of this is when pheophytin a, which was extracted from *E. prolifera*, was found to exhibit inhibitory effects on 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced superoxide radical ($O_2^{-\cdot}$) production and inflammatory responses in mice macrophages, as described in [49].

According to Heo *et al* [63] and Kim *et al* [86], the anti-inflammatory properties of fucoxanthin and astaxanthin derived from brown seaweeds have been observed. In a separate investigation, Shiratori *et al* [87] found that the anti-inflammatory properties of fucoxanthin were shown to be like those of the commercially available steroidal anti-inflammatory medication prednisolone when administered at comparable doses.

2.2.4 Anti-obesity activity

Obesity is a significant global public health concern that impacts individuals of all age groups, giving rise to a multitude of severe medical conditions such as type 2 diabetes, hypertension, hyperlipidemia, and cardiovascular disease [88]. Hence, it is imperative to explore other sources of safe anti-obesity medicines. According to Maeda H *et al* [64], the inclusion of fucoxanthin, a compound present in brown seaweeds, in dietary regimes may have the potential to mitigate obesity by impeding the process of adipocyte development.

Abidov *et al* [89] conducted a clinical trial whereby they observed that the administration of xanthogen, fucoxanthin, and pomegranate seed oil resulted in weight loss, decreased levels of body and liver fat, and enhanced liver function tests among a group of obese non-diabetic women. In addition, several noteworthy investigations [88-89] have demonstrated that the administration of fucoxanthin orally resulted in a significant decrease in the weight of abdominal white adipose tissue in both obese mice and normal mice fed a high-fat diet. However, no such reductions were observed in normal mice that were fed a regular diet.

2.2.5 Neuroprotective effect

It is projected that neurodegenerative disorders will exceed cancer and become the second leading cause of mortality among the elderly by the 2040s. Due to this rationale, a significant emphasis has been placed by scientists on ensuring neuroprotection that is both safe and efficacious [2].

Numerous studies have shed light on the neuroprotective characteristics of pigments generated from marine algae [2]. As an illustration, Okuzumi *et al* [90] reported the inhibitory effects of fucoxanthin, derived from the brown seaweed *Hijikia fusiformis*, on the expression of N-myc and the advancement of the cell cycle in a human neuroblastoma cell line. Thus, *in vitro* studies have demonstrated the potential neuroprotective properties of natural pigments.

But more research is warranted to examine the neuroprotective properties of pigments *in vivo* and in human subjects [2].

2.3 Extraction methods and analytical techniques

2.3.1 Extraction of photosynthesis pigments from marine algae

The extraction process is the first step in accessing bioactive chemicals from the initial raw material, enabling subsequent separation and characterization steps. The primary obstacle to separating photosynthetic pigments from the crude mixture is the simultaneous extraction of other components, including polyphenols, organic acids, pectin, and sugars. Hence, it is crucial to carefully choose the suitable extraction technique(s) to achieve optimal extraction yield and ensure stability throughout the processing and storage stages.

This section will discuss: 1) the prevailing procedures currently employed for the extraction of photosynthetic pigments from marine algae; 2) analytical methods for characterizing, isolating, and identifying pigments; and 3) antioxidant and prooxidant assays.

2.3.1.1 Conventional extraction methods

The conventional method for extracting photosynthetic pigments from marine algae involves the use of solvent extraction, which may or may not be combined with thermal treatment (either heating or cooling). The solvent extraction method involves the combination of two physical phases, namely the solvent and biomass. The process of extraction occurs when the solvent is absorbed into the cell wall, resulting in partial rupture and facilitating the release of cellular content for extraction. The process of total extraction can be time-consuming, and it is often observed that enhanced efficiency can be attained by applying stirring or mixing techniques. The mixture of solvent and solute that is obtained at the end of the method is commonly known as an "extract"[1], as depicted in **Figure 2.8**.

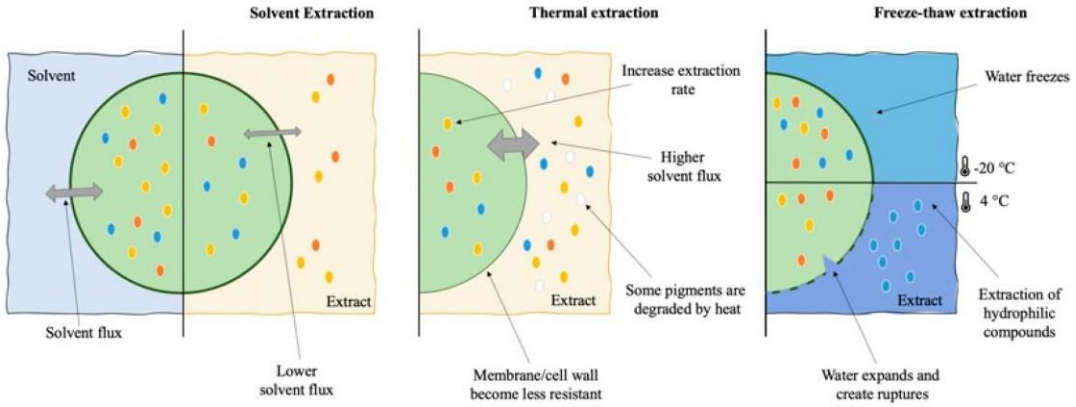


Figure 2.8 A schematic representation of classical extraction methods, including solvent extraction, thermal extraction, and freeze-thaw extraction [1].

Additionally, a potential method for increasing the efficiency of the extraction process involves a thermal treatment. The efficiency of extracting chlorophyll and carotenoids can be enhanced by conducting the process within a temperature range of 50 °C to 65 °C. Nevertheless, prolonged exposure to temperatures exceeding 70 °C can lead to the deterioration of thermally sensitive components such as carotenoids and chlorophylls [91]. In the context of phycobiliproteins, the freeze-thaw method is frequently employed as a prevalent extraction procedure, utilizing either phosphate buffer or water as a solvent. The freeze-thaw approach involves the freezing of the moist biomass to induce crystallization of intracellular water, followed by thawing at refrigeration temperatures (4 °C). This process leads to cell lysis because of ice crystal expansion [92]. The extraction process may encompass multiple freeze and thaw cycles, resulting in a time-intensive procedure and limited reproducibility, with the number of cycles exerting a significant influence [93]. The optimization of the process entails the modification of several parameters, such as solvent, thawing time, freezing temperature, and biomass-to-solvent ratio, among other considerations [94]. Additionally, the freeze-thaw approach has been employed as a pre-treatment technique for various novel extraction processes, such as homogenization techniques, ultrasonication and microwave-assisted extraction. [95, 96].

When choosing a solvent, it is crucial to consider the solubility of the molecule, the potential toxicity, and the ecological consequences of any residues [1]. The conventional method for extracting pigments involves the utilization of organic solvents, such as methanol, hexane and chloroform, or alternatively, more environmentally sustainable solvents like water, ethanol, ethyl acetate and acetone [97]. The utilization of acetone in an individual solvent extraction method has been found to result in the destruction of cell walls and the extraction of pigments. However, it is important to note that the efficiency of this procedure is rather poor, and it necessitates a significant amount of time [98, 99]. Therefore, it is feasible to employ a combination of solvents or extraction procedures to enhance efficacy. In the study Zou *et al* [100], a solvent mixture consisting of ethanol and ethyl acetate was employed in a 1:1 ratio to extract astaxanthin from *H. pluvialis*. The researchers conducted an optimization process by altering the ethanol percentage within the solvent mixture, from 30% to 70%. This manipulation allowed for the modification of the solvent mixture's polarity.

One significant benefit of this type of extraction is the decreased expenditure associated with infrastructure and operational protocols. In contrast, solvent extraction often requires significant quantities of organic solvents or a prolonged processing time, as observed in the extraction of phycobiliproteins, which involves cyclic thermal treatment. In both instances, the criterion of efficiency typically falls short in terms of suitability for industrial implementation [96].

2.3.1.2 Novel extraction methodologies

Numerous studies explore novel approaches to enhance and address the limitations associated with traditional extraction methods. Several methods, including **enzymatic extraction**, **supercritical fluid extraction**, **electroextraction**, and **wave-energy-based cell disruption**, have been suggested as potential approaches to enhance the extraction efficiency of photosynthetic pigments from marine algae. The following paragraphs will provide an overview

of the suitability of these approaches to the extraction of marine algae-derived products.

The enzymatic extraction method relies on the utilization of hydrolytic enzymes that possess the capability to disrupt the membrane and/or cell wall of marine algae. This disruption leads to the exposure of the intracellular components of these organisms to the solvent [96], as depicted in **Figure 2.9A**. Enzymatic extraction techniques used for marine algae focus primarily on extracting lipid compounds, including carotenoids, amphiphilic molecules such as chlorophyll, and protein pigments such as phycocyanin. The extraction process is carried out using an enzyme as a catalyst, such as cellulase and lysozyme, to break down the cell wall [96, 97]. Extractions of this type are commonly used as a pretreatment procedure, which is later followed by further solvent extraction [101]. As an example of this method, the use of enzymatic extraction of allophycocyanin from *Arthrospira platensis* improved the purity of allophycocyanin compared to conventional extraction, where the extraction process is carried out using lysozyme as the agent for 20 hours under conditions of 37 °C and a pH value of 7.0 [102].

One of the primary benefits associated with enzymatic extraction is the specificity of enzymes in breaking down the cell wall [96, 97]. Additionally, this method offers several other advantages, including the utilization of gentle reaction conditions in relation to pH and temperature, a high rate of extraction, and the elimination of the need for drying procedures [103]. Enzymatic reactions may exhibit prolonged reaction times, rendering them less desirable for implementation in industrial settings [104]. Also, there are several constraints that impede the utilization of this extraction procedure, including the elevated expense of enzymes and the imperative requirement of maintaining stable

circumstances throughout the process. This is due to the sensitivity of enzymes to variations in temperature and pH [105].

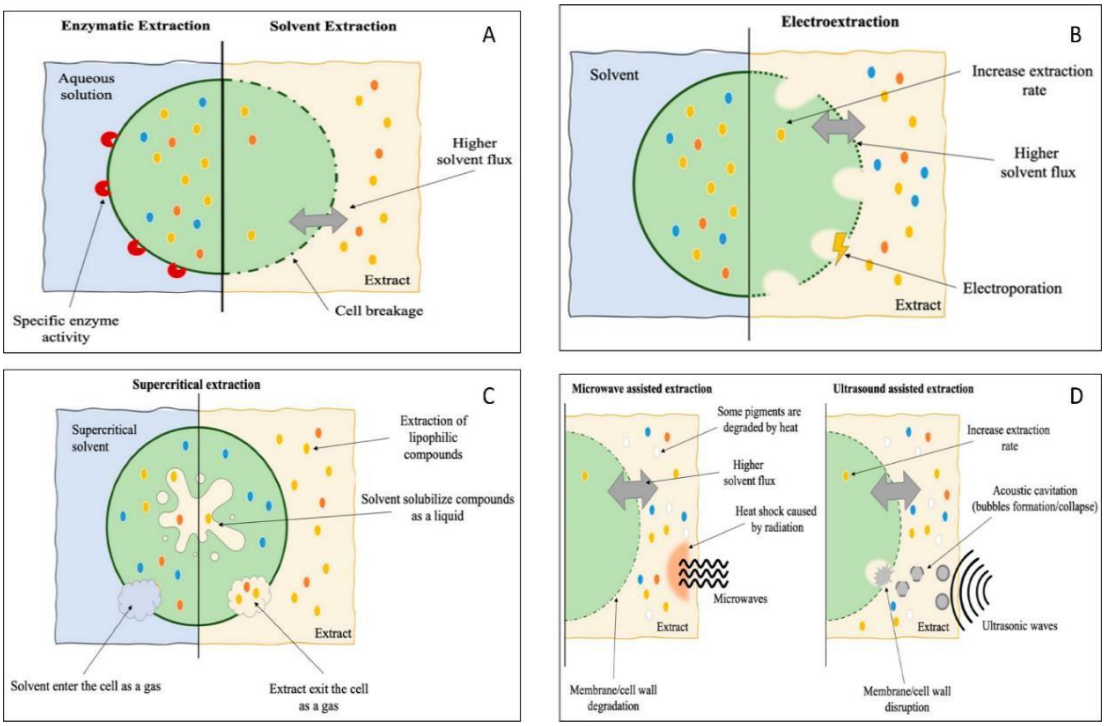


Figure 2.9 Schematic representation of the novel methods for extracting pigments from marine algae followed by solvent extraction. A) enzymatic extraction, B) electroextraction, C) supercritical fluid extraction, and D) wave-energy-based cell disruption techniques [1].

Electric field technologies have been found to be applicable to marine algae for various purposes, such as harvesting and bioproduct extraction. These technologies are also used to create stress in order to speed up the production of secondary metabolites such as total antioxidant compounds [106, 107]. The application of an electric field can be done either continuously or in pulses. This process results in the charging of the cell membrane, causing a destabilization of its structure. Ultimately, this leads to the occurrence of electroporation, wherein pores in the cell wall are opened either reversibly or irreversibly. These pores enable the penetration of solvents, thereby facilitating the extraction of intracellular compounds. Also, various factors, such as alterations in the intensity, waveform morphology, frequency, temperature,

extraction duration, number of pulses, and pulse duration, have the potential to impact the efficacy of the extraction process [108]. **Figure 2.9B** illustrates the impacts of electroextraction processes on cellular function.

Several studies have suggested that electric field technologies are viable extraction techniques for a wide range of pigments found in microalgae and cyanobacteria. Martinez *et al* [109] suggested using a pulsed electric field (PEF) to extract phycocyanin from *A. platensis*. The minimum electric field threshold observed was found to be 15 kV/cm, while the recommended optimal value for efficient processing was determined to be 25 kV/cm. This optimal value was achieved by utilizing a pulse duration of 3 μs and applying 50 pulses at a temperature of 40 °C. In another study, Luengo *et al* [110] optimized a method for the extraction of chlorophyll and carotenoids from *C. vulgaris* using the PEF technique. Utilizing pulse duration values ranging from microseconds to milliseconds, the application of an optimal therapy involving the administration of 25 pulses lasting 3 μs each at an electric field intensity of 20 kV/cm has shown significant efficacy in inducing cell permeabilization, cell disruption, and pigment extraction. In a separate investigation, Luengo *et al* [111] proposed the utilization of a regulated temperature range of 25 °C to 30 °C for the purpose of extracting lutein from *C. vulgaris* by the PEF technique.

Electric field technologies offer several notable benefits in comparison to conventional, enzymatic, and wave approaches. These advantages include significantly reduced energy input, faster extraction periods, and lower solvent requirements [104]. Compared to conventional mechanical procedures, these techniques provide several advantages in terms of operating processes. They exhibit scalability, are recognized as environmentally friendly due to their low solvent usage and may be run continuously [108, 112]. Conversely, these technologies may entail greater expenditures in terms of capital and installation. Furthermore, the utilization of organic solvents frequently remains indispensable, and it is crucial to adjust extraction parameters, including pulse number, duration, and electric field intensity, for each specific microorganism and intended product [113]. As an illustration, it has been observed that

cyanobacteria necessitate a much greater amount of energy to accomplish electroporation in comparison to marine algae. This discrepancy might be attributed to the smaller size and distinct membrane composition of cyanobacteria [112].

Supercritical Fluid Extraction (SFE) demonstrates efficacy in the extraction of pigments from marine algae, particularly carotenoids and chlorophylls. SFE relies on the utilization of a supercritical solvent, which exhibits gas-like behaviour to permeate the cell wall and liquid-like behaviour to dissolve molecules [114].

The SFE system comprises several key components, including a pump that generates high pressure using carbon dioxide (CO_2), a chamber for heating, a chamber for extraction, and a chamber for collecting the desired substances. Carbon dioxide is introduced into a heating chamber, where it is subjected to supercritical conditions. Subsequently, the carbon dioxide is transferred to the extraction vessel, where it permeates the biomass through diffusion. Extraction is initiated when the solvent infiltrates the biomass, leading to cellular growth and the concomitant dissolution of the desired chemicals. Subsequently, the extracted substance is transferred to a designated chamber, where the carbon dioxide (CO_2) undergoes a cooling process, followed by recompression and recycling. As a result, a concentrated extract with properties resembling oil is obtained, comprising carotenoids and chlorophyll [114, 115]. **Figure 2.9C** depicts a schematic illustration of SFE and its impact on cellular processes.

The utilization of supercritical CO_2 extraction has been employed for the purpose of extracting carotenoids and chlorophylls from various marine algae. Supercritical carbon dioxide is commonly employed as a solvent owing to its low polarity, non-flammable nature, and non-toxic features [116]. Hosseini *et al* [117] demonstrated in their study that the most favourable operational parameters for extracting carotenoids from *D. salina* were a pressure of 400 bar and a temperature of 55 °C. However, it is noteworthy that the highest ratio of carotenoids to chlorophylls was observed at a lower temperature of 30 °C. In

a separate investigation conducted by Jaime *et al* [118], it was determined that a pressure of 443 bar and a temperature of 27.5 °C were the optimal parameters for the extraction of β-carotene from *D. salina*.

The application of supercritical CO_2 extraction of carotenoids and chlorophylls in food processing has been investigated. The optimal conditions for obtaining the largest carotenoid yield and the highest ratio of total carotenoids to chlorophylls were found to be 250 bar and a temperature of 60 °C. Additionally, the use of ethanol as a cosolvent at a concentration of 7.7% (v/v) was shown to be the most effective in achieving these results [119].

Generally, SFE is acknowledged as an environmentally friendly technique owing to its reduced usage of organic solvents and shorter extraction duration in comparison to traditional solvent extraction methods. Nevertheless, the primary constraint of secure function evaluation (SFE) remains the financial implications associated with its implementation and ongoing functionality [29]. Supercritical extraction also has the potential to facilitate operations that operate under a "biorefining" framework, as it enables the additional processing and extraction of the residual biomass [120].

The extraction of high-value chemicals from marine algae has been the subject of research and optimization through **two wave-based extraction methods**, namely ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) [1]. In relation to the extraction of pigments, both approaches have been proposed, **Figure 2.9D** illustrates the impact of wave-based extraction methods on cellular activities. In the context of ultrasound-assisted extraction (UAE), the methodology involves the utilization of acoustic cavitation to generate cavitation bubbles, which then induce localized pressure augmentation upon their collapse. The subsequent breakdown of the cell wall enables the solvent to penetrate, resulting in increased extraction rates [121]. The utilization of UAE results in an enhanced extraction yield, leading to a reduction in both processing time and energy consumption. Additionally, UAE offers the advantages of ensuring consistent results, minimizing solvent usage, and operating within low temperature ranges (<70 °C). Consequently,

UAE can be regarded as an environmentally friendly extraction method [121, 122].

In a study conducted by Zou *et al* [100], the authors employed ultrasonic-assisted extraction (UAE) and a response surface approach to optimize the extraction of astaxanthin from *H. pluvialis*. The researchers determined that the ideal extraction conditions were achieved utilizing an ultrasound power of 200 W and a frequency of 40 kHz at a temperature of 41.1 °C for a duration of 16 minutes. This optimized condition resulted in a 35% greater yield of astaxanthin compared to traditional solvent extraction methods. Natarajan *et al* [123] suggested the implementation of a continuous system based on ultrasonic-assisted extraction (UAE) for the purpose of extracting lipids from *Chlorella sp*. This proposal indicates the potential for upscaling UAE operations. In their study, Dey and Rathod [121] conducted optimization of UAE for the purpose of extracting β-carotene from *A. platensis*. The researchers determined the ideal experimental parameters by employing heptane as the solvent, maintaining a temperature of 30 °C, and applying an electrical acoustic intensity of 167 $W.cm^{-2}$ for a duration of 8 minutes [121]. Furthermore, the experimental parameters employed in the ultrasound-assisted extraction method in the current study yielded favourable outcomes in the extraction of pigments from various matrices of marine algae, as documented in **Chapters 3 and 4**.

Microwave-assisted extraction (MAE) occurs because of the thermal shock induced by the application of radiation to the solution, leading to the destruction of the cell wall. The primary constrains associated with the utilization of thermal processes is the inherent poor thermostability of pigments. One of the primary advantages associated with MAE is a significant decrease in extraction time and the minimization of solvent usage. These facts establish MAE as an environmentally friendly extraction technique [124]. Additionally, due to the shorter duration of the extraction process, this method is not limited to molecules that are resistant to high temperatures. Also, employing low temperatures during microwave-assisted extraction in a reduced-pressure environment is a viable approach, but one that incurs a substantial rise in the

overall extraction costs [125, 126]. Pasquet *et al* [127] proposed the use of microwave extraction as a method for obtaining carotenoids and chlorophylls from *Cylindrotheca closterium*. They found that the ideal conditions for this extraction method were 5 minutes at 50 W, at 56 °C. Furthermore, they observed that microwave extraction resulted in a greater extraction rate compared to traditional thermal extraction. In a separate investigation, Juin *et al* [126] employed the method of MAE to extract phycobiliproteins from *Porphyridium purpureum*. The extraction of phycoerythrin was shown to be most effective when conducted at a temperature of 40 °C for 10 seconds [126]. Furthermore, novel approaches have been proposed to disrupt cellular structures and extract value-added compounds from marine algae. These methodologies encompass the utilization of cell milking, pressured systems, ohmic heating, lasers, flotation, hydrodynamic cavitation and high-voltage electrostatic fields [1]. **Table 2.3** provides a concise overview of the primary limitations and advantages associated with both conventional and novel techniques employed in the extraction of pigments from marine algae.

Table 2.3 Advantages and limitations of different methods for extracting pigments from marine algae [1].

Methodology	Advantages	Limitations
Classic solvent extraction	Reduced cost in terms of infrastructure and operating	Less efficiency High amount of organic solvents Time-consuming
Enzymatic	Specificity High cell disruption	High cost of enzymes Requires very controlled process. Need of separation of the enzymes
Electric fields	Lower energy input Reduced time of extraction Reduced need of solvent	High cost of infrastructure
Supercritical extraction	Eco-friendly Optimal temperature below degradation points for pigments	High cost of infrastructure Hard operating process
Wave energy-based	Eco-friendly Reduced time of extraction High extraction rate	High cost of infrastructure Hard control of temperature Hard up-scaling process
Pressure	High cell disruption Reduced need of solvent	High energy requirement High cost of infrastructure
Cell milking	Non-destructive method Can operate in continuous	Very sensible process to maintain the culture in continuous production
Hydrodynamic cavitation	Eco-friendly Reduced time of extraction Lower energy input High extraction rate	High cost of infrastructure Hard operating process
Laser	Eco-friendly Reduced time of extraction Lower energy input High extraction rate	Only applicable to small volumes
High voltage electrical discharge	Reduced time of extraction High cell disruption	High energy requirement
Ohmic heating	Reduced time of extraction Controlled and homogeneous temperature	High energy requirement High cost of infrastructure

Ultimately, the most favourable approach for the valorisation of marine algae involves the identification of extraction techniques that improve economic feasibility. Nevertheless, the existing literature primarily consists of small-scale experiments and theoretical investigations, which tend to yield unsatisfactory results when attempting to identify its applicability on a larger scale. Hence, a deficiency persists in the realm of technical feasibility investigations, as they remain crucial for enhancing the extraction of pigments and assessing the viability of implementing the procedure on an industrial scale.

2.3.2 Analytical techniques

2.3.2.1 Determination of photosynthetic pigments

Historically, researchers in marine chemistry were the first to use pigment analysis in oceanography. They used it as a global parameter to estimate phytoplankton biomass and productivity, study factors that affect phytoplankton pigment content, and study growth rates and grazing activities in the seas [128]. Due to the presence of chlorophyll *a* in all marine algae, it is the most measured biochemical parameter in oceanography, so its optical properties have been used to develop spectrophotometric and fluorometric techniques.

It is important to note that these methods either greatly underestimate or overestimate the concentration of chlorophyll *a*, which draws attention to the critical points in determining chlorophyll because of the overlapping bands of absorption and fluorescence with total chlorophyll (chlorophyll *a* epimer + divinyl chlorophyll *a* + mono chlorophyll *a* + chlorophyllide) and chlorophyll degradation products that were found in aqueous environments, which are pheophytins, pheophorbide, and chlorine ester [128, 129].

Also, degradation products have spectroscopic characteristics close to those of their chlorophyll. The spectra of chlorophyllides in the red wavelengths are so close to those of their chlorophylls that there is no way of differentiating the forms spectrophotometrically. The spectra of phaeopigments are also similar to those of chlorophylls, but they have a small red-shift, and their molar extinction coefficients are about 0.6 times lower than those of chlorophylls.

Although methods that consider phaeopigments in the extracts have been developed for chlorophyll *a*, none of the spectrophotometric methods is quite accurate in the presence of degradation products [130, 131].

On the other hand, high-performance chromatographic (HPLC) separation made it possible to determine the concentrations of a wide range of carotenoids, chlorophylls, and their degradation products at the same time [128, 132]. Many analytical studies of algal pigments using HPLC techniques have been published so far in the field of oceanography [132, 133] and in the field of nutrients [13, 134]. Among these methods, there is no single protocol suitable for all applications, and each method has advantages and disadvantages, given that the sensitivity of the method is affected by many factors such as sample collection, extraction and drying methods, sample storage, column type, detector type, mobile phase and column temperature [128].

However, spectrophotometric techniques are widely employed in various fields to routinely assess chlorophyll contents. These methods can be used to get accurate readings from extracts that have between 1 and 5 mg of chlorophyll per litter. The limitation of this evaluation is in the variability chlorophyll a content across different species, ranging from 0.1% to 9.7% of cell dry weight [136].

Louda and Monghkonsri [135] conducted a comparison between spectrophotometric estimates of chlorophyll contents and those acquired using high-performance liquid chromatography (HPLC). The researchers determined that the assessment of chlorophyll by spectrophotometry, utilizing the Wellburn [32] and Jeffrey and Humphrey [136] equations, yielded highly satisfactory outcomes. These authors confirmed that spectrophotometric analysis is considerably faster and less expensive than HPLC analysis. With no significant differences between the two referred methods, they concluded that spectrophotometric analysis is a suitable tool for routine chlorophyll evaluation.

This thesis employed spectrophotometric analysis to estimate the total concentration of photosynthetic pigments in the examined algal extracts

(Chapters 3 and 4), through preliminary determination of chlorophylls and carotenoids in 80% of acetone, phycocyanin and phycoerythrin in water. Besides spectrophotometric methods, the present study also conducted HPLC-DAD analysis **(Chapter 3**) to detect individual photosynthetic pigments in the examined algal extracts.

2.3.2.2 Purification of photosynthetic pigments

Purification of photosynthetic pigments is a crucial step to eliminate potential interferences from various components, including polysaccharides, polyphenols, soluble dietary fiber, and other soluble phytochemicals. In addition to separating the chlorophyll pigments and carotenoids from each other and obtaining them individually in pure form, it is crucial to evaluate their biological activities, as they can also be impacted by the choice of purification method [128, 137].

Chromatography is a biophysical methodology that facilitates the isolation, characterization, and purification of the constituents within a mixture [138]. Several chromatographic techniques based on the stationary phase have been developed and used for the analytical and preparative separation of carotenoids, chlorophylls, and their derivatives. These techniques include paper chromatography [139], thin layer chromatography [140], conventional column chromatography [141], high-performance liquid chromatography [142], and counter-current chromatography (CCC) [143].

The purification of phycobiliproteins can be achieved by utilizing many features, including size and shape, total charge, the presence of hydrophobic groups on the surface, and binding ability with the stationary phase. There are four separation approaches that rely on a molecule’s properties and interaction type, including ion exchange, surface adsorption, partition, and size exclusion processes [138].

The principle behind the chromatography technique to purify pigments is based on the polar interaction between the mixture and the solid (or

stationary) phase and the mobile phase, which can be a liquid or a gas. The mixture of pigments to be separated first becomes adsorbed on the surface of the solid phase through polar interactions. More polar samples adhere (or bond) more strongly than less polar samples. Then the mobile phase comes in and sweeps away the stationary phase, competing for the mixture of pigments. The more polar the solvent, the more it can carry components of the mixture with it, “snatching” them away from the solid phase as it moves. Different components with different polarities will move at different rates as the solvent moves, causing them to separate [144].

Among these chromatographic techniques, column chromatography is considered the most common technique for separating compounds. The popularity of column chromatography as a method for compound purification can be attributed to its relatively lower cost compared to HPLC and counter current chromatography (CCC) procedures. Despite the numerous advantageous attributes of HPLC technology, such as its exceptional sensitivity, expeditious turnover rate, and utility as a quantitative approach [138].

The present study employed column chromatography to achieve the purification of photosynthetic pigments derived from marine algae **(Chapter 4)**. Additionally, this approach has proven to be effective in the conversion and isolation of chlorophyll *a* into its stable derivatives, obviating the necessity for the acidification and heating procedures outlined in **Section 1.2.2**.

2.3.2.3 Identification of pigments

Spectroscopic methodologies are used to identify pigments by studying the quantitative interaction of energy (usually electromagnetic energy) with pigments using combinations of key techniques: 1) Ultraviolet-visible (UV-Vis) absorption spectroscopy; 2) Fourier transfer infrared analysis; 3) Nuclear magnetic resonance (NMR) spectroscopy; and 4) Mass spectroscopy (MS) [145].

Each spectroscopic method has unique features that help identify pigments. For example, the use of UV-Vis spectroscopy makes it possible to study the optical properties of pigments by analysing electronic transitions. On the other hand, the use of IR enables the functional groups present in pigments to be distinguished by examining molecular vibrations. NMR spectroscopy is used to understand the chemical composition of pigments by examining transitions involving specific nuclei, such as ^{1}H proton and ^{13}C carbon. In contrast, mass spectrometry offers an accurate method to estimate the partial weight of pigments by analyzing fragmentation ions and measuring the mass/charge ratio (m/e) [145].

This section will provide an overview of the different spectroscopy techniques that were used in this thesis (**Chapters 3 and 4**), explaining the basic principles behind each technique.

UV-Vis absorption spectroscopy

Ultraviolet-visible (UV-Vis) absorption spectroscopy is an extensively employed method in various scientific domains, encompassing bacterial culture, drug identification, nucleic acid purity assessment and quantification, as well as quality control applications in the beverage industry and chemical research.

In general, a spectrophotometer is comprised of four primary components: a light source, a monochromator, a sample component, and a detector. The UV-vis spectrophotometer employs a light source consisting of a deuterium lamp and a tungsten/halogen lamp. The deuterium lamp emits light within the ultraviolet wavelength range of 150 nm to 400 nm, while the tungsten lamp emits light within the visible and near-infrared regions of 340 nm to 3500 nm. A monochromator disperses the radiation released by the light source (polychromatic radiation, which encompasses a wide range of wavelengths), into an array of monochromatic radiation, characterized by a single or restricted range of wavelengths. This monochromatic radiation is then directed towards the sample, which is positioned within the cuvettes (**Figure 2.10**) [145, 146].

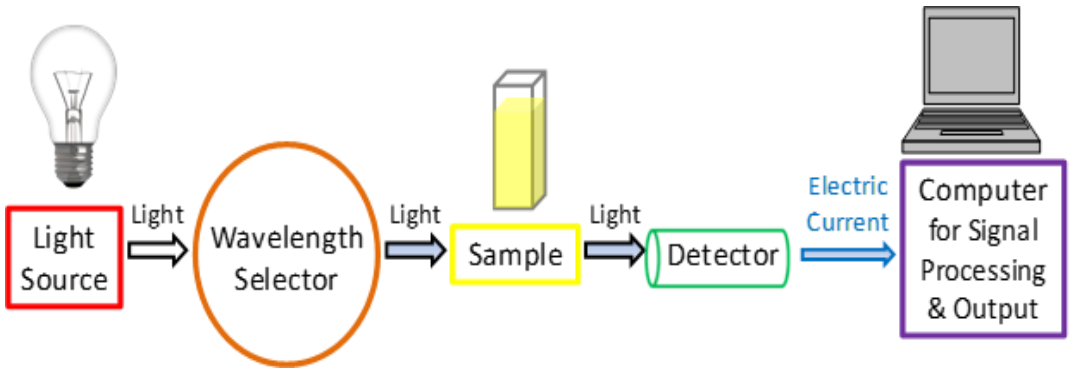


Figure 2.10 A brief diagram illustrating the primary constituents of a UV-Vis spectrophotometer.

Cuvettes are optically transparent cells that are commonly constructed from materials like glass, plastic, or quartz. Plastic and glass materials are not suitable for measurements in the ultraviolet (UV) area due to their inherent absorption of UV light at a wavelength of 310 nm. Quartz cuvettes are very suitable for conducting experiments within the UV-Vis-IR range since they absorb light below 190 nm [146].

Fourier transfer infrared analysis (FT-IR)

In the field of analytical procedures, infrared (IR) spectroscopy holds significant importance and enjoys extensive utilization. The acquisition of the infrared spectrum often involves the transmission of infrared electromagnetic radiation through a material that exhibits a permanent or induced dipole moment. The objective is to ascertain the proportion of incident radiation that is absorbed at a specific energy level. The energy associated with each peak observed in an absorption spectrum is directly related to the frequency of molecular vibrations. This enables the qualitative identification of certain bond types present in the analyzed material. The primary function of an infrared spectrometer is to measure the energy of electromagnetic radiation passing through a sample, often represented as a function of wavenumber or frequency [145].

The central component of each fourier transform infrared (FTIR) piece of equipment is an optical instrument known as an interferometer, as depicted in

Figure 2.11. The Michelson interferometer is often regarded as the oldest and most prevalent form. Typically, the infrared source consists of a ceramic material that is heated to approximately 1200 °C. The collimating mirror functions by gathering light emitted from the source and subsequently aligning the rays in a parallel manner. The beamsplitter composed of potassium bromide (KBr) exhibits a transmission efficiency of roughly 50%, allowing approximately half of the incident light to pass through while reflecting the remaining half. A portion of the transmitted light is directed towards a stationary mirror, while the remaining portion is directed towards a mirror in motion. The incident light is reflected by the two mirrors and subsequently directed back to the beamsplitter, where it undergoes recombination to form a unified light beam. The laser beam undergoes interaction with the sample, specifically exhaust gas, within a gas cell, ultimately reaching the detector. The utilization of a multireflection cell enables the acquisition of an extended optical path length while minimizing the overall volume of the cell [147].

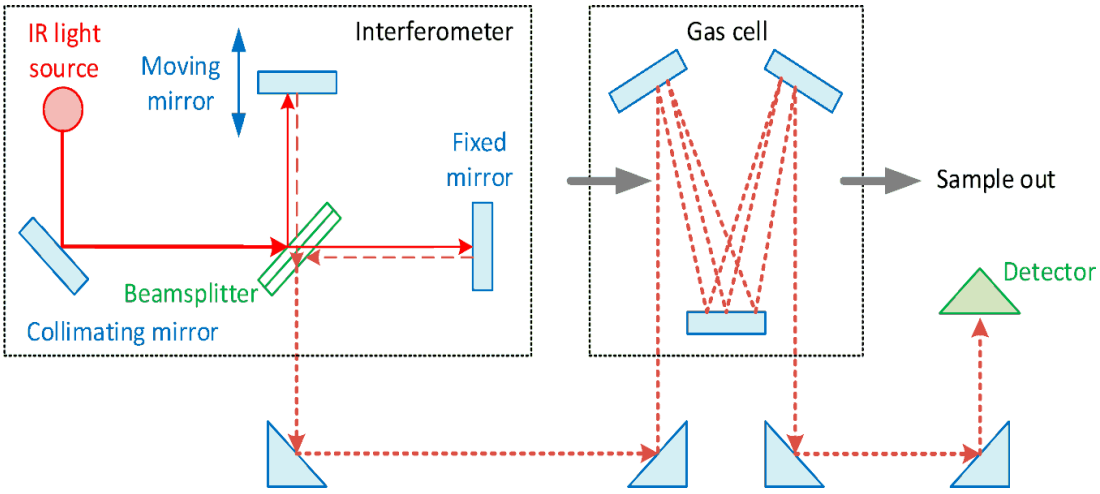


Figure 2.11 Principles of the Fourier-Transform Infrared Spectroscopy (FT-IR) [147].

Nowadays, the comprehensive range of electromagnetic frequencies is examined using an interference mechanism and subsequently transformed into the domain of frequency or wavenumber using a mathematical approach referred to as the Fourier transform. The utilization of fourier-transform infrared (FTIR) spectroscopy has significantly enhanced the accuracy and resolution

of infrared spectra while simultaneously reducing the duration necessary for data acquisition [147].

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a highly consequential analytical technology that has emerged as a prominent scientific advancement in recent decades. The fundamental principles of nuclear magnetic resonance (NMR) involve the utilization of nuclei for determining the structural and chemical constitution of diverse substances, each possessing its own unique magnetic field. The fundamental NMR spectrometer employs a magnetic field and a specialized detector to evaluate changes (see **Figure 2.12**) [145].

A nuclear magnetic resonance (NMR) spectrometer includes fundamental components, specifically the magnet, magnetic field, and detector **(Figure 2.12**). The NMR signal is generated by the interaction of electromagnetic radiation with the nuclei, resulting in their transition to a higher energy level (E1/E2) at a specific frequency (v). When the cessation of electromagnetic radiation occurs, it induces the nuclei to undergo relaxation and attain a state of thermal equilibrium. The emission of energy from atomic nuclei is detected and represented as spectra. These spectra are unique to each nucleus and correspond to the energy differences between two specific states, E2 and E1 [145].

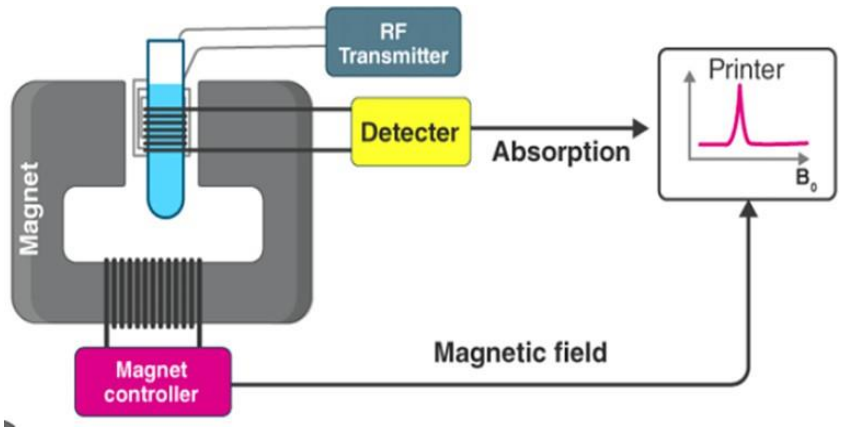


Figure 2.12 A schematic representation of the standard configuration of a NMR spectrometer, highlighting the interconnections between its key elements, namely the magnet, magnetic field, and detector.

The nuclear magnetic resonance (NMR) technique, in addition to elucidating the molecular structure, may help to ascertain the composition and level of impurities in the sample. NMR spectroscopy allows for the detection of many nuclei, including ^{1}H (proton), ^{13}C (carbon-13), ^{15}N (nitrogen-15) and ^{19}F (fluorine-19). ^{1}H and ^{13}C are widely used in the identification of pigments [145].

Accurate mass spectroscopy (MS)

Mass spectrometry (MS) is a widely employed analytical technique that facilitates the identification of chemicals within a given sample by utilizing their mass to charge ratio (m/z) and relative abundance. The primary procedure of mass spectrometry (MS) entails the conversion of the sample into ions in the gas phase through the utilization of a high-energy electron beam (known as electron ionization). The gaseous ions undergo fragmentation, resulting in the formation of several charged ions that can be either positively or negatively charged. The charged ions thereafter traverse a magnetic and electric field, where they are subsequently segregated according to their mass-to-charge ratio (see **Figure 2.13**) [145].

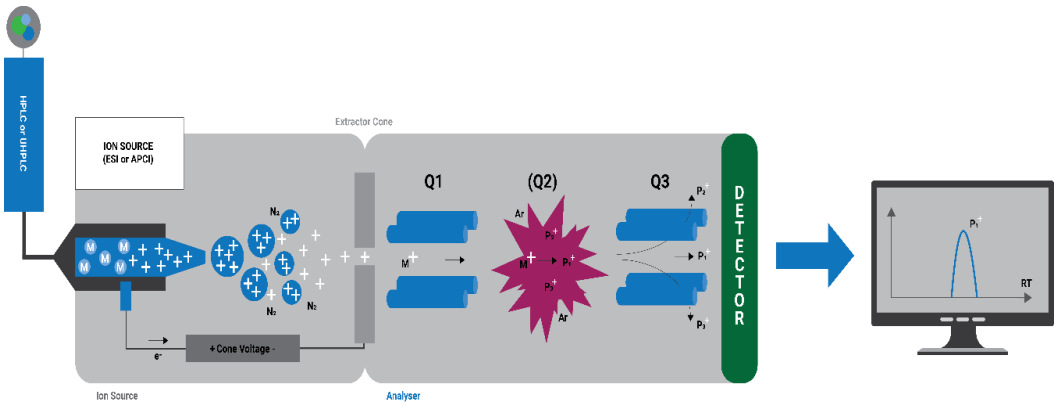


Figure 2.13 A schematic illustration of the fundamental principles behind mass spectrometry.

The trajectory deviation of ions is contingent upon their respective mass and charge. As an illustration, it can be observed that lighter ions experience a greater degree of deflection compared to heavier ions, whereas ions possessing a charge of two positive units undergo a greater degree of deflection in comparison to ions carrying a charge of one positive unit. Subsequently, the isolated ions are detected through the utilization of specific devices, such as an electron multiplier, which enables the generation of a graphical representation depicting the spectrum of relative abundance in relation to the mass-to-charge ratio [145].

2.4 Assessment of antioxidant and pro-oxidant activities

Various reactive oxygen species (ROS) are generated as natural by-products by cellular components such as mitochondria (through the electron transport chain), NADPH oxidase, peroxisomes, and the endoplasmic reticulum during metabolic processes. These ROS include superoxide radical/anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2) [148].

Reactive oxygen species (ROS) are crucial in various biological processes, including metabolism, cellular signalling, and cellular growth and development, when present in controlled and reduced quantities. The production of reactive oxygen species (ROS) is markedly increased by exposure to external stressors such as pollutants, ionizing radiation, heavy metals, and xenobiotics (e.g., cytotoxic medications). This heightened ROS creation leads to considerable harm to cellular DNA and other biomolecules. The condition characterized by an imbalance between the creation of reactive oxygen species (ROS) and their buildup, along with the cells' incapacity to effectively detoxify these detrimental molecules, is commonly referred to as oxidative stress [149].

Chlorophylls and carotenoids are primarily acknowledged for their antioxidant and cytoprotective characteristics. However, it is important to note that under certain circumstances, such as elevated concentrations and abnormal conditions like imbalanced and heightened intracellular oxidative stress (typically observed in cancer cells), increased oxygen levels (found in the lungs of

smokers), reduced levels of endogenous enzymes and antioxidants, and elevated levels of reactive metal ions (e.g., Fe (III) and Cu (II)), carotenoids can exhibit pro-oxidant properties [150, 151]. Besides carotenoids, other widely recognized antioxidative phytochemicals, such as polyphenols [152], ascorbate [153], and tocopherols, have demonstrated pro-oxidant properties under specific physiological and biochemical conditions [154]. Also, chlorophylls exhibit pro-oxidant activity in lipophilic environments under light conditions (see **Figure 2.14**) [155].

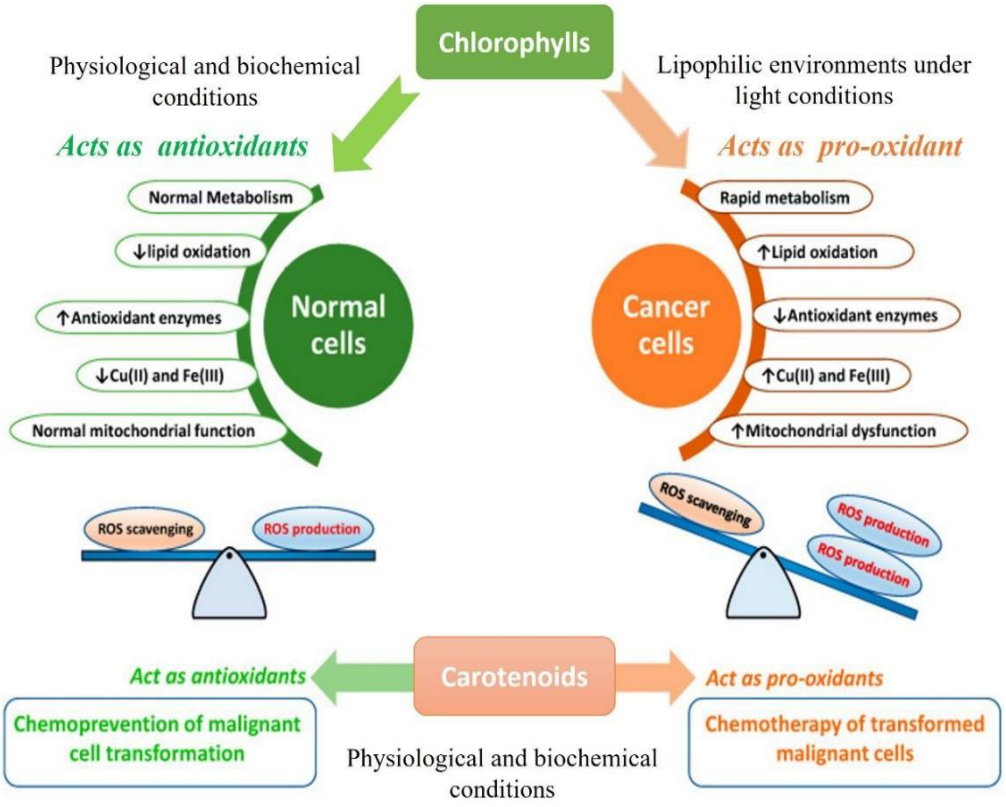


Figure 2.14 .An overview of the key distinctions in cellular metabolism between normal cells and malignant cells. In instances characterized by elevated levels of oxidative stress inside cancer cells, carotenoids predominantly exhibit pro-oxidant properties within biological settings, hence inducing death in cancer cells because of reactive oxygen species (ROS). On the other hand, it is noteworthy that chlorophyll functions as pro-oxidant agents in lipid-rich environments when exposed to light.

This section will discuss both antioxidant and prooxidant activities in terms of concept, reaction mechanism, and method of measurement.

2.4.1 Antioxidant activities

The term “antioxidant” has various definitions. From a chemical point of view, an antioxidant is defined as “any substance that, when present in concentrations lower than those of an oxidizable substrate, retards or significantly prevents the oxidation of that substrate” [156]. From a biological perspective, they are defined as “any natural or synthetic substances that may prevent or delay oxidative cell damage caused by physiological oxidants that have clear positive redox potential, covering reactive oxygen species (ROS), reactive nitrogen species (RNS), and free radicals” [157].

The concept of antioxidant activity (capacity) refers to the reaction rate between the reactive species (extreme and non-extreme) and the antioxidants. Therefore, the activity is linked to the interaction of this pair of reactive compounds, and the relationship between structure and activity is estimated. Therefore, the measurement of antioxidant activity is primarily conducted in model systems, namely in homogeneous solutions. This methodology is valuable in elucidating the underlying reaction mechanism(s) and categorizing the antioxidant activity exhibited by a group of chemicals [158]. In addition to *in vitro* procedures (outside the normal biological context, commonly called test tube experiments), *ex vivo* antioxidant activity measurement has also been performed (assays in cells/tissues/organs grown outside the biological environment for up to 24 hours and minimal changes in natural conditions) and at *in vivo* levels (experiments developed using the entire organism) [48].

Numerous procedures have been published to assess the antioxidant potential of chlorophylls, carotenoids and phycobiliproteins [48, 159]. This section focuses on the methodologies that are frequently employed for pigments. The classification of approaches was initially based on the distinction between protocols that prioritized main antioxidants and those that prioritized secondary antioxidants. The term "primary" is used to describe chain-breaking

antioxidants, which are evaluated using the hydrogen atom transfer-based assay (HAT), single-electron-transfer-based assay (SET), ROS-scavenging activities, metal chelation, or inhibition of lipid peroxidation. On the other hand, the term "secondary" is used to describe preventive antioxidants that function through a neutralization reaction [158].

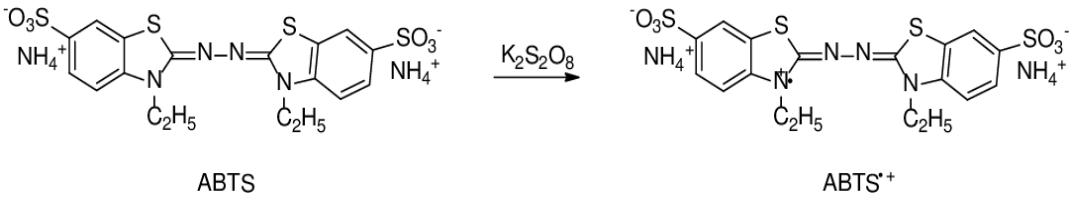
The choice of an appropriate protocol is contingent upon various criteria, including the compound's polarity and structure, the properties of the medium in which the analysis is conducted, the practicality of the method, etc [48].

Ferric reducing antioxidant power (FRAP) and oxygen radical antioxidant capacity (ORAC) assays are SET and HAT-based assays, respectively. On the other hand, Trolox equivalent antioxidant capacity (TEAC) and (2,2-diphenyl-1-picrylhydrazyl) (DPPH) assays are recognized as mixed-mode assays, incorporating both HAT and SET mechanisms. The presence of antioxidants in the sample can potentially engage in either single or numerous chemical reaction processes, which is contingent upon the specific reaction system and the many sources of radicals or oxidants. Phenolics exhibit a higher capacity for scavenging peroxyl radicals compared to carotenoids, but carotenoids demonstrate superior efficacy in quenching singlet oxygen when compared to many polyphenols. Hence, it is imperative to conduct several antioxidant assays, as each methodology yields distinct insights into the antioxidant potential of the samples [160].

This study involved the performance of various antioxidant activity assays to assess the potential radical scavenging activity of photosynthetic pigment extracts and pure pigments, as described in **Chapters 3** and **4**. All assays used in this study were based on colorimetric methods, and the principles of each assay are described in **sections 4.1.1, 4.1.2, and 4.1.3.**

2.4.1.1 Trolox equivalent antioxidant activity (TEAC) assay

The TEAC assay is a straightforward and practical technique used to assess the overall antioxidant capacity of a given sample. The $ABTS^{+}$ (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radicals, which exhibit a high level of absorption at a wavelength of 734 nm, are produced through the incubation of ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) with potassium peroxodisulfate. The $ABTS^{+}$ radicals have a blue-green hue, which undergoes a reduction in color intensity upon interaction with antioxidants **(**see **Figure 2.15)**. The extent to which color bleaching is enhanced is directly proportional to the concentration of antioxidants. The measurement of radical scavenging activity involves the combination of ABTS radicals with the sample for a specific duration of time, followed by a comparison with the Trolox standard curve.



ABTS ABTS$^{\bullet+}$

Figure 2.15 Reaction mechanism of TEAC assay

In comparison to the alternative oxidase (AOX), the TEAC assay offers a straightforward approach that enables the evaluation of radical scavenging activity for antioxidants with both hydrophilic and hydrophobic properties. One disadvantage of this approach is that the ABTS compound does not accurately mimic the physiological radical source [161].

2.4.1.2 Ferric reducing ability of plasma (FRAP) assay

The FRAP assay is based on the reduction of the ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex to ferrous tripyridyltriazine (Fe^{2+}-TPTZ) by antioxidants under acidic conditions (pH 3.6). The Fe^{2+}-TPTZ complex exhibits a blue color and

shows a maximum absorption at 593 nm. The change in absorbance is directly proportional to the concentration of antioxidants in the sample, as shown in **Figure 2.16** [161]. The FRAP assay is a rapid, uncomplicated, and highly consistent procedure in comparison to alternative approaches.

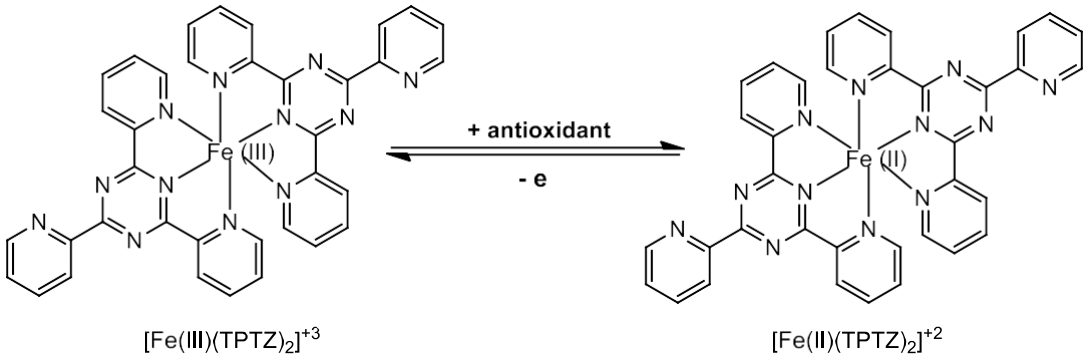


Figure 2.16 Mechanism of the FRAP assay.

2.4.1.3 DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

DPPH is a widely used *in vitro* antioxidant activity assay that is popular among researchers. The DPPH radical exhibits a purple color and demonstrates its highest absorption at a wavelength of 517 nm. Upon reacting with antioxidants, these radicals undergo a process in which DPPH molecules abstract hydrogen atoms. The colour changes from purple to yellow (**Figure 19**). The scavenging activity of DPPH is mostly influenced by the solvent characteristics and pH of the medium [162].

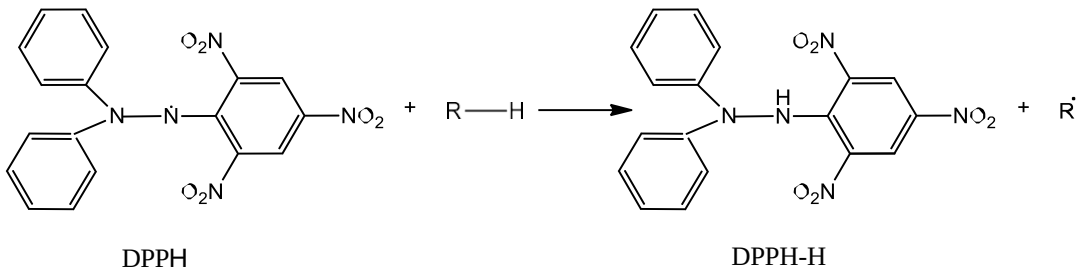


Figure 2.17. Mechanism of DPPH assay

The DPPH test is regarded as a simple, accurate, and cost-effective technique for assessing the antioxidant activity of substances. Moreover, the obtained results may be replicated and can be compared to those obtained using

alternative methods. There are several significant constraints associated with this approach, namely the limited solubility of DPPH in organic solvents. The presence of colored samples may pose a challenge to quantitative analysis due to interference with other compounds in the sample [163].

2.4.2 Pro-oxidant activities for chlorophyll

Chlorophylls are pigments that can interact with light at a specific wavelength. This reaction generates destructive photoreactions of disease-causing microbes and cancer cells. This process is called photodynamic therapy (PDT), which depends on the presence of three components: the photosensitizer (pigments=PS), the light at a specific wavelength, and molecular oxygen in cells. The mechanism by which the photosensitiser is activated includes the absorption of light by the photosensitiser, which initiates the excitation of the molecule, where the electron moves from the ground state to the excited singlet state [164].

During the relaxation process back to the ground state, the singlet excited state can transit into the triplet excited state, where it can be trapped for some time as it involves the forbidden spin-spin transition [164, 165]. The photosensitiser in the excited triplet state participates in the photoreactions to produce reactive oxygen species (ROS), which can destroy the pathogenic bacteria through two mechanisms (**Figure 2.18**). The first mechanism is the interaction between the excited triplet state and the receptor substrate by transferring an electron to produce free radicals. After that, these free radicals interact with oxygen to produce ROS, initially forming O_2*. This generates more ROS within the cell. The initiation of these reactions leads to cell-destructive oxidative stress [166]. In contrast, the second mechanism is a direct interaction between the triplet state of the photosensitiser and the oxygen in the biological environment to produce electronically singlet oxygen. Singlet oxygen produced in a specific area makes it ideal to damage the vital substrates without affecting neighboring and distant cells. Thus, the second mechanism is most common, as it is the main destructive pathway for microbial cells [167].

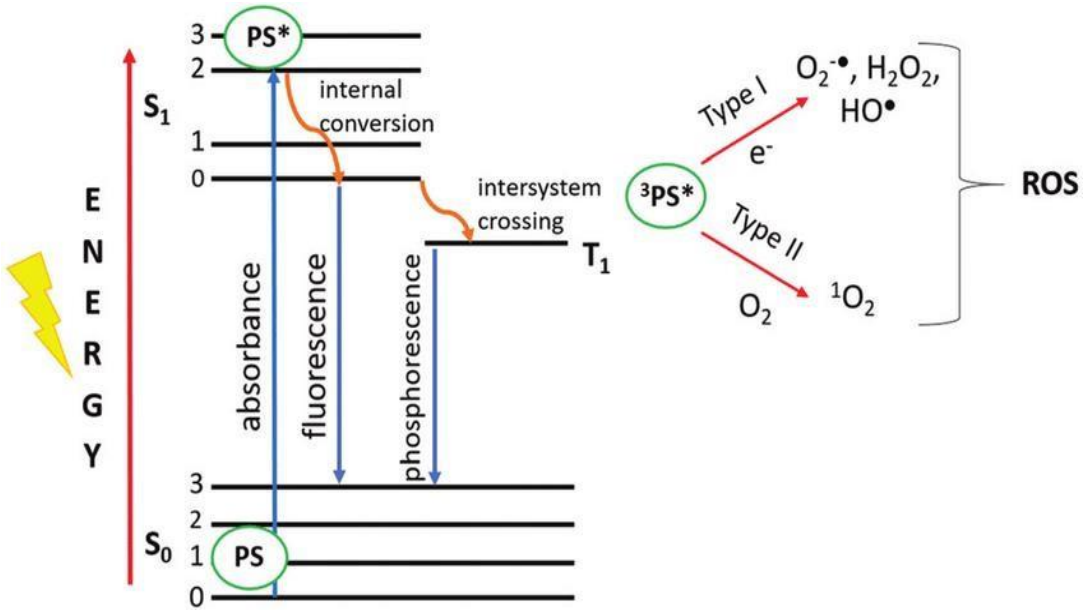


Figure 2.18 The Jablonski diagram to illustrate the mechanism by which photochemical reaction are carried out for photosensitizer [166].

This study included evaluating the ability of pure pigments to produce singlet oxygen ($^1O_2$) and measuring their quantum yield (ΦΔ), as described in **Chapter 4**. The methods used to evaluate the ability of photosensitizers to produce singlet oxygen are described in the next section.

Measurement of Singlet Oxygen ($^1O_2$)

Singlet molecular oxygen ($^1O_2$) refers to the excited state of molecular oxygen when the electrons have antiparallel spins in the same p* antibonding orbital. The investigation of singlet oxygen generation and reactivity has become a complicated and wide field that has impacted various disciplines, including synthetic chemistry, polymer chemistry, and photodynamic therapy. There are many known protocols for generating singlet oxygen, along with a range of techniques utilized for its detection [168]. These methods include photophysical methods, where photogenerated singlet oxygen is directly measured at 1270 nm, or photochemical (indirect) methods, where molecular traps for singlet oxygen are employed. In this project, photochemical methods were used to evaluate the ability of pure pigments to produce singlet oxygen. This method is based on measuring the decrease in the light absorption of

molecules of known specificity to quench singlet oxygen, such as 1,3-diphenylisobenzofuran (DPBF). The interaction of singlet oxygen generates photosensitive compounds because DPBF readily undergoes ring-opening reactions **(Figure 2.19),** resulting in a decrease in absorption intensity at 410 nm with an increase in singlet oxygen production induced by photosensitive compounds over time [168].

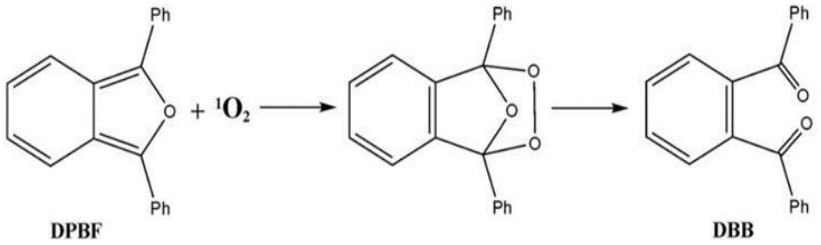


Figure 2.19. Ring-opening reaction of DPBF in the presence of singlet oxygen.

The determination of the quantum yield of singlet molecular oxygen generation (ΦΔ) by a photosensitizer is commonly done using relative approaches, such as measuring the reactivity of $^1O_2$ or phosphorescence. The experimental results obtained for an unknown sensitizer (sample) are compared with those of a well-known standard or reference sensitizer, which has a preferably high and accurately calculated efficiency (ΦΔ) under proper experimental settings. Phenalene is an aromatic ketone that is referred to as the universal reference compound for ΦΔ determination. In addition to phenalene, other aromatic ketones, heterocycles, carbon nanoforms, or coordination compounds with precisely determined ΦΔ values can serve as references for $^1O_2$ generation. These references can be used not only in solutions but also in solid materials such as dye-doped monoliths, films, micro-/nanofibers, and particles, both organic and inorganic [169].

2.5 Conclusions and future perspectives

The pigments produced by macroalgae and cyanobacteria have great potential for use in several industries. This is since β-carotene, astaxanthin, and phycocyanin already have a commercial market. However, the process of extraction and current technologies are still coupled with a high cost of implementation and operation. In addition, energetic requirements are a very major aspect of the sustainability of the process [170].

As shown in this review, the standardization of an extraction protocol for the pigments of interest is still a challenge since pigments are easily degraded due to temperature, light, or ways to disrupt the cell wall. Therefore, the laboratory and small-scale levels have been utilized to investigate the techniques of extracting and purifying pigments. However, the recovery of pigments on a large scale is still laborious since not every cell disruption, extraction, or purification procedure is scalable. Furthermore, these technologies have the potential to consume a significant amount of energy [171, 172]. As stated by Grima *et al* [173], extraction can represent as much as 60% of total expenses. To reduce costs, it is still necessary to streamline existing operations and create new ones. Moreover, employing an appropriate extraction technique might decrease the need for purification. Purification may or may not be necessary, depending on the specific application, such as in the case of food, feed, nutraceuticals, and cosmetics. Purification requirements can cause the final price to increase by a factor of a hundredfold [10]. Finally, there is a need for economic and environmental studies on the production of pigments from algae. Phycoerythrin and astaxanthin, which are high-value chemicals, have recently undergone economic evaluation and life cycle studies [172, 174, 175]. In conclusion, efforts should focus on reducing product loss, used equipment and energy expenses related to the extraction and purification procedures. Furthermore, it is necessary to enhance the advancement of extensive downstream processing to achieve economically feasible and ecologically sustainable procedures.

2.6 Reference

1. Pagels, F., et al., *Extraction of pigments from microalgae and cyanobacteria—A review on current methodologies.* Applied Sciences, 2021. **11**(11): p. 5187.

2. Pangestuti, R. and S.-K. Kim, *Biological activities and health benefit effects of natural pigments derived from marine algae.* Journal of functional foods, 2011. **3**(4): p. 255-266.

3. Patel, A.K., et al., *Algae as an emerging source of bioactive pigments.* Bioresource Technology, 2022. **351**: p. 126910.

4. Wright, S., S. Jeffrey, and R. Mantoura, *Phytoplankton pigments in oceanography: guidelines to modern methods*. 2005: Unesco Pub. Paris, France.

5. Ye, K.X., et al., *A review of pigments derived from marine natural products.* Israel Journal of Chemistry, 2019. **59**(5): p. 327-338.

6. Di Salvo, E., et al., *Natural Pigments Production and Their Application in Food, Health and Other Industries.* Nutrients, 2023. **15**(8): p. 1923.

7. Bandaranayake, W.M., *The nature and role of pigments of marine invertebrates.* Natural Product Reports, 2006. **23**(2): p. 223-255.

8. Mulders, K.J., et al., *Phototrophic pigment production with microalgae: biological constraints and opportunities.* Journal of phycology, 2014. **50**(2): p. 229-242.

9. Choi, W.Y. and H.Y. Lee, *Enhancement of chlorophyll a production from marine spirulina maxima by an optimized ultrasonic extraction process.* Applied Sciences, 2017. **8**(1): p. 26.

10. de Morais, M.G., et al., *Phycocyanin from microalgae: properties, extraction and purification, with some recent applications.* Industrial Biotechnology, 2018. **14**(1): p. 30-37.

11. Płotka-Wasylka, J., et al., *Extraction with environmentally friendly solvents.* TrAC Trends in Analytical Chemistry, 2017. **91**: p. 12-25.

12. Talarico, L. and G. Maranzana, *Light and adaptive responses in red macroalgae: an overview.* Journal of Photochemistry and Photobiology B: Biology, 2000. **56**(1): p. 1-11.

13. Yalçın, S., et al., *HPLC Detection and antioxidant capacity determination of brown, red and green algal pigments in seaweed extracts.* Journal of Chromatographic Science, 2021. **59**(4): p. 325-337.

14. Freitas, M.V., et al., *Red seaweed pigments from a biotechnological perspective.* Phycology, 2021. **2**(1): p. 1-29.

15. Pereira, L. and J.M. Neto, *Marine algae: biodiversity, taxonomy, environmental assessment, and biotechnology*. 2014: CRC Press.

16. Mörschel, E., *The light-harvesting antennae of cyanobacteria and red algae.* Photosynthetica (Praha), 1991. **25**(1): p. 137-144.

17. Mishra, T., et al., *Spirulina: The Beneficial Algae.* International Journal of Applied Microbiology Science., 2013. **2**.

18. Holdt, S.L. and S. Kraan, *Bioactive compounds in seaweed: functional food applications and legislation.* Journal of applied phycology, 2011. **23**: p. 543-597.

19. Mišurcová, L., *Chemical composition of seaweeds.* Handbook of marine macroalgae: biotechnology and applied phycology, 2011: p. 171-192.

20. Morançais, M., J.-L. Mouget, and J. Dumay, *Proteins and pigments*, in *Microalgae in health and disease prevention*. 2018, Elsevier. p. 145-175.

21. Ito, K. and K. Hori, *Seaweed: chemical composition and potential food uses.* Food reviews international, 1989. **5**(1): p. 101-144.

22. Hosikian, A., et al., *Chlorophyll extraction from microalgae: A review on the process engineering aspects.* International journal of chemical engineering, 2010. **2010**.

23. Büchel, C., *Light harvesting complexes in chlorophyll c-containing algae.* Biochimica et Biophysica Acta (BBA)-Bioenergetics, 2020. **1861**(4): p. 148027.

24. Larkum, A.W. and M. Kühl, *Chlorophyll d: the puzzle resolved.* Trends in plant science, 2005. **10**(8): p. 355-357.

25. Nonomura, Y., et al., *Spectroscopic properties of chlorophylls and their derivatives. Influence of molecular structure on the electronic state.* Chemical physics, 1997. **220**(1-2): p. 155-166.

26. Manivasagan, P., et al., *Marine natural pigments as potential sources for therapeutic applications.* Critical reviews in biotechnology, 2018. **38**(5): p. 745-761.

27. Ferruzzi, M.G. and J. Blakeslee, *Digestion, absorption, and cancer preventative activity of dietary chlorophyll derivatives.* Nutrition research, 2007. **27**(1): p. 1-12.

28. Batista, A.P., et al., *Rheological characterization of coloured oil-in-water food emulsions with lutein and phycocyanin added to the oil and aqueous phases.* Food hydrocolloids, 2006. **20**(1): p. 44-52.

29. Guedes, A.C., H.M. Amaro, and F.X. Malcata, *Microalgae as sources of carotenoids.* Marine drugs, 2011. **9**(4): p. 625-644.

30. Maoka, T., *Carotenoids as natural functional pigments.* Journal of natural medicines, 2020. **74**(1): p. 1-16.

31. Lichtenthaler, H.K. and C. Buschmann, *Chlorophylls and carotenoids: Measurement and characterization by UV-VIS spectroscopy.* Current protocols in food analytical chemistry, 2001. **1**(1): p. F4. 3.1-F4. 3.8.

32. Wellburn, A.R., *The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution.* Journal of plant physiology, 1994. **144**(3): p. 307-313.

33. Rønsholdt, B. and E. McLean, *Determination of total carotenoid content in rainbow trout muscle by multivariate calibration of VIS reflectance spectra.* Journal of Food Composition and Analysis, 2001. **14**(4): p. 345-357.

34. Singh, N.K., et al., *The phycobilisomes: an early requisite for efficient photosynthesis in cyanobacteria.* EXCLI journal, 2015. **14**: p. 268.

35. Marx, A. and N. Adir, *Allophycocyanin and phycocyanin crystal structures reveal facets of phycobilisome assembly.* Biochimica et Biophysica Acta (BBA)-Bioenergetics, 2013. **1827**(3): p. 311-318.

36. Sonani, R.R., et al., *Recent advances in production, purification and applications of phycobiliproteins.* World journal of biological chemistry, 2016. **7**(1): p. 100.

37. Izadi, M. and M. Fazilati, *Extraction and purification of phycocyanin from spirulina platensis and evaluating its antioxidant and anti-inflammatory activity.* Asian Journal of Green Chemistry, 2018. **2**(4): p. 364-379.

38. Feng, Y., et al., *Crystal structure of geranylgeranyl pyrophosphate synthase (CrtE) involved in cyanobacterial terpenoid biosynthesis.* Frontiers in plant science, 2020. **11**: p. 589.

39. Dammeyer, T. and N. Frankenberg-Dinkel, *Function and distribution of bilinbiosynthesis enzymes in photosynthetic organisms.* Photochemical & Photobiological Sciences, 2008. **7**(10): p. 1121-1130.

40. Beale, S.I., *Enzymes of chlorophyll biosynthesis.* Photosynthesis research, 1999. **60**: p. 43-73.

41. Falkowski, P.G. and J.A. Raven, *Aquatic photosynthesis*. 2013: Princeton University Press.

42. Wang, Y. and T. Chen, *The biosynthetic pathway of carotenoids in the astaxanthin-producing green alga Cehlorella zofingiensis.* World Journal of Microbiology and Biotechnology, 2008. **24**: p. 2927-2932.

43. Baroli, I., et al., *Zeaxanthin accumulation in the absence of a functional xanthophyll cycle protects Chlamydomonas reinhardtii from photooxidative stress.* The Plant Cell, 2003. **15**(4): p. 992-1008.

44. Lohr, M. and C. Wilhelm, *Algae displaying the diadinoxanthin cycle also possess the violaxanthin cycle.* Proceedings of the National Academy of Sciences, 1999. **96**(15): p. 8784-8789.

45. Khan, S.B., et al., *Protective effect of Amphiroa dilatata on ROS induced oxidative damage and MMP expressions in HT1080 cells.* Biotechnology and Bioprocess Engineering, 2010. **15**: p. 191-198.

46. Pisani, P., F. Bray, and D.M. Parkin, *Estimates of the world-wide prevalence of cancer for 25 sites in the adult population.* International journal of cancer, 2002. **97**(1): p. 72-81.

47. Le Tutour, B., et al., *Antioxidant and pro-oxidant activities of the brown algae, Laminaria digitata, Himanthalia elongata, Fucus vesiculosus, Fucus serratus and Ascophyllum nodosum.* Journal of Applied Phycology, 1998. **10**: p. 121-129.

48. Pérez-Gálvez, A., I. Viera, and M. Roca, *Carotenoids and chlorophylls as antioxidants.* Antioxidants, 2020. **9**(6): p. 505.

49. Okai, Y., et al., *Identification of antimutagenic substances in an extract of edible red alga, Porphyra tenera (Asadusa-nori).* Cancer letters, 1996. **100**(1-2): p. 235-240.

50. Cho, M., et al., *Antioxidant properties of extract and fractions from Enteromorpha prolifera, a type of green seaweed.* Food Chemistry, 2011. **127**(3): p. 999-1006.

51. Lanfer-Marquez, U.M., R.M. Barros, and P. Sinnecker, *Antioxidant activity of chlorophylls and their derivatives.* Food research international, 2005. **38**(8-9): p. 885-891.

52. Cahyana, A.H., Y. Shuto, and Y. Kinoshita, *Pyropheophytin a as an antioxidative substance from the marine alga, Arame (Eisenia bicyclis).*

Bioscience, biotechnology, and biochemistry, 1992. **56**(10): p. 1533-1535.

53. Ina, A., et al., *Pheophytin a, a low molecular weight compound found in the marine brown alga Sargassum fulvellum, promotes the differentiation of PC12 cells.* International Journal of Developmental Neuroscience, 2007. **25**(1): p. 63-68.

54. Ina, A. and Y. Kamei, *Vitamin B 12, a chlorophyll-related analog to pheophytin a from marine brown algae, promotes neurite outgrowth and stimulates differentiation in PC12 cells.* Cytotechnology, 2006. **52**: p. 181-187.

55. Hiqashi-Okaj, K., S. Otani, and Y. Okai, *Potent suppressive effect of a Japanese edible seaweed, Enteromorpha prolifera (Sujiao-nori) on initiation and promotion phases of chemically induced mouse skin tumorigenesis.* Cancer letters, 1999. **140**(1-2): p. 21-25.

56. Okai, Y. and K. Higashi-Okai, *Potent anti-inflammatory activity of pheophytin a derived from edible green alga, Enteromorpha prolifera (Sujiao-nori).* International Journal of Immunopharmacology, 1997. **19**(6): p. 355-358.

57. Hoshina, C., K. Tomita, and Y. Shioi, *Antioxidant activity of chlorophylls: Its structure-activity relationship*, in *Photosynthesis: mechanisms and effects*. 1998, Springer. p. 3281-3284.

58. Hsu, C.-Y., et al., *The antioxidant and free radical scavenging activities of chlorophylls and pheophytins.* 2013.

59. Ganesan, P., et al., *Anti-angiogenic effect of siphonaxanthin from green alga, Codium fragile.* Phytomedicine, 2010. **17**(14): p. 1140-1144.

60. Nomura, T., et al., *Proton-donative antioxidant activity of fucoxanthin with 1, 1-diphenyl-2-picrylhydrazyl (DPPH).* IUBMB Life, 1997. **42**(2): p. 361-370.

61. Sasaki, K., et al., *Effects of fucoxanthin addition to ground chicken breast meat on lipid and colour stability during chilled storage, before*

and after cooking. Asian-Australasian Journal of Animal Sciences, 2008. **21**(7): p. 1067-1072.

62. Hosokawa, M., et al., *Fucoxanthin induces apoptosis and enhances the antiproliferative effect of the PPARγ ligand, troglitazone, on colon cancer cells.* Biochimica et Biophysica Acta (BBA)-General Subjects, 2004. **1675**(1-3): p. 113-119.

63. Heo, S.-J., et al., *Evaluation of anti-inflammatory effect of fucoxanthin isolated from brown algae in lipopolysaccharide-stimulated RAW 264.7 macrophages.* Food and chemical toxicology, 2010. **48**(8-9): p. 2045-2051.

64. Maeda, H., et al., *Effect of medium-chain triacylglycerols on anti-obesity effect of fucoxanthin.* Journal of Oleo Science, 2007. **56**(12): p. 615-621.

65. Das, S.K., et al., *Fucoxanthin induces apoptosis in osteoclast-like cells differentiated from RAW264. 7 cells.* Journal of Agricultural and Food Chemistry, 2010. **58**(10): p. 6090-6095.

66. Shimoda, H., et al., *Anti-pigmentary activity of fucoxanthin and its influence on skin mRNA expression of melanogenic molecules.* Journal of Pharmacy and Pharmacology, 2010. **62**(9): p. 1137-1145.

67. Yabuta, Y., et al., *Antioxidant activity of the phycoerythrobilin compound formed from a dried Korean purple laver (Porphyra sp.) during in vitro digestion.* Food Science and Technology Research, 2010. **16**(4): p. 347-352.

68. Gabr, G.A., S.M. El-Sayed, and M.S. Hikal, *Antioxidant activities of phycocyanin: A bioactive compound from Spirulina platensis.* J. Pharm. Res. Int, 2020. **32**: p. 73-85.

69. Renugadevi, K., et al., *Antioxidant activity of phycocyanin pigment extracted from marine filamentous cyanobacteria Geitlerinema sp TRV57.* Biocatalysis and agricultural biotechnology, 2018. **16**: p. 237-242.

70. Jadaun, P., et al., *Elucidation of Antiviral and Antioxidant Potential of C-Phycocyanin against HIV-1 Infection through In Silico and In Vitro Approaches.* Antioxidants, 2022. **11**(10): p. 1942.

71. Cherng, S.-C., et al., *Anti-inflammatory activity of c-phycocyanin in lipopolysaccharide-stimulated RAW 264.7 macrophages.* Life sciences, 2007. **81**(19-20): p. 1431-1435.

72. Lobo, V., et al., *Free radicals, antioxidants and functional foods: Impact on human health.* Pharmacognosy reviews, 2010. **4**(8): p. 118.

73. Je, J.-Y., P.-J. Park, and S.-K. Kim, *Antioxidant activity of a peptide isolated from Alaska pollack (Theragra chalcogramma) frame protein hydrolysate.* Food Research International, 2005. **38**(1): p. 45-50.

74. Pradhan, B., et al., *Bioactive metabolites from marine algae as potent pharmacophores against oxidative stress-associated human diseases: A comprehensive review.* Molecules, 2020. **26**(1): p. 37.

75. Fung, A., N. Hamid, and J. Lu, *Fucoxanthin content and antioxidant properties of Undaria pinnatifida.* Food chemistry, 2013. **136**(2): p. 1055-1062.

76. Lourenço-Lopes, C., et al., *Biological action mechanisms of fucoxanthin extracted from algae for application in food and cosmetic industries.* Trends in Food Science & Technology, 2021. **117**: p. 163-181.

77. Wu, Q., et al., *The antioxidant, immunomodulatory, and anti-inflammatory activities of Spirulina: an overview.* Archives of toxicology, 2016. **90**(8): p. 1817-1840.

78. Pagels, F., et al., *Phycobiliproteins from cyanobacteria: Chemistry and biotechnological applications.* Biotechnology Advances, 2019. **37**(3): p. 422-443.

79. Goodman, G.E., et al., *The association between lung and prostate cancer risk, and serum micronutrients: results and lessons learned*

from β-carotene and retinol efficacy trial. Cancer Epidemiology Biomarkers & Prevention, 2003. **12**(6): p. 518-526.

80. Parker, R.S., *Carotenoids in human blood and tissues.* The Journal of nutrition, 1989. **119**(1): p. 101-104.

81. Toniolo, P., et al., *Serum carotenoids and breast cancer.* American Journal of Epidemiology, 2001. **153**(12): p. 1142-1147.

82. Hosokawa, M., et al., *Apoptosis-inducing effect of fucoxanthin on human leukemia cell line HL-60.* Food Science and Technology Research, 1999. **5**(3): p. 243-246.

83. Kotake-Nara, E., A. Asai, and A. Nagao, *Neoxanthin and fucoxanthin induce apoptosis in PC-3 human prostate cancer cells.* Cancer letters, 2005. **220**(1): p. 75-84.

84. Kaewkroek, K., et al., *Anti-inflammatory activity of compounds from Kaempferia marginata rhizomes.* Songklanakarin Journal of Science & Technology, 2017. **39**(1).

85. Pan, M.-H., et al., *Anti-inflammatory activity of traditional Chinese medicinal herbs.* Journal of traditional and complementary medicine, 2011. **1**(1): p. 8-24.

86. Kim, E.J., et al., *Fucoidan present in brown algae induces apoptosis of human colon cancer cells.* BMC gastroenterology, 2010. **10**: p. 1-11.

87. Shiratori, K., et al., *Effects of fucoxanthin on lipopolysaccharide-induced inflammation in vitro and in vivo.* Experimental eye research, 2005. **81**(4): p. 422-428.

88. Hasani-Ranjbar, S., Z. Jouyandeh, and M. Abdollahi, *A systematic review of anti-obesity medicinal plants-an update.* Journal of Diabetes & Metabolic Disorders, 2013. **12**: p. 1-10.

89. Abidov, M., et al., *The effects of Xanthigen™ in the weight management of obese premenopausal women with non-alcoholic fatty*

liver disease and normal liver fat. Diabetes, obesity and metabolism, 2010. **12**(1): p. 72-81.

90. Okuzumi, J., et al., *Inhibitory effects of fucoxanthin, a natural carotenoid, on N-myc expression and cell cycle progression in human malignant tumor cells.* Cancer letters, 1990. **55**(1): p. 75-81.

91. Aguilar-Machado, D., et al., *Effect of ohmic heating processing conditions on color stability of fungal pigments.* Food Science and Technology International, 2017. **23**(4): p. 338-348.

92. Prabakaran, G., et al., *Extraction and characterization of phycocyanin from Spirulina platensis and evaluation of its anticancer, antidiabetic and antiinflammatory effect.* International Journal of Biological Macromolecules, 2020. **153**: p. 256-263.

93. Tavanandi, H.A., et al., *Simple and efficient method for extraction of C-Phycocyanin from dry biomass of Arthospira platensis.* Algal research, 2018. **31**: p. 239-251.

94. Jaeschke, D.P., et al., *Phycocyanin from Spirulina: A review of extraction methods and stability.* Food Research International, 2021. **143**: p. 110314.

95. Aarthy, A., et al., *An insight on algal cell disruption for biodiesel production.* Asian J Pharm Clin Res, 2018. **11**(2): p. 21-26.

96. Salinas-Salazar, C., et al., *Methods for extraction of valuable products from microalgae biomass.* Microalgae biotechnology for development of biofuel and wastewater treatment, 2019: p. 245-263.

97. Kumar, S.J., et al., *Sustainable green solvents and techniques for lipid extraction from microalgae: A review.* Algal Research, 2017. **21**: p. 138-147.

98. Kim, D.-Y., et al., *Cell-wall disruption and lipid/astaxanthin extraction from microalgae: Chlorella and Haematococcus.* Bioresource technology, 2016. **199**: p. 300-310.

99. Mendes-Pinto, M., et al., *Evaluation of different cell disruption processes on encysted cells of Haematococcus pluvialis: effects on astaxanthin recovery and implications for bio-availability.* Journal of Applied Phycology, 2001. **13**: p. 19-24.

100. Zou, T.-B., et al., *Response surface methodology for ultrasound-assisted extraction of astaxanthin from Haematococcus pluvialis.* Marine drugs, 2013. **11**(5): p. 1644-1655.

101. Zuorro, A., G. Maffei, and R. Lavecchia, *Optimization of enzyme-assisted lipid extraction from Nannochloropsis microalgae.* Journal of the Taiwan Institute of Chemical Engineers, 2016. **67**: p. 106-114.

102. Tavanandi, H.A., P. Vanjari, and K. Raghavarao, *Synergistic method for extraction of high purity Allophycocyanin from dry biomass of Arthrospira platensis and utilization of spent biomass for recovery of carotenoids.* Separation and Purification Technology, 2019. **225**: p. 97-111.

103. Sierra, L.S., C.K. Dixon, and L.R. Wilken, *Enzymatic cell disruption of the microalgae Chlamydomonas reinhardtii for lipid and protein extraction.* Algal Research, 2017. **25**: p. 149-159.

104. Gong, M. and A. Bassi, *Carotenoids from microalgae: A review of recent developments.* Biotechnology advances, 2016. **34**(8): p. 1396-1412.

105. Vernès, L., et al., *Biorefinery concept as a key for sustainable future to green chemistry—the case of microalgae.* Plant Based “Green Chemistry 2.0” Moving from Evolutionary to Revolutionary, 2019: p. 15-50.

106. Sastry, S.K., et al., *Why ohmic heating? Advantages, applications, technology, and limitations*, in *Ohmic heating in food processing*. 2014, CRC Press. p. 7-16.

107. Geada, P., et al., *Electrotechnologies applied to microalgal biotechnology–Applications, techniques and future trends.* Renewable and Sustainable Energy Reviews, 2018. **94**: p. 656-668.

108. Costa, J.A.V., et al., *Progress in the physicochemical treatment of microalgae biomass for value-added product recovery.* Bioresource technology, 2020. **301**: p. 122727.

109. Martínez, J.M., et al., *C-phycocyanin extraction assisted by pulsed electric field from Artrosphira platensis.* Food Research International, 2017. **99**: p. 1042-1047.

110. Luengo, E., et al., *Effect of pulsed electric field treatments on permeabilization and extraction of pigments from Chlorella vulgaris.* The Journal of membrane biology, 2014. **247**: p. 1269-1277.

111. Luengo, E., et al., *A comparative study on the effects of millisecond- and microsecond-pulsed electric field treatments on the permeabilization and extraction of pigments from Chlorella vulgaris.* The Journal of membrane biology, 2015. **248**: p. 883-891.

112. Martínez, J.M., et al., *Pulsed electric field permeabilization and extraction of phycoerythrin from Porphyridium cruentum.* Algal Research, 2019. **37**: p. 51-56.

113. Saini, R.K. and Y.-S. Keum, *Carotenoid extraction methods: A review of recent developments.* Food chemistry, 2018. **240**: p. 90-103.

114. Abrahamsson, V., et al., *Multicomponent inverse modeling of supercritical fluid extraction of carotenoids, chlorophyll A, ergosterol and lipids from microalgae.* The Journal of Supercritical Fluids, 2018. **139**: p. 53-61.

115. Machmudah, S., et al., *Supercritical fluids extraction of valuable compounds from algae: Future perspectives and challenges.* Engineering Journal, 2018. **22**(5): p. 13-30.

116. Macías-Sánchez, M.D., et al., *Extraction of carotenoids and chlorophyll from microalgae with supercritical carbon dioxide and ethanol as cosolvent.* Journal of separation science, 2008. **31**(8): p. 1352-1362.

117. Hosseini, S.R.P., O. Tavakoli, and M.H. Sarrafzadeh, *Experimental optimization of SC-CO2 extraction of carotenoids from Dunaliella salina.* The Journal of Supercritical Fluids, 2017. **121**: p. 89-95.

118. Jaime, L., et al., *β-Carotene isomer composition of sub-and supercritical carbon dioxide extracts. Antioxidant activity measurement.* Journal of agricultural and food chemistry, 2007. **55**(26): p. 10585-10590.

119. Guedes, A.C., et al., *Supercritical fluid extraction of carotenoids and chlorophylls a, b and c, from a wild strain of Scenedesmus obliquus for use in food processing.* Journal of Food Engineering, 2013. **116**(2): p. 478-482.

120. Gallego, R., M. Bueno, and M. Herrero, *Sub-and supercritical fluid extraction of bioactive compounds from plants, food-by-products, seaweeds and microalgae–An update.* TrAC Trends in Analytical Chemistry, 2019. **116**: p. 198-213.

121. Dey, S. and V.K. Rathod, *Ultrasound assisted extraction of β-carotene from Spirulina platensis.* Ultrasonics sonochemistry, 2013. **20**(1): p. 271-276.

122. Fernandes, A.S., et al., *Identification of chlorophyll molecules with peroxyl radical scavenger capacity in microalgae Phormidium autumnale using ultrasound-assisted extraction.* Food Research International, 2017. **99**: p. 1036-1041.

123. Natarajan, R., et al., *Lipid releasing characteristics of microalgae species through continuous ultrasonication.* Bioresource technology, 2014. **158**: p. 7-11.

124. Lee, S.Y., et al., *Cell disruption and lipid extraction for microalgal biorefineries: A review.* Bioresource technology, 2017. **244**: p. 1317-1328.

125. Tsubaki, S., et al., *Microwave-assisted hydrothermal extraction of sulfated polysaccharides from Ulva spp. and Monostroma latissimum.* Food chemistry, 2016. **210**: p. 311-316.

126. Juin, C., et al., *Microwave-assisted extraction of phycobiliproteins from Porphyridium purpureum.* Applied biochemistry and biotechnology, 2015. **175**: p. 1-15.

127. Pasquet, V., et al., *Study on the microalgal pigments extraction process: Performance of microwave assisted extraction.* Process Biochemistry, 2011. **46**(1): p. 59-67.

128. Andersen, R.A., *Algal culturing techniques*. 2005: Elsevier.

129. Porra, R.J., *The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b.* Photosynthesis research, 2002. **73**: p. 149-156.

130. Aminot, A. and F. Rey, *Standard procedure for the determination of chlorophyll a by spectroscopic methods.* International Council for the Exploration of the Sea, 2000. **112**: p. 25.

131. Eijckelhoff, C. and J.P. Dekker, *A routine method to determine the chlorophyll a, pheophytin a and β-carotene contents of isolated photosystem II reaction center complexes.* Photosynthesis research, 1997. **52**: p. 69-73.

132. Hooker, S.B., et al., *The third seawifs HPLC analysis round-robin experiment (seaharre-3)*. 2009.

133. Wright, S., et al., *Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton.* Marine ecology progress series, 1991: p. 183-196.

134. Lourenço-Lopes, C., et al., *A HPLC-DAD method for identifying and estimating the content of fucoxanthin, β-carotene and chlorophyll a in brown algal extracts.* Food Chemistry Advances, 2022. **1**: p. 100095.

135. Louda, J.W., *HPLC-based chemotaxonomy of Florida Bay phytoplankton: Difficulties in coastal environments.* Journal of liquid chromatography & related technologies, 2007. **31**(3): p. 295-323.

136. Jeffrey, S.t. and G. Humphrey, *New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton.* Biochemie und physiologie der pflanzen, 1975. **167**(2): p. 191-194.

137. Rowland, S.M., S. Van Wychen, and L.M. Laurens, *Identification and Quantification of Photosynthetic Pigments in Algae. Laboratory Analytical Procedure (LAP), Issue Date: October 27, 2022*. 2022, National Renewable Energy Lab.(NREL), Golden, CO (United States).

138. Coskun, O., *Separation techniques: chromatography.* Northern clinics of Istanbul, 2016. **3**(2): p. 156.

139. Sestak, Z., *PAPER CHROMATOGRAPHY OF CHLOROPLAST PIGMENTS (CHLOROPHYLLS AND CAROTENOIDS). III.* 1980.

140. Foote, J., *Thin Layer Chromatography (TLC) of Chlorophyll Pigments.* American Biology Teacher, 1984. **46**(4): p. 226-29.

141. Omata, T. and N. Murata, *Preparation of chlorophyll a, chlorophyll b and bacteriochlorophyll a by column chromatography with DEAE-Sepharose CL-6B and Sepharose CL-6B.* Plant and cell physiology, 1983. **24**(6): p. 1093-1100.

142. Jeffrey, S., S. Wright, and M. Zapata, *Recent advances in HPLC pigment analysis of phytoplankton.* Marine and Freshwater Research, 1999. **50**(8): p. 879-896.

143. Jubert, C. and G. Bailey, *Isolation of chlorophylls a and b from spinach by counter-current chromatography.* Journal of Chromatography A, 2007. **1140**(1-2): p. 95-100.

144. Ettre, L., *Chromatography: The separation technique of the 20th century.* Chromatographia, 2000. **51**: p. 7-17.

145. Pretsch, E., et al., *Structure determination of organic compounds*. 2000: Springer.

146. Rojas, F.S. and C.B. Ojeda, *Recent development in derivative ultraviolet/visible absorption spectrophotometry: 2004–2008: A review.* Analytica chimica acta, 2009. **635**(1): p. 22-44.

147. Giechaskiel, B. and M. Clairotte, *Fourier transform infrared (FTIR) spectroscopy for measurements of vehicle exhaust emissions: A review.* Applied Sciences, 2021. **11**(16): p. 7416.

148. Shin, J., et al., *Pro-oxidant actions of carotenoids in triggering apoptosis of cancer cells: A review of emerging evidence.* Antioxidants, 2020. **9**(6): p. 532.

149. Srinivas, U.S., et al., *ROS and the DNA damage response in cancer.* Redox biology, 2019. **25**: p. 101084.

150. Ribeiro, D., et al., *Antioxidant and pro-oxidant activities of carotenoids and their oxidation products.* Food and Chemical Toxicology, 2018. **120**: p. 681-699.

151. Arathi, B.P., et al., *Enhanced cytotoxic and apoptosis inducing activity of lycopene oxidation products in different cancer cell lines.* Food and chemical toxicology, 2016. **97**: p. 265-276.

152. León-González, A.J., C. Auger, and V.B. Schini-Kerth, *Pro-oxidant activity of polyphenols and its implication on cancer chemoprevention and chemotherapy.* Biochemical pharmacology, 2015. **98**(3): p. 371-380.

153. Chen, Q., et al., *Pharmacologic doses of ascorbate act as a prooxidant and decrease growth of aggressive tumor xenografts in mice.* Proceedings of the National Academy of Sciences, 2008. **105**(32): p. 11105-11109.

154. Carocho, M. and I.C. Ferreira, *A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives.* Food and chemical toxicology, 2013. **51**: p. 15-25.

155. Usuki, R., Y. Endo, and T. Kaneda, *Prooxidant activities of chlorophylls and pheophytins on the photooxidation of edible oils.* Agricultural and Biological Chemistry, 1984. **48**(4): p. 991-994.

156. Halliwell, B., *How to characterize a biological antioxidant.* Free radical research communications, 1990. **9**(1): p. 1-32.

157. Apak, R.a., et al., *Antioxidant activity/capacity measurement. 1. Classification, physicochemical principles, mechanisms, and electron transfer (ET)-based assays.* Journal of agricultural and food chemistry, 2016. **64**(5): p. 997-1027.

158. Sun, Y., C. Yang, and R. Tsao, *Nomenclature and general classification of antioxidant activity/capacity assays.* Measurement of Antioxidant Activity & Capacity: Recent Trends and Applications, 2018: p. 1-19.

159. Dagnino-Leone, J., et al., *Phycobiliproteins: Structural aspects, functional characteristics, and biotechnological perspectives.* Computational and Structural Biotechnology Journal, 2022.

160. Prior, R.L., X. Wu, and K. Schaich, *Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements.* Journal of agricultural and food chemistry, 2005. **53**(10): p. 4290-4302.

161. Rubio, C.P., et al., *Spectrophotometric assays for total antioxidant capacity (TAC) in dog serum: an update.* BMC Veterinary Research, 2016. **12**(1): p. 166.

162. Romulo, A. *The principle of some in vitro antioxidant activity methods*. in *IOP Conference Series: Earth and Environmental Science*. 2020. IOP Publishing.

163. Arnao, M.B., *Some methodological problems in the determination of antioxidant activity using chromogen radicals: a practical case.* Trends in Food Science & Technology, 2000. **11**(11): p. 419-421.

164. Robertson, C.A., D.H. Evans, and H. Abrahamse, *Photodynamic therapy (PDT): a short review on cellular mechanisms and cancer*

research applications for PDT. Journal of Photochemistry and Photobiology B: Biology, 2009. **96**(1): p. 1-8.

165. Castano, A.P., T.N. Demidova, and M.R. Hamblin, *Mechanisms in photodynamic therapy: part two—cellular signaling, cell metabolism and modes of cell death.* Photodiagnosis and photodynamic therapy, 2005. **2**(1): p. 1-23.

166. Kwiatkowski, S., et al., *Photodynamic therapy–mechanisms, photosensitizers and combinations.* Biomedicine & pharmacotherapy, 2018. **106**: p. 1098-1107.

167. Nowak-Stepniowska, A., P. Pergoł, and A. Padzik-Graczyk, *Photodynamic method of cancer diagnosis and therapy--mechanisms and applications.* Postepy biochemii, 2013. **59**(1): p. 53-63.

168. Li, B., et al., *Photosensitized singlet oxygen generation and detection: Recent advances and future perspectives in cancer photodynamic therapy.* Journal of biophotonics, 2016. **9**(11-12): p. 1314-1325.

169. Fresnadillo, D.G. and S. Lacombe, *Reference photosensitizers for the production of singlet oxygen.* Singlet Oxygen: Applications in Biosciences and Nanosciences, 2016. **1**: p. 105-143.

170. Acién, F.G., et al., *Economics of microalgae production*, in *Microalgae-based biofuels and bioproducts*. 2017, Elsevier. p. 485-503.

171. Munir, N., et al., *Harvesting and processing of microalgae biomass fractions for biodiesel production (a review).* Sci Tech Dev, 2013. **32**(3): p. 235-243.

172. Ruiz-Ruiz, F., J. Benavides, and M. Rito-Palomares, *Scaling-up of a B-phycoerythrin production and purification bioprocess involving aqueous two-phase systems: Practical experiences.* Process Biochemistry, 2013. **48**(4): p. 738-745.

173. Molina Grima, E., E. Belarbi, and F. Acién-Fernández, *Recovery of 430 microalgal biomass and metabolites: process options and economics.*

431 Biotechnol Adv 20: 491–515. doi: 10.1016. S0734-9750 (02), 2003: p. 00050-2.

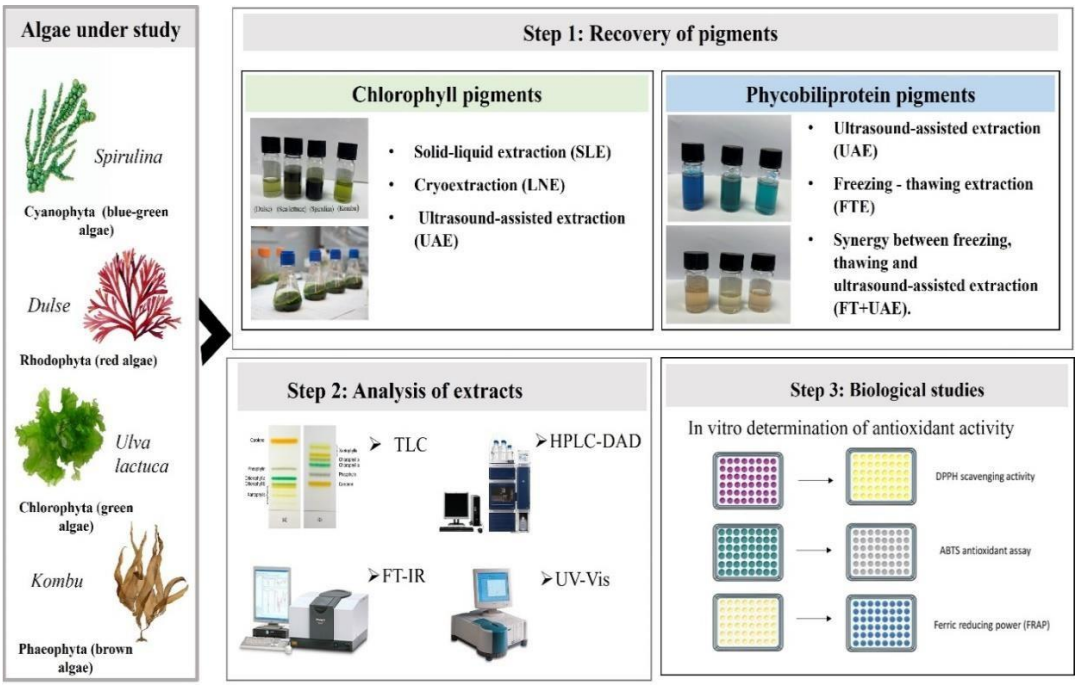
174. Pérez-López, P., et al., *Life cycle assessment of the production of bioactive compounds from Tetraselmis suecica at pilot scale.* Journal of Cleaner Production, 2014. **64**: p. 323-331.

175. Deenu, A., S. Naruenartwongsakul, and S.M. Kim, *Optimization and economic evaluation of ultrasound extraction of lutein from Chlorella vulgaris.* Biotechnology and bioprocess engineering, 2013. **18**: p. 1151-1162.

Chapter 3 Extraction and Characterization of Photosynthetic Pigments from Four Types of Marine Algae and Evaluation of their Antioxidant Activity.

Highlights

- The extraction of chlorophyll pigments and phycobiliproteins (C-PC and R-PC) from the pigment biomass of four different algae species was performed using different techniques.
- Under ultrasonic extraction conditions, the highest chlorophyll *a* content was obtained.
- The combination of ultrasonic with the main extraction procedures (freezing and thawing) results in a synergistic effect, leading to enhanced production and purity of phycobiliproteins.
- Optimizing extraction procedures can result in both increased extraction yield and the preservation of high radical scavenging efficacy in extracts containing high levels of intact chlorophyll *a* and phycobiliproteins.



Abstract

Marine algae are widely used in the food industry as pharmaceuticals and cosmetics due to their high phytochemical content and beneficial effects on human health. However, extracting bioactive chemicals remains a challenge due to the complex chemical nature of marine algae. This study focused on investigating photosynthetic pigments in several algae species, such as *Spirulina*, *Dulse*, *Sea lettuce* and *Kombu*, with particular emphasis on the following pigments: chlorophyll *a* and phycobiliproteins. This study assessed the extraction yield and amount of chlorophyll *a* in extracts by employing three different approaches to disrupt the cell wall: solid-liquid extraction, cryoextraction, and ultrasonic extraction, while using acetone as a solvent in all these methods. Ultrasound-assisted extraction resulted in the highest quantities of total chlorophyll *a* for all algae species. Among the studied algae, *Spirulina* extract exhibited the highest concentration of chlorophyll *a*, measuring around 34.2 mg/g. This was followed by *Dulse* extract at around 8.8 mg/g and *Sea lettuce* at approximately 2.7 mg/g. The lowest concentration observed in the *Kombu* extract was 1.5 mg/g. TLC and HPLC-DAD analysis of the extracts, revealed the presence of pheophytin *a* and pheophorbide *a*, which are the degradation products of chlorophyll *a*. The *Spirulina* extract exhibited the lowest percentage of degradation, while the highest percentage of degradation was in the *Kombu* extracts. To extract phycocyanin from *Spirulina* and phycoerythrin from *Dulse*, ultrasound-assisted extraction and six cycles of freezing/thawing were used. Water was used as a solvent in all these methods. The combination of ultrasonication and freezing/thawing (four cycles) resulted in the highest yield of phycocyanin (10.5 mg/g) with a purity of 0.87, as well as phycoerythrin (4.55 mg/g) with a purity of 0.34. The comparative analysis of the extracts demonstrated an improvement in the radical scavenging activity (DPPH, TEAC, and FRAP) of the extracts subjected to ultrasound, in contrast to the extracts obtained using the other approaches.

3.1 Introduction

Marine algae have been utilized since ancient times as a highly nutritious food and have been extensively researched for various applications, such as a dietary supplement, a medicinal functional food, cosmetics, and other biomaterials [1, 2]. However, in most cases, it is more cost-effective to utilize dried marine algae directly instead of extracting the biologically active elements, such as phycocyanin, xanthophyll, chlorophylls, etc. This is due to the challenging extraction procedures involved and the high instability of these components when exposed to light, elevated temperatures, extraction solvents, etc. [3, 4]. Therefore, the consumption of marine algae powder is more common than extract forms, despite its unpalatable taste [2].

The extracts of phycobiliproteins (phycocyanin and phycoerythrin) and xanthophyll, which are unique components found in marine algae, have been extensively researched due to their bioactive properties. These substances have demonstrated various beneficial biological functions, such as anticancer and anti-inflammatory activities, as well as their use as natural pigments [2, 5]. Nevertheless, the extraction of chlorophyll *a* from marine algae has not been studied excessively due to the alternative resources containing natural chlorophylls [6]. More specifically, the demand for natural chlorophyll *a* has increased because of its many biological qualities, such as potent antioxidant, anti-obesity, anti-cancer, and anti-ageing benefits [1, 2].

Obtaining pure chlorophyll *a* from natural sources has been a challenge due to the complexity of its purification procedures and the need for careful handling to prevent damage from heat and light during the process. That is why the cost of natural chlorophyll *a* is significantly higher and has experienced a substantial increase in comparison to other chlorophylls, such as chlorophyll *b* and *c.* [6, 7]. The current methods of obtaining chlorophyll *a* and phycobiliprotein pigments from algae involve extracting them from wet biomass, which has a high susceptibility to spoilage, for example, microbial spoilage is caused by microorganisms such as fungi (molds, yeasts) and bacteria. Drying biomass extends its shelf-life, benefiting small-scale industries by eliminating the need for extensive space and skill to cultivate biomass. Nevertheless, there

have been reports indicating that dry biomass is unsuitable for the extraction of pigments from algae due to its significant resistance to cell walls. In practice, there is a scarcity of reports that attempt to identify an appropriate technique for extracting pigments from dried algal biomass [8-10].

Current research on extracting chlorophyll *a* from marine algae has primarily focused on utilizing the supercritical extraction method. This involves experimenting with different factors, such as solvent ratio, temperature, and pressure. There are multiple publications showing that the significant quantities of chlorophyll from plant sources can be extracted by the optimization of these variables [11, 12]. Nevertheless, most ideal conditions were observed at elevated temperatures ranging from 60 to 80 °C. However, this would lead to a significant decrease in effectiveness due to the low levels of intact chlorophyll present in the extracts. Furthermore, it should be noted that the extraction solvents commonly employed in these studies like methanol and ether, are not suitable for the extraction due to their inherent toxicity. However, they have been utilized to demonstrate a superiority in extracting chlorophyll compared to ethanol or acetone. Nevertheless, the utilization of certain solvents that are not suitable for consumption in food related products may also impose restrictions on their usage in certain sectors such as drug-cosmetic industries [13]. In contrast, the present research on extracting phycobiliprotein mostly evolves around the utilization of the freezing and thawing technique, which takes a long time to perform. This involves conducting experiments with several variables, such as the type of solvents, the ratio of solvents, the duration and the number of freezing and thawing cycles [14].

The process of ultrasonic extraction is commonly employed to efficiently disrupt cell walls. However, if performed without the application of additional heat, it can result in the deterioration of even thermostable bioactive substances present in biomass. This is attributed to the high frequency of intense ultrasonic vibrations. The introduction of the extraction solvent will induce the formation of numerous cavitation bubbles. The subsequent rupture of these bubbles near the cell walls will produce shock waves and liquid jets, which can efficiently dismantle solid cell membranes in a shorter duration [15, 16]. Nevertheless, the utilization of ultrasonic extraction techniques in industrial

settings has several challenges, including the need for sizable equipment and increased process controls [17].

The aim of this study is to improve the extraction of chlorophyll *a* and phycobiliprotein pigments (phycocyanin and phycoerythrin) from the dried edible algae biomass by using a simple ultrasound technique, either by itself or in combination with other techniques, while keeping the temperature low. In addition, unlike many existing studies on enhancing extraction procedures, this research assesses the antioxidant activity of the extracts obtained from the enhanced method by comparing them with the extracts obtained from the conventional extraction technique.

3.2 Materials and methods

3.2.1 Algae samples and Chemicals.

In this study, four different samples of marine algae were used: 1) green algae (*Chlorophyta*: *Ulva lactuca*, common name: *Sea lettuce*); 2) red algae (*Rhodophyta*: *Palmaria palmata*, common name: *Dulse*); 3) brown algae (*Phaeophyta*: *Laminaria japonica*, common name: *Kombu*); and 4) blue-green algae (*Cyanophyta*: *Arthrospira platensis*, common name: *Spirulina*) to extract pigments and perform analytical characterization of their extracts. Three marine algae, *Phaeophyta*, *Rhodophyta*, and *Chlorophyta*, were purchased from the Oemine Nature brand. These samples were received without natural residues and in large, dry forms. The samples were crushed until they obtained a homogeneous powder. As for the *Cyanophyta* alga, which was purchased from the Nukraft brand, it was received without natural residues and in powder and dry form. All chemicals and solvents were obtained from Fisher Scientific (Loughborough, UK) and Sigma Aldrich (Dorset, UK) with an HPLC analytical grade.

3.2.2 Pigment standards

In a study by Shorog *et al* [18], lutein, pheophytin *a*, pheophorbide *a* and β-carotene were separated from *Spirulina* and used as standards in this study.

The powders were stored in the dark at -20 °C in tightly sealed, opaque glass containers for further analysis.

3.2.3 Pigments Extraction

3.2.3.1 Recovery of chlorophyll pigments

Solid-liquid extraction (SLE)

All algae species were subjected to solid-liquid extraction (SLE). In all cases, 10 g of algae powder samples were mixed with 40 mL of acetone. The mixture was heated at +50 °C under reflux and stirred at 800 rpm for 24 h. Then, the mixture was filtered by centrifugation at 2,012 × g (6,000 rpm) for 15 minutes. The green acetone extract was evaporated under reduced pressure, yielding 300 mg, 100 mg, 35 mg, and 20 mg of solid residue for *Spirulina*, *Dulse*, *Sea lettuce*, and *Kombu*, respectively. These samples were analysed while they were fresh.

Cryoextraction

All algal species were subjected to cryoextraction by liquid nitrogen **(LNE)**. Approximately 10 g of dry algae was mixed with 40 mL of acetone in a 100 mL beaker. Then liquid nitrogen was added until the algae mixture was frozen. After that, the frozen algae were transferred to a two-necked flask with a capacity of 250 mL, and the mixture was heated under reflux and stirred for 3 h under a nitrogen atmosphere. Then, the mixture was filtered by centrifugation at 2,012 × g (6,000 rpm) for 15 minutes. The green acetone extract was evaporated under reduced pressure, yielding 500 mg, 200 mg, 50 mg, and 35 mg of solid residue for *spirulina*, *dulse, sea lettuce,* and *kombu,* respectively. These samples were analysed while they were fresh.

Ultrasound-assisted extraction (UAE)

All algae species were subjected to ultrasound-assisted extraction **(UAE)**. 10 g of powdered algae samples were mixed with 40 mL of acetone. The mixture was sonicated in a sonication bath at +50 °C and 40 kHz for 3 h. After that, the mixture filtrate was centrifuged at 2012× g (6000 rpm) for 15 min. The

green acetone extract was evaporated under reduced pressure, yielding 740 mg, 400 mg, 100 mg, and 85 mg of solid residue for *Spirulina*, *Dulse, Sea lettuce,* and *Kombu,* respectively. These samples were analysed while they were fresh.

3.2.3.2 Recovery of phycobiliprotein pigments

Ultrasound-assisted extraction (UAE)

Dulse (red algae) and *Spirulina* (cyanobacteria) were subjected to ultrasound-assisted extraction (UAE). 10 g of powdered algae samples were mixed with 40 mL of water. The mixture was sonicated through a sonication bath at +50 °C and 40 kHz for 3 h. After that, the mixture filtrate was centrifuged at 2012× g (6000 rpm) for 15 min. This yields a blue aqueous solution (30 mL) from *Spirulina* containing phycocyanin and a red aqueous solution from *Dulse* containing phycoerythrin. These solutions were subjected to spectroscopic analysis.

Freezing - thawing extraction (FTE)

Dulse (red algae) and *Spirulina* (cyanobacteria) were exposed to six cycles of freezing and thawing. Each cycle consisted of 24 hours of freezing at a temperature of -20 ± 2 °C, followed by 3 hours of thawing at a temperature of +50 °C using a water bath. Approximately 10 g of each alga was dispersed in 40 mL of distilled water and allowed to freeze for 24 h in the freezer (− 20 ◦C). Then the mixture was thawed at +50 ◦C using a water bath. The mixture reached +50 °C after two hours of thawing time. The mixture was centrifuged at 1207 × ‘g force’ for 15 min, yielding a blue aqueous solution (30 mL) from *Spirulina* containing phycocyanin and a red aqueous solution from *Dulse* containing phycoerythrin. These solutions were subjected to spectroscopic analysis.

Combined method utilizing freezing, thawing and ultrasound-assisted extraction (FT-UAE).

Dulse (red algae) and *Spirulina* (cyanobacteria) were subjected to ultrasonication (for 30 min) followed by freezing and thawing (6 cycles, 24 h of freezing and 3 h of thawing). Approximately 10 g of each of the algae powders was dispersed in 40 mL of distilled water. The mixture was sonicated in a sonication bath at +50 °C and 40 kHz for 3 h. After that, the mixture was allowed to freeze for 24 hours at −20 °C, followed by 2 hours of thawing at a temperature of +50 °C using a water bath. The mixture was centrifuged at 1207 × 'g force' for 15 min, yielding a blue aqueous solution (30 mL) from *Spirulina* containing phycocyanin and a red aqueous solution from *Dulse* containing phycoerythrin. These solutions were subjected to spectroscopic analysis.

3.2.4 Characterisation of extracts

UV-vis spectroscopy of photosynthetic pigments

A Cary 50 UV-Vis (Shimadzu, UK) (≤ 1.5 nm optical resolution) was used to perform the identification and quantification of pigments in the prepared extracts. Stock solutions from the pigment extracts were prepared at a concentration of 1 mg/mL. For chlorophyll pigment extracts, 40 µL of a stock solution was placed into a quartz cuvette with a track length of 1 cm. This was mixed with 3 mL of 80% acetone, and for phycobiliprotein pigment extracts, water was used as solvents. The absorbance of the chlorophyll extracts was checked at 663 nm, 645 nm, and 470 nm compared to a blank reagent (80% acetone). For phycobiliprotein pigment extracts, the absorbance was checked at 652 nm and 620 nm compared to a blank reagent (water). Welburn equations (1, 2, and 3) were used to find the concentration of chlorophyll *a*, chlorophyll *b*, and total carotenoids in chlorophyll pigment extracts [19].

$$C_{chlorophyll\,a}\ (\underline{\mu g}_{ml}) = 12.25 \times A_{663} - 2.79 \times A_{645} \quad (1)$$

$$C_{chlorophyll\,b}\ (\underline{\mu g}_{ml}) = 21.5 \times A_{645} - 5.10 \times A_{663} \quad (2)$$

$$C_{carotenoids}\left(\frac{\mu g}{ml}\right) = \frac{1000 \times A_{470} - 1.82\, C_{chlorophyll\ a} - 85.02\, C_{chlorophyll\ b}}{198} \quad (3)$$

The concentration of phycocyanin and phycoerythrin in water extracts was determined using the formulae provided by Bennett and Bogorad (1973) [20].

$$C_{\text{phycocyanin}}\left(\frac{\text{mg}}{ml}\right) = \frac{A_{620} - 0.474 \times A_{652}}{5.34}$$

$$C_{\text{allophycocyanin}}\left(\frac{mg}{ml}\right) = \frac{A_{652} - 0.208 \times A_{620}}{5.09}$$

$$C_{\text{phycoerythrin}}\left(\frac{\text{mg}}{ml}\right) = \frac{[A_{652} - 2.41[C_{\text{phycocyanin}}]] - 0.89[C_{\text{allophycocyanin}}]}{9.62}$$

where *A* is absorbance (a.u.) at the maximum wavelength of the corresponding pigment. The concentration of each pigment is expressed as mg of pigment per g of powdered algae (mg/g).

The purity of the extracted phycocyanin (C-PC) and phycoerythrin (R-PC) was determined as follows:

$$\text{Purity (phycocyanin)} = \frac{A_{620}}{A_{280}}$$

$$\text{Purity (phycoerythrin)} = \frac{A_{562}}{A_{280}}$$

where A620 nm and A562 nm represent the highest absorption of phycocyanin and phycoerythrin, while A280 nm represents protein absorption.

3.2.5 Thin Layer Chromatography (TLC)

Pigment initial separation was achieved using thin-layer chromatography (TLC). The experiment utilized Merck silica gel 60 F254 precoated aluminum plates (Darmstadt, Germany) as the stationary phase. The chlorophyll pigment extracts were dissolved in acetone and applied individually as spot along a line approximately 1 cm above the bottom of the TLC plate using a micropipette. Subsequently, the TLC plate was positioned in a vertical way within a sealed container that contained the mobile phase. Approximately 0.5 cm of the solvent system was added to the container. This study used mobile-phase hexane and acetone in different ratios (70:30, v/v), (80:20, v/v), and (90:10, v/v). The solvents ascended the plate gradually through capillary action. The procedure ceased when the solvent reached its maximum capacity. After completing the elution process, the pigments were determined based on their color and the retention factors' RF values.

The retention factors (RF) were computed for each spot using the following formula:

$$R\text{F}(spot) = (distance\ the\ spot\ has\ moved)/\ (distance\ solvent\ front\ moved)$$

3.2.6 High performance liquid chromatography pigment Analysis

Identification and quantification of chlorophyll pigments in the acetone extracts was performed using high-performance liquid chromatography coupled to a diode array detector (HPLC-DAD) using the method described in Lorenço-Lopes *et al* [21]. The Agilent 1260 Infinity HPLC equipment (Santa Clara, CA, USA) equipped with a 4.6×100 mm, 3.5 µm Poroshell 120 EC column C18 was used for the analysis. The solvents were: A) 5 mM ammonium acetate in water; B) 5 mM ammonium acetate in MeOH; and C) ethyl acetate. The run conditions were that the flow rate was 0.5 mL/min and the injection volume was 50 µL. The gradient was as follows (minutes; % solvent A; % solvent B; % solvent C): (0.01; 30; 70; 0), (2; 5; 95; 0), (8; 5; 95; 0), (9; 0; 50; 50), (20; 0; 50; 50), (20.01; 50; 50; 0) (35; 70; 30; 0). The UV wavelength was set at 254 nm, 410 nm, 450 nm, and 670 nm to detect pigments in extracts. The DAD chromatogram peak area at 410 nm and 450 nm was used to determine the amount of the main pigments in algae extracts.

3.2.7 Fourier transfer infrared analysis (FT-IR)

The phycocyanin and phycoerythrin in aqueous extracts were analyzed by recording their FT-IR spectra on a Bruker Alpha Platinum (ATR FTIR) spectrometer with specific vibrational frequencies in the 1 and 100 cm^{-1} scans to elucidate the chemical structure and the type of molecular bonding in phycocyanin and phycoerythrin.

3.2.8 Assessment of antioxidant activities

3.2.8.1 DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The antioxidant activity of the pigment extracts was evaluated using a modified method previously described by Hsu *et al* [22]. A fresh stock solution of DPPH (120 µM) in EtOH and a series of the standard Trolox solution covering a range of 0-750 µM in ethanol/DMSO were prepared. A 96-well plate was used to perform the DPPH assay. Briefly, 10 µL of sample or Trolox standard were mixed with 300 µL of DPPH reagent and incubated for 30 min at room temperature. Subsequently, absorbance was measured at 515 nm. The inhibition ratio (scavenging activity) of the DPPH was calculated by the equation:

$$Scavenging\ activity\ (\%) = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

where $A_{control}$ is the absorbance of the DPPH in ethanol and $A_{samples}$ is the absorbance of the samples containing DPPH. In this assay, samples and standards were run in triplicate, and the results were expressed as mean ± standard deviation as in mg Trolox equivalents per gram of sample.

3.2.8.2 ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) decolorization assay

The pigment extracts were evaluated for their ability to inhibit the radical 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+}$), according to Fernando *et al* [23]. $ABTS^{+}$ stock solution (14 mM) was mixed in a 1:1 ratio (v/v) with potassium sulfate peroxide (4.9 mM) in the dark at room temperature for 12 or 24 h to form radicals. The $ABTS^{+}$ working solution was prepared by

diluting the $ABTS^+$ solution with ethanol to reach an initial absorbance of 0.700 ± 0.020 at 734 nm. A standard solution of Trolox (10 mM) was prepared to cover a range of 0-750 µM in ethanol. A 96-well plate was used to perform the ABTS assay. Then, 10 µL of standard Trolox or pigment extract samples were mixed with 300 µL of $ABTS^+$ solution and incubated for 10 min at room temperature in the dark. Then, the absorbance was measured at 734 nm. The decolorization effect of each sample or standard was calculated for the absorbance of the control ($ABTS^{\bullet+}$ solvent) at 734 nm by the equation:

$$Decolorization(\%) = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

In this assay, samples and standards were run in triplicate, and the results were expressed as mean ± standard deviation as in mg Trolox equivalents per gram of sample.

3.2.8.3 Ferric-reducing antioxidant power (FRAP) assay.

Ferric-reducing antioxidant power (FRAP) was determined by the spectrophotometric method previously described by Govindappa et al. (2014) [24]. Three stock solutions were prepared: A) 20 mM ferric chloride solution; B) 10 mM TPTZ (2,4,6-tri(2-pyridyl)-1,3,5-triazine) solution in 40 mM HCl; and C) 300 mM acetate buffer (3.1 g sodium acetate and 16 mL glacial acetic acid), pH 3.6. The working solution was prepared by mixing A, B, and C in a 1:1:10 ratio (v/v). A standard solution of Trolox (10 mM) was prepared to cover a range of 0-1000 µM in ethanol. Briefly, 10 µL of standard Trolox or pigment extract samples were mixed with 300 µL of FRAP reagent and incubated for 15 min at 37 °C. Subsequently, the absorbance of the colored product was taken at 593 nm. The reducing power was calculated by the equation:

$$Reducing\ power\ (\%) = \frac{(A_{sample} - A_{control})}{(A_{-\ \text{Highest Abs of Standard}} - A_{control})} \times 100$$

In this assay, samples and standards were run in triplicate, and the results were expressed as mean ± standard deviation as in mg Trolox equivalents per gram of sample.

3.2.9 Data analysis

All experiments were carried out in triplicate. All results were expressed as the mean value ± standard deviation (SD). The graphs were plotted with Origin Pro 2020 for Windows, and Chem Draw Prime 2019 for Windows was used to represent chemical structures.

3.3 Results and discussion

3.3.1 The effect of different techniques on extraction yields and purity of photosynthetic pigments.

The yield of chemical extraction depends on various factors, including the polarity of solvents, pH, extraction duration, temperature, cell wall disruption method, and the chemical content of the material. The solvent, the mechanism of cell wall degradation, and the chemical characteristics of the sample are the important factors under identical time and temperature conditions. Frequently employed solvents for extracting pigments from algae in the literature include methanol, ethanol, butanol, acetone, chloroform, and water. Researchers also utilized various procedures to disrupt the cell wall, such as ultrasound, microwave, cryoextraction, and enzymatic extraction, to maximize the extraction yield [25-29].

In the present study, the acetone solvent was selected to obtain chlorophyll extracts from the algae. According to the literature, acetone can be the best solvent for extracting chlorophyll for several reasons. The degradation of chlorophyll in acetone may be less than in polar solvents such as ethanol, which may increase the activity of chlorophyllase. It is an enzyme whose activity increases mainly with disruption of the cell wall. This enzyme is not desirable for its activity during the extraction process because it leads to the degradation of chlorophyll during or after extraction [30, 31]. Also, acetone might be more selective at extracting chlorophyll than ethanol as ethanol also extracts proteins and phenolic compounds and acts as a precipitation agent for sugars, which make up many chemical parts of algae [27, 32].

To enhance the efficiency of the extraction process in this investigation, mechanical and non-mechanical techniques were employed to disrupt the cell wall. The utilization of an ultrasonic bath has been employed to extract chlorophyll and phycobiliprotein pigments. This is since these mechanical procedures promote the breakage of cell walls, the infiltration of organic solvents into the cell membrane, and the dissolution of lipids and lipoproteins [16]. Extraction has also been conducted by subjecting algae to freezing using liquid nitrogen to obtain the chlorophyll pigments and freezing at a temperature of -20 °C to obtain the phycobiliprotein pigments. Cryoextraction operates as a non-mechanical process and induces substantial thermal shock to the material, leading to a more effective breakdown of cell walls [31, 33]. As a control experiment, solid-liquid extraction was conducted to extract chlorophyll pigments, while freezing and thawing cycles were performed to extract phycobiliprotein pigments. Freezing and subsequent thawing is a commonly employed technique for extracting phycobiliproteins [34].

The ratio of solid to liquid (S/L) in the extraction procedures for chlorophyll and phycobiliproteins has been standardized to 1:4 in the present study. This is because when extracting pigments, the selection of the solid-to-liquid S/L ratio must be done carefully, as a higher S/L ratio is preferred for achieving a better yield, while a lower S/L ratio is chosen for obtaining a higher level of purity [10]. The ultrasound parameters used for extracting chlorophyll from the algae in this investigation were adjusted according to the method conditions outlined by Choi and Lee [35] for extracting chlorophyll from dried *Spirulina*, with certain adjustments. The characteristics were as follows: frequency of 40 kHz, temperature of +50 °C, and duration of 3 h. The parameters for the ex- traction process of phycobiliproteins were adjusted according to the work con- ducted by Tavanandi *et al* [10] with some modifications, which involved the extraction of phycocyanin from dry *Spirulina*. These parameters included the duration of freezing (24 h) and thawing (3 h) and the number of freeze-thaw treatment cycles (6 cycles), as well as the ultrasound parameters (frequency of 40 kHz, temperature of +50 °C, and duration of 3 h). The results of the methods used in this study to extract chlorophyll and phycobiliproteins (phy- cocyanin (C-PC) and phycoerythrin (R-PC)) from the algae species were an- alyzed based on the extraction yield (mg/g), target pigment content (mg/g) in

extracts, and purity of the phycobiliproteins using the spectrophotometric methods, as shown in **Table 3.1** and **Figure 3.1**.

Table 3.1. A comparison between different methods for extracting chlorophyll and phycobiliproteins from marine algae under study: *Spirulina* (S), *Dulse* (D), *Sea lettuce* (U) and *Kombu* (K).

Target pigment	**Extraction method**	**Extraction yield (mg/g)**				**Chlorophyll content (mg/g)**				**Purity**	
Algae species		S	D	U	K	S	D	U	K	S C-PC	D R-PC
Chlorophyll	Solid/liquid (SLE)	30	10	3.5	2	9.5	1.1	0.13	0.16		
	Cryoextraction (LNE)	50	20	5	3.5	16.4	3.9	0.59	0.33		
	Ultrasound-assisted (UAE)	74	40	10	8.5	34.2	8.8	2.7	1.5		
Carotenoids	Solid/liquid (SLE)	30	10	3.5	2	3.25	0.009	0.11	0.011		
	Cryoextraction (LNE)	50	20	5	3.5	6.42	0.279	0.19	0.070		
	Ultrasound-assisted (UAE)	74	40	10	8.5	2.57	0.004	0.04	0.034		
Phycobiliprotein	1 cycle (FTE)	30	5			1.35	0.94			0.23	0.093
	2 cycles FT	53	15			2.73	0.96			0.35	0.093
	3 cycles FT	60	20			4.52	1.04			0.39	0.13
	4 cycles FT	63	33			6.3	1.94			0.45	0.14
	5 cycles FT	61	35			5.35	2.01			0.41	0.19
	6 cycles FT	57	34			5.3	1.80			0.38	0.23
	Ultrasound-assisted (UAE)	50	10			3.50	1.89			0.30	0.05
	Synergy between 1 cycle of FTE+ UAE	40	12			2.9	1.99			0.6	0.05
	UAE + 2 cycles FT	66	35			7.45	3.16			0.75	0.23
	UAE + 3 cycles FT	70	50			8.93	4.32			0.81	0.33
	UAE + 4 cycles FT	69	55			10.5	4.55			0.87	0.34
	UAE + 5 cycles FT	68	53			10.154	4.214			0.89	0.33
	UAE + 6 cycles FT	66	53			9.9	4.12			0.89	0.33

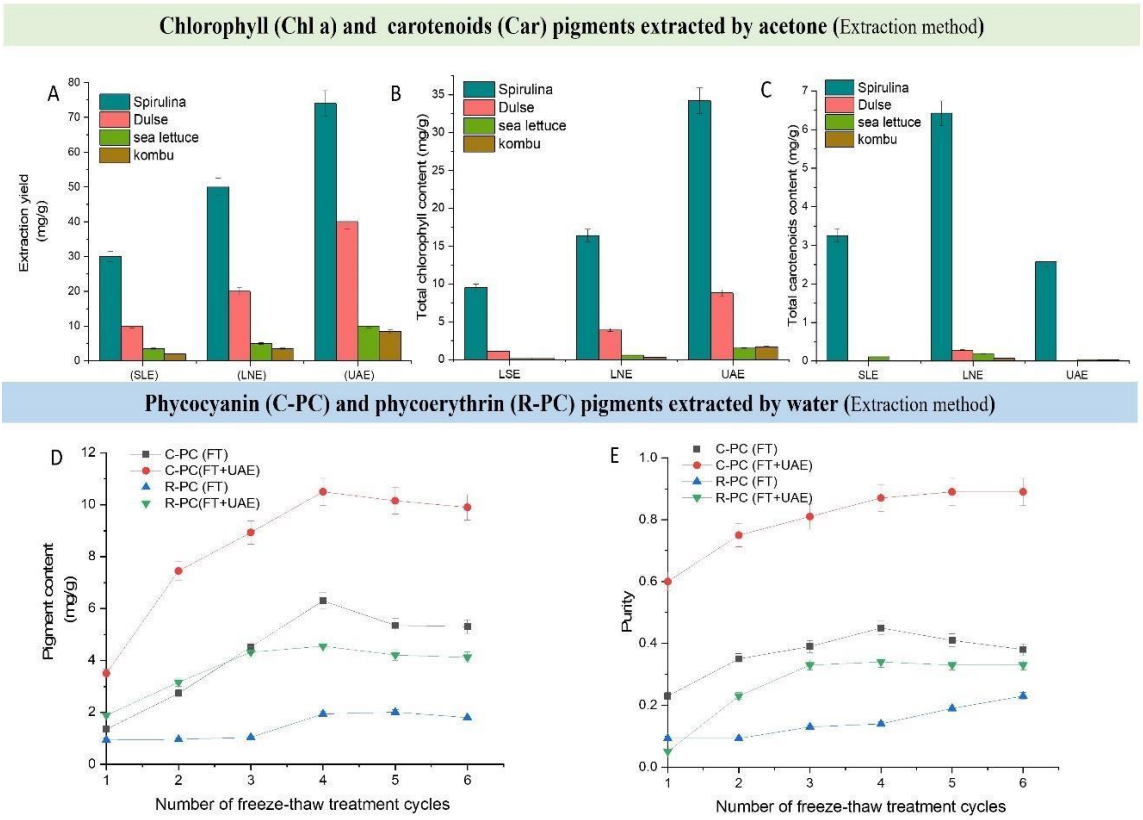


Figure 3.1 A) The effect of the three extraction techniques on the extraction yield (mg/g) to extract chlorophyll: solid-liquid (SLE), cryoextraction (LNE), and ultrasound-assisted extraction (UAE). **B, C)** The effect of the three extraction techniques on the chlorophyll and carotenoids pigment content (mg/g) in extracts. **D)** The impact of three different extraction methods on the amount of phycobiliprotein pigments (phycocyanin (C-PC) and phycoerythrin (R-PC) that were extracted: ultrasound-assisted extraction (UAE), freezing-thawing extraction (FTE), and FT-UAE, which combines freezing, thawing, and ultrasound-assisted extraction. **E**) Degree of purity of phycobiliprotein in aqueous extracts according to the three methods

Indeed, ultrasound-assisted extraction showed superior performance in this study to extract chlorophyll pigments from algae under study compared to cryoextraction and solid-liquid extraction, as ultrasound helped increase the chlorophyll extraction yield and reduce the processing time, as shown in **Figures 3.1A** and **Table 3.1**. Furthermore, the extracts obtained by ultrasound-assisted extraction exhibited lower contamination with carotenoids compared to the extracts obtained by cryoextraction, as depicted in **Figure 3.1B**. This result is consistent with the results of previous studies conducted on ultrasound-

assisted extraction of chlorophyll from dried *Spirulina* [15] and dried seaweed [36].

While ultrasound enhanced the production of phycobiliproteins (C-PC and R-PC), it simultaneously reduced their purity. However, using both ultrasound and freezing-thawing together had a synergistic effect, leading to an increased yield and purity of phycobiliproteins (C-PC and R-PC) as shown in Figure 22 and Table 3.1, while reducing the number of freezing and thawing cycles by two, from six to four. This modification cut the processing time by about 48 hours overall. This result is consistent with the study conducted by Tavanandi *et al* [10] to extract phycocyanin from dried *Spirulina* through the synergistic effects of ultrasound and freezing-thawing method.

When ultrasonic treatment was used at 40 kHz and +50 °C for 3 hours to extract chlorophyll, *Spirulina* extract had the highest amount of chlorophyll *a* (34.2 mg/g), followed by *dulse* (8.8 mg/g), *sea lettuce* (1.5 mg/g), and *Kombu* (1.7 mg/g). The concentrations were much higher than what was found with a solid-liquid extraction at +50 °C for 24 hours, where the chlorophyll content was 9.5 mg/g for *Spirulina*, 1.1 mg/g for *Dulse*, 0.13 mg/g for *Sea lettuce*, and 0.16 mg/g for *Kombu*. In this study, the amount of chlorophyll extracted by ultrasonic extraction from dry *Spirulina* samples was close to what was reported in the study conducted by Majid *et al* [17], where they used acetone and ultrasound to extract chlorophyll from *Spirulina*. However, the chlorophyll extracted from *Spirulina* using ultrasound in this study is two times higher than the amount of chlorophyll reported in the study conducted by Han Jeong *et al* [3] using high-pressure homogenization at 650 bar and a shear stress of approximately 20,000 (1/s).

The content of phycocyanin (C-PC) in aqueous extracts was higher than the content of phycoerythrin (R-PC) in aqueous extracts in all methods used. **Figures 3.1D and E** and **Table 3.1** illustrate the disparity in the efficacy of the three techniques for extracting phycocyanin from *Spirulina* and phycoerythrin from *Dulse*. The figure clearly demonstrates that the yield progressively increases with each cycle, reaching a plateau after the fifth cycle. During the fourth cycle of freezing and thawing, the cell membrane of the biomass was

sufficiently disrupted to release C-PC while minimizing the release of other contaminating proteins from the cytoplasm and other organelles. This led to the achievement of maximal purity and productivity. For freeze-thaw cycles above 4, there was a little increase in the yield of C-PC, but the purity decreased, suggesting the presence of contaminating proteins.

When ultrasound and freeze-thaw cycles are combined, there is an improvement in extraction yield for C-PC from 6.3 to 10.5 mg/g and R-PC from 2.01 to 4.55 mg/g, along with an increase in purity for C-PC from 0.49 to 0.87 and R-PC from 0.141 to 0.34. The improved extraction efficiency might be because of how ultrasound-assisted freezing and thawing work together. This is because sonoporation and the sonocapillary effect work together to disrupt cell membranes and allow fluid to pass through them. Sonoporation would lead to the liberation of cellular contents into the extraction medium. At the same time, the sonocappillary effect is turned on, which increases both the depth and speed at which the solvent can enter the canals through pores. After the freezing process, the development of ice crystals within the cells leads to additional rupture of the cell membrane after thawing, as the membrane is already disturbed by ultrasonication. This leads to an increased leakage of intracellular molecules [37].

The difference in extraction yield and pigment content between the algae under study may be due to differences in the composition of the cell wall. Macroalgae, also known as seaweed, have a more complex and tough cell wall composed of cellulose, sulfur polysaccharides, and alginate. Polysaccharides provide macroalgae with mechanical and osmotic support and protect against environmental stress. On the other hand, the cell wall of microalgae, which are unicellular organisms, is less complex and consists mainly of polysaccharides, including cellulose, hemicellulose, and pectin. The cell wall of some microalgae also contains protein and lipid components [3,4] (**Figure 3.2**).

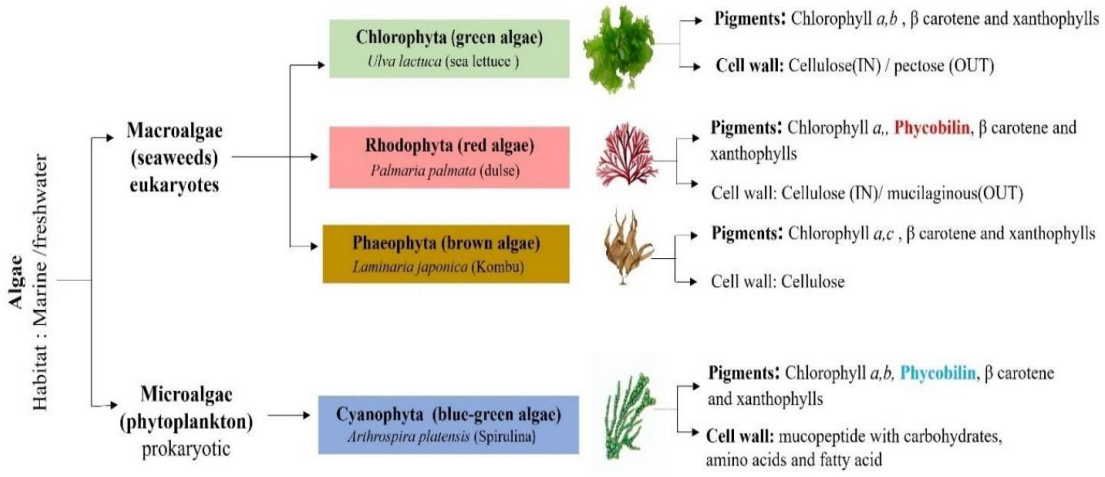


Figure 3.2. Shows the classification of algae under study.

3.3.2 Characterization of chlorophyll extracts from marine algae

A further aim of this study was to characterize and identify chlorophyll pigments in extracts using the selected method that provided the highest concentration of chlorophyll. The ultrasound-assisted extraction procedure, conducted at a frequency of 40 kHz and a temperature of +50 °C for a duration of 3 hours, yielded the highest chlorophyll pigment concentration for all the algae examined in this study. The following sections will therefore provide a detailed description of the chlorophyll extracts from the four algae obtained from ultrasound-assisted extraction by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC-DAD).

Thin-layer Chromatography (TLC)

The pigments extracted from *Spirulina* (S), *Dulse* (D), *Sea lettuce* (U), and *Kombu* (K) were initially separated using thin-layer chromatography (TLC) with varying ratios of hexane and acetone solvents. The identification of pigment classes contained in the four algae extracts is based on the color patterns formed on the TLC plate and the Rf values. The chromatographic profiles were observed using visible light, and the Rf values are provided in **Table 3.2** and **Figure 3.3**. The solvent system was hexane-acetone (70:30, v/v),

which yielded the most effective separation of pigments compared to other ratios (80:20, v/v) and (90:10, v/v). The TLC plate exhibited the following color sequences: green, yellow, yellowish green, bluish green, grey, and orange (**Figure 3.3**). According to Pasang *et al* [38], Chl *a* expressed a blue-green color, Chl *b* expressed a yellow-green color, and carotenoids expressed yellow, orange, and red colors on TLC plates. In addition, the grey spots have been suggested to be derivatives of chlorophyll, such as pheophytin *a* and pheophorbide *a*.

Table 3.2. Summary of the retention factor (RF) values for the pigments separated from *Spirulina* (S), *Dulse* (D), *Sea lettuce* (U), and *Kombu* (K) on the TLC plate eluted with hexane-acetone (70:30, v/v).

Spot	**Color**	**RF**	***Spirulina***	***Sea lettuce***	***Kombu***	***Dulse***
1	Gray	0.09	-	+	-	+
2	Yellow	0.15	-	+	+	+
3	Yellow	0.25	+	+	+	+
4	Blue green	0.33	+	-	-	-
5	Gray	0.47	-	+	+	+
6	Gray	0.5	+	-	+	+
7	Orang	0.92	+	-	-	-

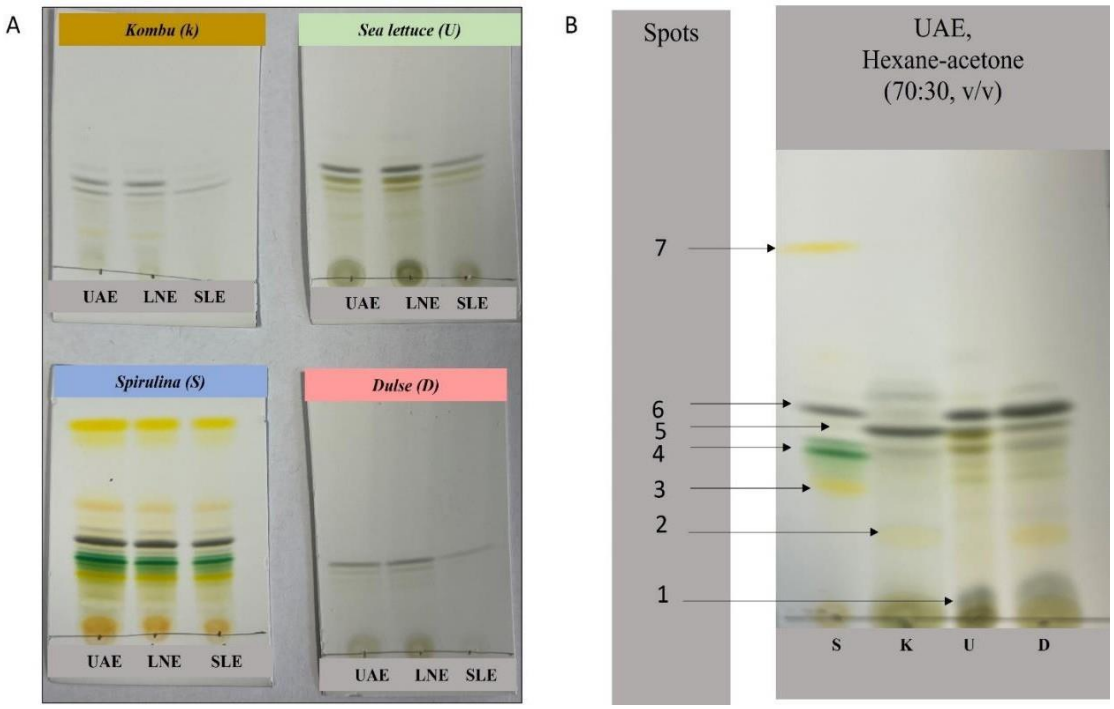


Figure 3.3 Thin-layer chromatography plates for extracted pigments from *Spirulina* (S), *Dulse* (D), *Sea lettuce* (U), and *Kombu* (K) with the solvent system hexane-acetone (70:30, v/v) under visible light.

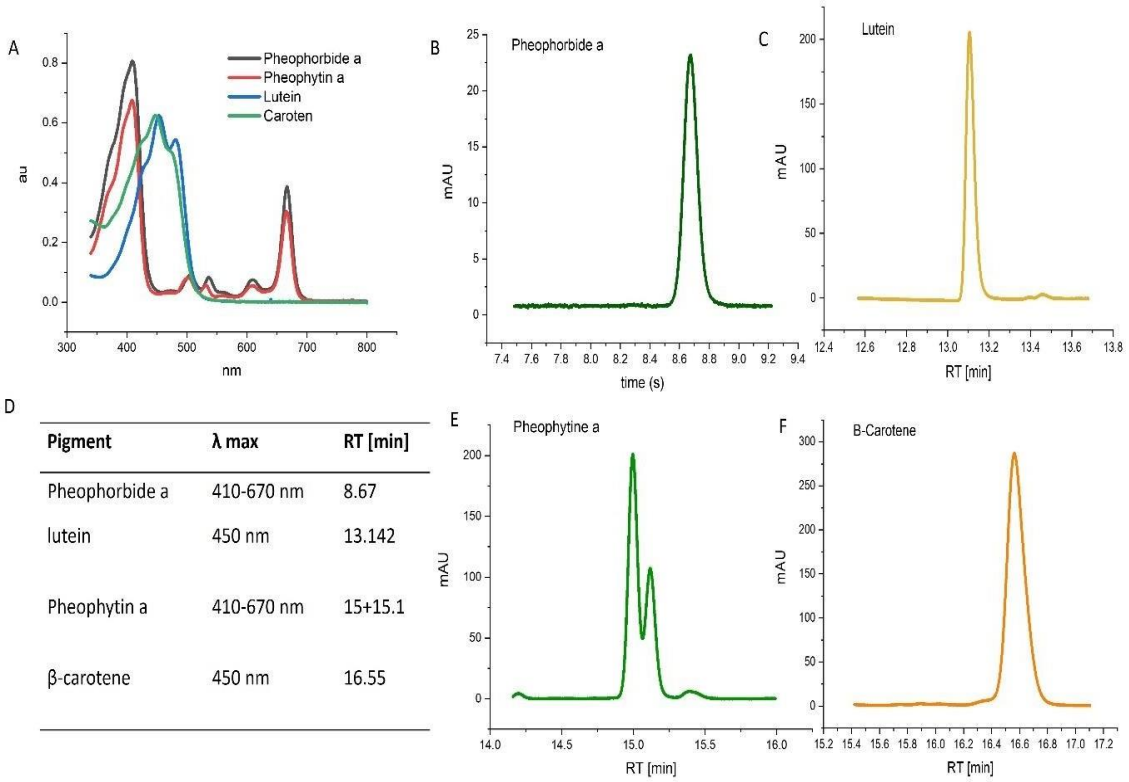
In general, the order of elution of pigments with hexane-acetone (70:30, v/v) went from the pigment with the highest polarity to the pigment with the lowest polarity: xanthophyll (yellow), chlorophyll *a* (blue green), and beta-carotene (yellow-orange). Moreover, the elution of chlorophyll derivatives appeared higher than that of chlorophyll.

The eluted hexane-acetone (70:30, v/v) on TLC for *Spirulina* extract yielded five distinct colored spots. These spots included a yellow spot (RF = 0.25) corresponding to xanthophylls, a distinct green band (RF = 0.33) corresponding to Chl *a*, a grey spot (RF = 0.5) indicative of chlorophyll derivatives, and an orange spot (RF =0.92), consistent with carotene. In the three remaining algae species, *Dulse*, *Sea lettuce*, and *Kombu*, the distinct green band was not present at RF = 0.33, and the orange band was also absent at RF = 0.92. Only two-color spots, yellow and grey, were observed in *dulse* and *kombu*. Regarding *Sea lettuce*, it was noted that there were three distinct color patches (yellow, yellowish green, and grey). The yellowish-green spot indicates the presence of Chl *b* at RF = 0.35.

3.3.3 HPLC-DAD analysis

In this study, HPLC-DAD was used to isolate and characterize photosynthetic pigments based on their polarity, ranging from polar to nonpolar pigments such as chlorophyllide to pheophytin in the chlorophyll group or xanthophyll to carotene in the carotenoid group [39]. Analysis of standard pigments helped to determine the selectivity of the method by comparing their retention times and overlaying the UV spectra with those of the pigment peaks of the extracts at 410 nm, 450 nm, and 670 nm. The amount of pigment in the four extracts being studied was shown by the DAD chromatogram peak area at 410 nm for chlorophyll and its derivatives and 450 nm for carotene and xanthophyll.

The pigments involved in photosynthesis are categorized into three groups based on their metabolic origin: chlorophylls, xanthophylls, and carotenoids. For that reason, four pigment standards were used before the HPLC-DAD separation of the pigments in the extracts: lutein for the xanthophyll group, β-carotene for the carotene group, pheophytin *a* for the chlorophyll *a* group, and pheophorbide *a* for chlorophyll *a* derivatives without a phytyl group. This was done to acquire an initial understanding of the optical characteristics and ascertain the absorption ranges, maximum absorption, and retention times. The UV-vis spectra, chromatograms, and retention times obtained for the four standards are shown in **Figure 3.4**.



Pigment	λ max	RT [min]
Pheophorbide a	410-670 nm	8.67
lutein	450 nm	13.142
Pheophytin a	410-670 nm	15+15.1
β-carotene	450 nm	16.55

Figure 3.4. UV-vis spectra in HPLC-DAD, chromatograms, and retention times for pigment standards eluded by the solvent system: A) 5 mM ammonium acetate in water; B) 5 mM ammonium acetate in MeOH; and C) ethyl acetate and detected at 410 nm for pheophorbide *a* and pheophytin *a*, at 450 nm for lutein and β- carotene.

The UV-vis spectra of β-carotene and lutein exhibited double-peak patterns, with the highest wavelengths occurring between 440 nm and 480 nm, respectively. Pheophytin *a* and pheophorbide *a* exhibited a Qy peak band in the red area of the spectrum at 670 nm, accompanied by a strong Soret band at approximately 410 nm. The λ_{max} at 450 nm appears to be the best detection wavelength for carotenoids and xanthophylls in algae extracts, while the λ_{max} at 410 nm is a suitable wavelength for detecting chlorophyll and its derivatives. Also, chlorophyll and its derivatives can be selectively detected at a wavelength of 670 nm. However, the peaks of maximum absorbance may slightly change with respect to those found in the literature because of the influence of the solvent on the spectroscopic characteristics of the detected molecules [40-42]. Additionally, the relative proportions of the different derivates of each pigment in each sample prepared or obtained using different methods may also have natural variations, e.g., depending on the sources, age, etc. of the

algae [40]. Following spectroscopic detection, the standards were subjected to high-performance liquid chromatography (HPLC-DAD) analysis. The standards were chromatographically separated using a C18 column at a wavelength of 450 nm for the detection of lutein and β-carotene and at wavelengths of 410 nm and 670 nm for the detection of chlorophyll and its derivatives. The retention times for the standard pigments, which are pheophorbide *a*, lutein, pheophytin *a*, and β-carotene, were 8.67 min, 13.1 min, 15.1 min, and 16.55 min, respectively, using the solvent system: A) 5 mM ammonium acetate in water; B) 5 mM ammonium acetate in MeOH; and C) ethyl acetate (**Figure 3.4**). The chromatogram of pheophytin a (**Figure 25, E**) appeared bimodal under these conditions, perhaps due to the racemization facilitated by the solvent system.

Table 3.3. provides a summary of HPLC-DAD data for the photosynthetic pigments separated from the four algal extracts, as well as their corresponding retention times and peak areas. The chromatograms of the four algae extracts obtained are shown in **Appendix A** (**Figures A.1, A.2, A.3**, and **A.4**)**.** In general, the results obtained from HPLC-DAD for the four algal extracts: *Spirulina* (S), *Dulse* (D), *Sea lettuce* (U), and *Kombu* (K) showed the presence of pigments: carotenoids and chlorophyll and their derivatives in different concentrations. The chromatograms of algal extracts contained many overlapping peaks and perhaps several shoulders of pigment isomers, some of which had non-identifiable peaks. When these chromatograms of the studied extracts are compared with the published literature, the reported studies, recorded sim- ilar observations [41-43]. For example, Schmid and Stich (1995) [44] demonstrated that marine algae have a very complex composition containing many different molecules. So, it is expected that the chromatograms of algae extracts will not be as sharp and clear as the standards, so a method for the separation of algae pigments by HPLC cannot be generalized, because each method has its limits.

Table 3.3. HPLC-DAD data of four algae extracts: *Spirulina* (S), *Dulse* (D), *Sea lettuce* (U), and *Kombu* (K) at 410 nm and 450 nm according to the acetone ultrasound-assisted extraction method

	Chlorophylls pigment separated from the four algal extracts at 410 nm and 670 nm						
				Spirulina	*Dulse*	*Sea lettuce*	*Kombu*
Peak	Retention time (min)	Pigment	Symbol	Peak area %			
1	8.42	Pheophorbide a isomer	Pheo 1	nd	3.70	6.40	nd
2	8.67	Pheophorbide a	Pheo 2	nd	12.59	13.63	nd
3	14.19	UNK* Chla derivative	C1	2.47	1.64	2.01	nd
4	14.44	UNK Chla derivative	C2	16.58	5.69	4.48	9.80
4	14.62	UNK Chla derivative	C3	4.52	1.59	11.83	nd
6	15.01	pheophytin a isomer	C4	0.89	2.61	4.76	nd
7	15.12	pheophytin a	C5	2.21	4.43	6.30	36.87
8	15.81	Chl a	C6	60.12	55.84	43.64	38.68
9	16.04	Chl a isomer	C7	13.21	11.91	6.90	14.65
	Carotenoids pigment separated from the four algal extracts at 450 nm						
1	9.82	fucoxanthin	X1	nd	3.77	nd	37.22
2	13.14	Lutein	X2	20.19	nd	6.65	nd
3	16.55	β-carotene	B1	43.15	nd	nd	nd

*UNK: unknow , nd – not detected

The analysis of the *Spirulina* extract by HPLC-DAD indicates the presence of nine distinct peaks (see **Table 3.3**). These peaks were divided into three groups: chlorophylls, xanthophylls, and carotenoids, based on their absorption regions and corresponding standards. At a wavelength of 450 nm, two peaks appeared: X2 and B1. X2, at RT 13.14 min, indicates the presence of lutein when compared to the standard (lutein, RT 13.14 min), while B1, at RT 16.55 min, corresponds to β-carotene compared to the standard (β-carotene, RT 16.55 min). At a wavelength of 410 nm and 670 nm, with a retention time between 14.01 and 16.05 min, seven peaks appeared (C1, C2, C3, C4, C5, C6 and C7). These seven peaks refer to the total chlorophyll *a*, which represents derivatives of chlorophyll and conjugate isomers that cannot be

distinguished spectroscopically despite their structural differences. The C6 peak was identified as Chl *a* according to its absorption spectrum, which showed two bands at 665 nm, and 430 nm. It is also the highest peak in the total chlorophyll *a* with RT 15.8 min. The C7 peak at RT 16.04 min was identified as an isomer of Chl *a* based on most research, which considers this isomer to be typically a small peak that occurs next to the main chlorophyll peak [46]. While the C5 peak at 15.1 min was considered pheophytin *a* based on their comparison with the absorption spectrum and retention time of the corresponding standard (pheophytin *a*, RT 15.1 min). The C4 peak at RT 15.01 min was identified as isomers of pheophytin *a* [46].

C1, C2, and C3 eluted at retention times of 14.19 min, 14.44 min, and 14.62 min, respectively. Determining their structural characteristics was challenging due to the absence of any alterations in their absorption properties compared to other total chlorophyll *a* peaks. Therefore, it has been suggested that it is either diphenyl chlorophyll *a* (DV Chl *a*) and its isomers or chlorophyllids and their isomers based on literature reports [47-48]. Their identification as pheophorbide *a* was excluded because the standard used for this compound showed a retention time of 8.96 min, while these pigments were detected between 14 and 15 min, as shown in **Table 3.3.** When pigments cannot be identified spectroscopically, they can be identified structurally by isolation and purification, and then identified by various spectroscopic methods such as NMR and MS.

This pattern of total chlorophyll *a* containing seven peaks was also observed in the chromatograms for *Dulse*, *Kombu*, and *Sea lettuce*, with retention times close to those recorded for the *Spirulina* sample (see **Table 3.3).** This indicates that the total chlorophyll *a* is similar in all algae species under this study. This complex chlorophyll content creates a challenge for the determination of the individual chlorophyll derivatives in the extracts by spectroscopic methods [49], as there is no drastic difference in the optical properties of chlorophyll, its derivatives or degradation products despite the varied structural features of side chains [47].

In addition to total chlorophyll *a* with a retention time between 14.01 and 16.05 min (detection at wavelengths of 410 nm and 670 nm), the analysis of the HPLC-DAD of *Sea lettuce* and *Dulse* extracts showed a peak with RT 8.67 min. This was identified as pheophorbide *a* based on the standard (pheophorbide a, RT 8.67 min). The xanthophyll content in the macroalgae (*Sea lettuce*, *Dulse*, and *Kombu*) was identified using a detection wavelength of 450 nm. HPLC-DAD analysis of *Sea lettuce* showed a peak belonging to lutein at RT 13.12 min. While *Dulse* and *Kombu* extracts showed a peak at RT 9.82 min (see **Table 3.3**). This peak had a broad and distinct absorption spectrum, with a maximum at 453 nm and by comparison with the literature, it was identified as fucoxanthin. According to the previous reports [50], fucoxanthin is the dominant xanthophyll in brown algae and responsible for its colour. Also, these results were consistent with many other studies of brown and red algae [51, 52].

Overall, the HPLC-DAD analysis showed that xanthophylls, β-carotene, and chlorophyll *a* were present, but only in *Spirulina*. While *Sea lettuce*, *Dulse*, and *Kombu* contained xanthophylls and chlorophyll *a*, β-carotene was not detected. These findings are in line with a study on *Spirulina* by Davani *et al* [55], which reported the presence of lutein, β-carotene, and chlorophyll a as the main pigments in *Spirulina*. On the other hand, a study conducted by Yalçın *et al* [51] on brown, red, and green algae reported that the main pigments generally found in the brown and red species were fucoxanthin, chlorophyll *a*, and pheophytin *a*, but the amount in red algae was generally much lower than that in brown algae. The pigments present in the green algae groups were chlorophyll *a*, chlorophyll *b*, siphonoxanthin, and pheophytin *a*. Additionally, HPLC-DAD analysis confirmed that chlorophyll a is the main pigment in algae extracts, as its peak area in the *Spirulina* extract reached 60.12%, 55.84% in *Dulse* extract, 43.64% in *Sea lettuce* extract and 38.68 % in *Kombu* as shown in **Table 3.3**. Moreover, the *Spirulina* extract contains the lowest amount of degradation products, such as pheophytin *a*. The peak area of pheophytin *a* in the *spirulina* extract was about 2.2%, compared to 36.3% for *kombu*, 6.3% for *Sea lettuce*, and 4.4% for *Dulse*. Other studies [21, 56, 57] have also supported this finding.

3.3.4 Characterization of phycobiliprotein extracts from marine algae

This investigation focused on extracting blue phycocyanin and red phycoerythrin through the following processes: (i) freezing-thawing (6 cycles); (ii) ultrasonic treatment; and (iii) a combination of freezing-thawing and ultrasonic treatment. As discussed in **Section 3.1**, freezing, thawing, and ultrasonic treatment (4 cycles) achieved the optimal content and purity of both phycocyanin and phycoerythrin. UV-vis absorption and FT-IR spectroscopy were used to characterize these two extracts. The phycobiliprotein (PBP) family is categorized into three classes according to their spectral characteristics: phycoerythrin (with a maximum wavelength of 565 nm), phycocyanin (with a maximum wavelength of 620 nm), and allophycocyanin (with a maximum wavelength of 650 nm) [58]. In this study, the blue phycocyanin extract exhibits a peak at 620 nm, whereas the red phycocyanin extract shows an absorption peak at 565 nm, as shown in **Figure 3.5**.

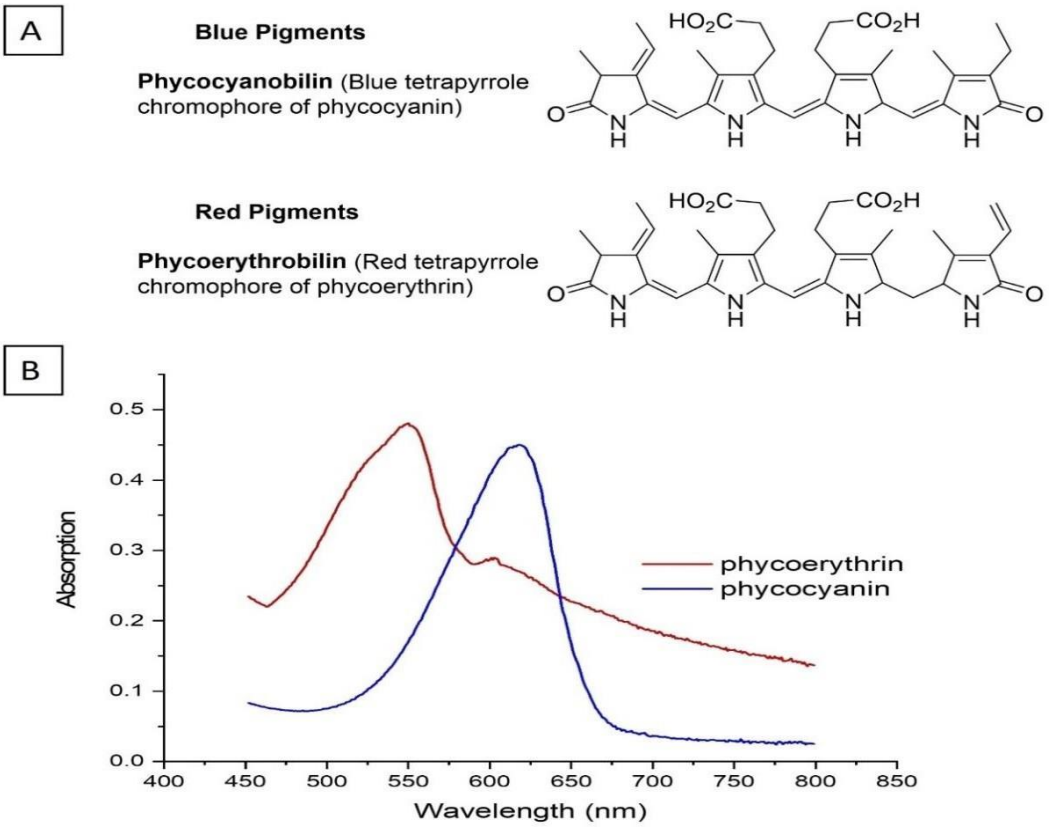


Figure 3.5. (A) General structures of red and blue pigments; (B) The visible absorption spectra of phycocyanin and phycoerythrin

FT-IR spectroscopy was employed to further analyse phycocyanin and phycoerythrin. The FTIR spectra of the two pigment extracts were very similar, as shown in **Figure 3.6**. The main functional groups of phycocyanin and phycoerythrin were identified by comparing the recorded peaks with the literature [14]. The spectrum exhibits peaks at specific wavenumbers: (1) a peak at 3466 cm^{-1} corresponding to stretching vibrations of N–H and O–H; (2) a peak at 1638 cm^{-1} corresponding to stretching vibrations of C–O (amide); (3) a peak at 1460 cm^{-1} corresponding to C—C and O–C–O stretches, and (4) a peak at 1101 cm^{-1} corresponding to C–O stretch.

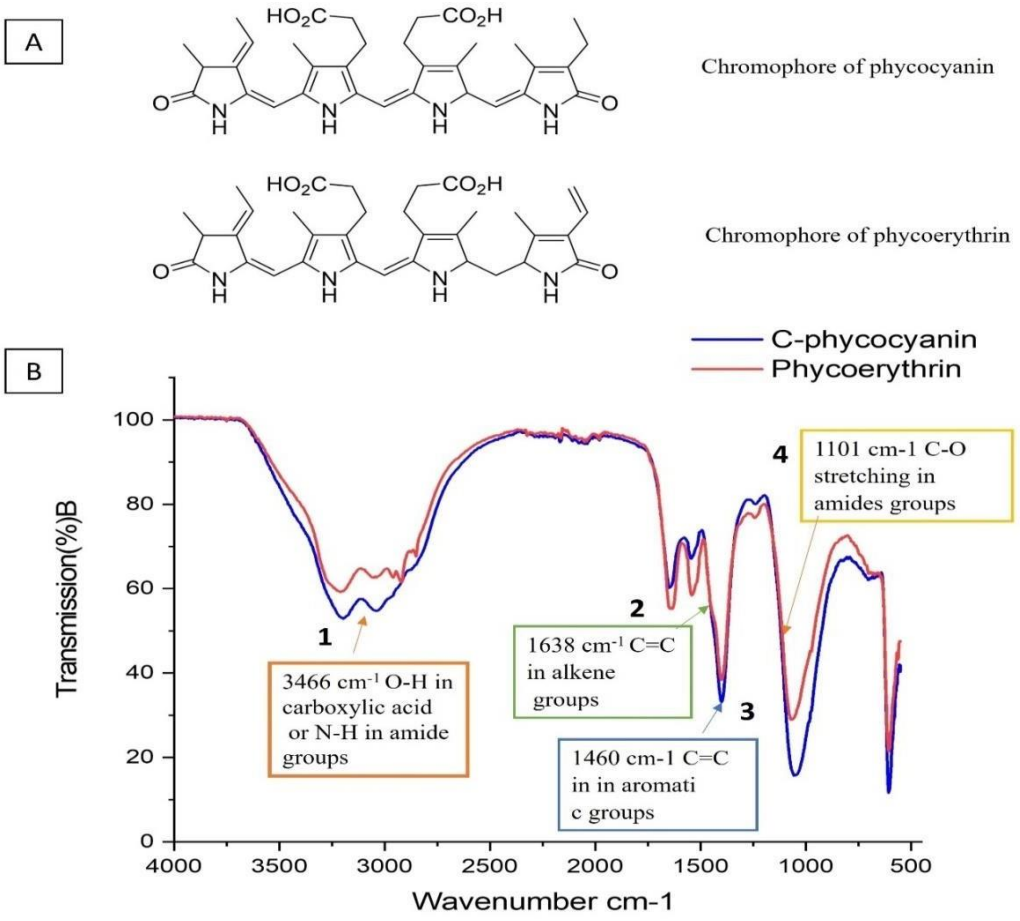


Figure 3.6 (A) General structures of red and blue pigments; (B) distinct absorption regions of phycocyanin and phycoerythrin in FT-IR.

3.3.5 Evaluation of the antioxidant properties of pigments extracts

Over 100 distinct assays have been employed for measuring the antioxidant activity and free radical scavenging capacity of compounds [59]. Various compounds exhibit varying sensitivities to different assays, meaning that a single assay cannot assess the overall antioxidant capacity of all compounds present in the extract, and it is necessary to use several assays with different mechanisms of action to evaluate the entire antioxidant capacity of an extract or molecule [60]. To assess the antioxidant potential of the pigment extracts for the algae species, the current study utilized three commonly used methods: ABTS, DPPH, and FRAP assays.

These methods were chosen due to the complicated chemical compositions of the algae assays. These three approaches for assessing antioxidant activity possess distinct features, including a clear principle and a simple procedure, which demonstrate the capacity of substances to reduce or remove many free radicals, as well as exhibit strong biological relevance [61]. The FRAP assay assesses antioxidant capacity by directly reducing ferric (III) ions to ferrous (II) ions through a single electron transfer (SET) process. On the other hand, DPPH and ABTS evaluate antioxidant capacity by scavenging free radicals using both SET and hydrogen atom transfer (HAT) [62].

Trolox (10 mg/mL) was used as a standard to cover a range of 0-1000 µg/mL, diluted in an ethanol / DMSO mixture (60:40, v/v) **as shown in Figure 3.7, 3.8 and 3.9**. In addition, the results are expressed as mean ± standard deviation as µg Trolox Equivalent Antioxidant Capacity per mL of extracts (µg TE/mL) **as shown in Figure 3.10**.

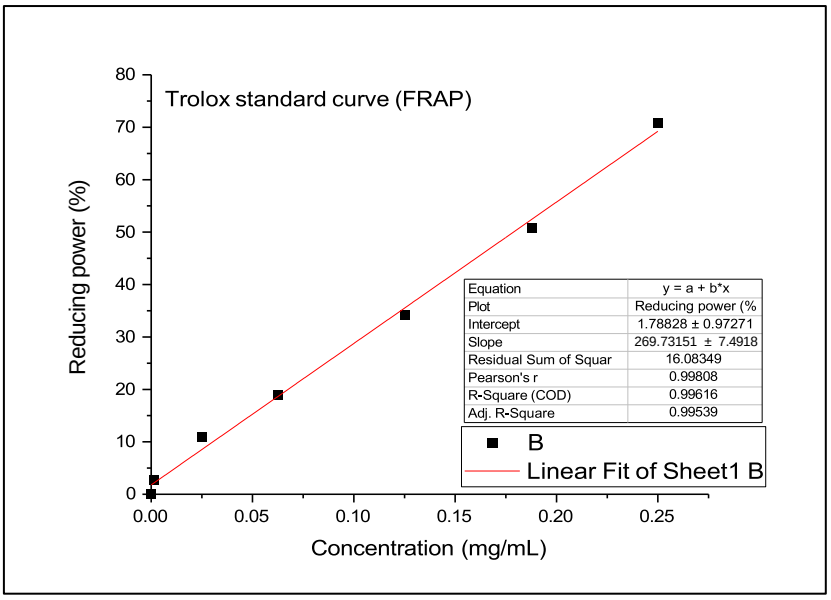


Figure 3.7 Trolox standard curve representing decolorization (%) at different concentrations for Trolox in the range 0-1000 Mm to evaluate of the antioxidant efficacy of pigments extracted from algae under study by FRAP assay.

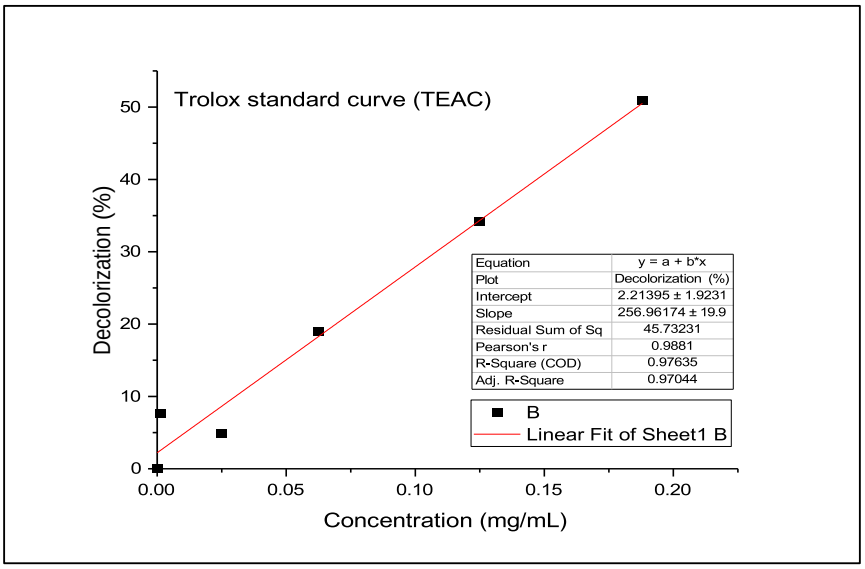


Figure 3.8. Trolox standard curve representing decolorization (%) at different concentrations for Trolox in the range 0-1000 Mm to evaluate of the antioxidant efficacy of pigments extracted from algae under study by TEAC assay.

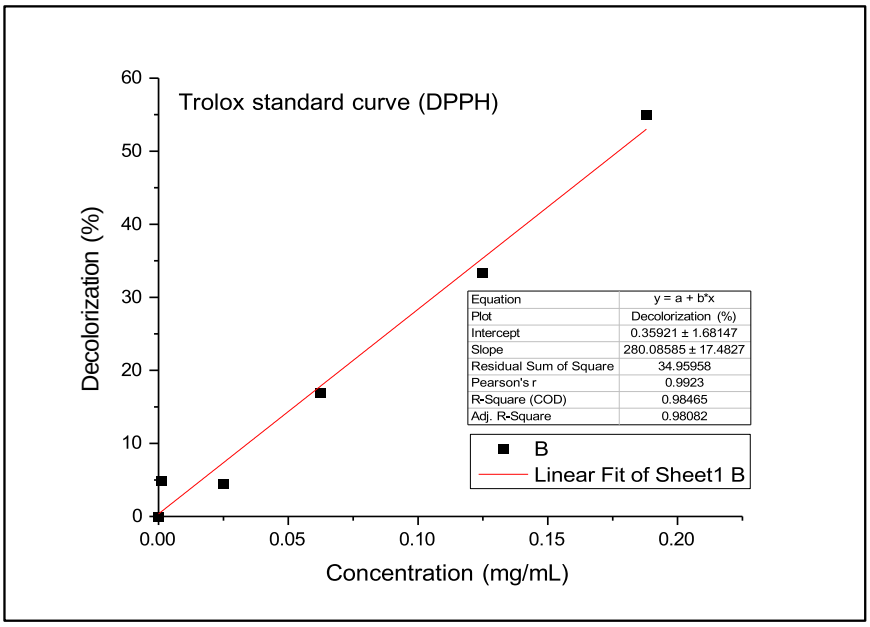


Figure 3.9. Trolox standard curve representing decolorization (%) at different concentrations for Trolox in the range 0-1000 Mm to evaluate of the antioxidant efficacy of pigments extracted from algae under study by DPPH assay.

Figure 3.10 presents the antioxidant activity of chlorophyll, phycocyanin, and phycoerythrin extracts from the algae species. The antioxidant effects of chlorophyll extracts from *Spirulina* (S), *Dulse* (D), *Sea lettuce* (U), and *Kombu* (K) appear in **Figures 3.10 A, B, and C**. These extracts were obtained using solid-liquid extraction (SLE), cryoextraction (LNE), and ultrasound-assisted extraction (UAE). The antioxidant effects of the phycocyanin extracts from *Spirulina* and the phycoerythrin extracts from *Dulse* are apparent in **Figures 3.10 D, E, and F**. These extracts were obtained using the freeze-thaw method (FTE 6 cycles) as well as the combination of freezing and thawing with ultrasound (FT+UAE 4 cycles). The antioxidant activity of these extracts was compared to the extracts obtained from a single freeze-thaw cycle (FTE 1 cycle).

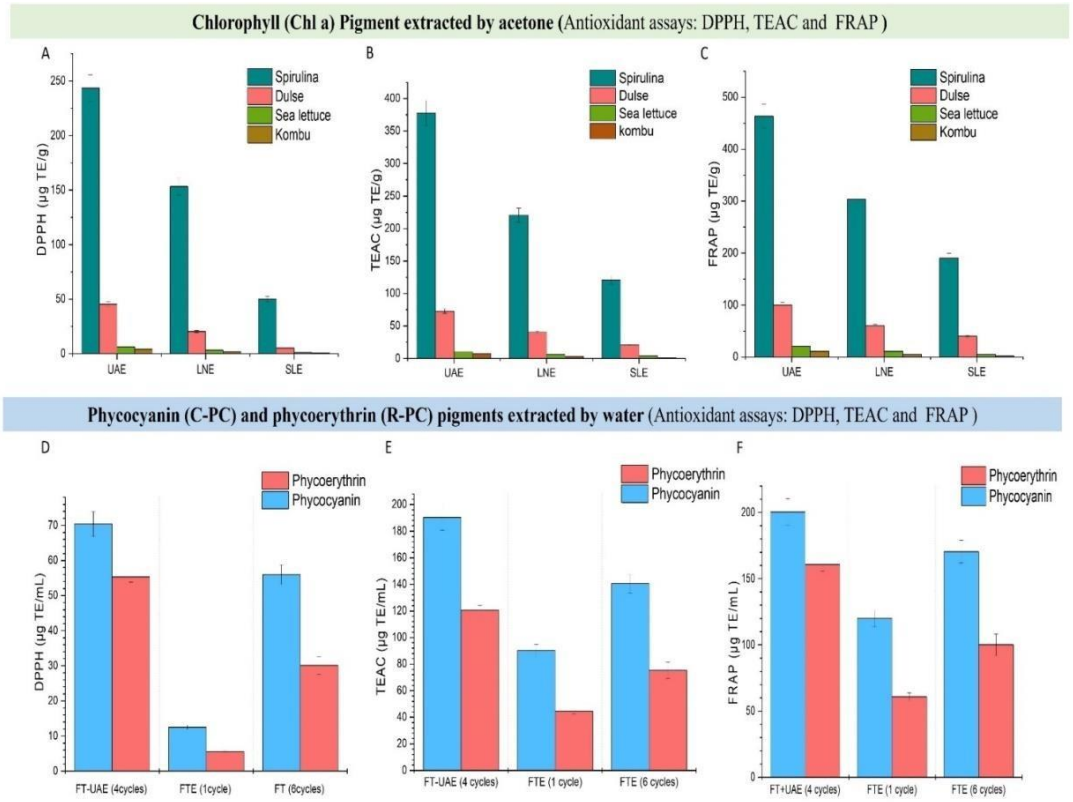


Figure 3.10 Antioxidant activity of chlorophyll, phycocyanin, and phycoerythrin extracts from the algae under study. A, B, and C) The antioxidant effects of chlorophyll extracts from *Spirulina* (S), *Dulse* (D), *Sea lettuce* (U), and *Kombu* (K); D, E, and F) The antioxidant effects of the phycocyanin extracts from *Spirulina* and the phycoerythrin extracts from *Dulse*.

In this study, the three assays showed a similar trend for all samples. In general, the DPPH assay exhibits the lowest activity, while the FRAP assay showed a higher radical reduction capacity similar to that of the TEAC assay. The results demonstrated that UAE chlorophyll extracts from all four algal extracts had superior performance in scavenging radicals compared to LNE and SLE in all three assays. In the FRAP assay, the UAE *Spirulina* extract exhibited the most significant radical activity, estimated to be 463.51 µg TE/g. *Dulse* exhibited the second level of radical activity, with an estimated value of 100.16 µg TE/g. *Sea lettuce* followed with a value of 20.24 µg TE/g, and *Kombu* had the lowest value at 11.25 µg TE/g. The extracts obtained from SLE gave the lowest values for radical scavenging activity, ranging from 40 µg TE/g to 2 µg TE/g.

The results demonstrated that the combination of ultrasonic and freeze-thaw (FT+UAE, 4 cycles) improved the ability of phycoerythrin and phycocyanin extracts to scavenge free radicals in DPPH, ABTS, and FRAP experiments in comparison to extracts obtained only through freeze-thaw (1 and 6 cycles). The FRAP assay showed superior radical-scavenging capability compared to other assays. Based on the FRAP assay, the phycocyanin extract (FT+UAE, 4 cycles) had the most radical scavenging activity, about 200.40 µg TE/g. The phycoerythrin extract (FT+UAE, 4 cycles) came in second, with about 160.68 µg TE/g.

The improvement in biological activity shown in **Figure 3.10** can be attributed to the synergistic impact of elevated quantities of chlorophyll a, phycoerythrin, phycocyanin, and other bioactive compounds present in the extracts. Nevertheless, this outcome suggests that the *Spirulina* extract obtained from the UAE has potential as a subject for further investigations into the isolation and purification of pigments due to its high content of pigments. Therefore, **Chapter 4** contains comprehensive investigations of *Spirulina* extracts, including the isolation and purification of chlorophyll pigments and phycocyanin, along with the evaluation of antioxidant activity for individual pigments.

3.4 Conclusion

To extract the chlorophyll pigments, the algae were subjected to solid-liquid extraction, cryoextraction, and ultrasound-assisted extraction. Acetone was used as the solvent for all the extraction processes. It was shown that the extraction process using simple ultrasound can deliver high amounts of chlorophyll *a* when optimizing the input frequency, process time, temperature, etc. The improved process is expected to reduce purification steps and obtain chlorophyll *a* of a high quality since this approach reduces the degradation of chlorophyll as demonstrated by HPLC-DAD. The extraction of phycobiliproteins from *Spirulina* and *Dulse* was performed in water as a solvent, along with the use of freezing, thawing, and ultrasonication to disrupt the cell wall. This work also demonstrated that the combination of ultrasound, freezing, and thawing improved the extraction yield and purity of phycobiliproteins. A comparison of these extracts' antioxidant activities showed that the improved extraction methods can not only produce high yields of extracts but also maintain the high effectiveness of extracts that contain high amounts of intact chlorophyll and phycobiliprotein. The overview and comparison of the antioxidant activity of these extracts, along with the accessibility of extraction techniques, could change the perspective of the broader use of these pigments.

3.5 References

1. Wells, M.L., et al., *Algae as nutritional and functional food sources: revisiting our understanding.* Journal of applied phycology, 2017. **29**: p. 949-982.

2. García, J.L., M. De Vicente, and B. Galán, *Microalgae, old sustainable food and fashion nutraceuticals.* Microbial biotechnology, 2017. **10**(5): p. 1017.

3. Han Jeong, A., et al., *Studies on Simultaneous Determination of Chlorophyll a and b, Pheophorbide a, and $\beta-Carotene $ in Chlorella and Spirulina Products.* Journal of Food Hygiene and Safety, 2005. **20**(3): p. 141-146.

4. Yamada, T. and K. Sakaguchi, *Comparative studies on Chlorella cell walls: induction of protoplast formation.* Archives of Microbiology, 1982. **132**: p. 10-13.

5. Hernández-Corona, A., et al., *Antiviral activity of Spirulina maxima against herpes simplex virus type 2.* Antiviral Research, 2002. **56**(3): p. 279-285.

6. Bhat, S., *Chlorophyll: The wonder pigment.* 2005.

7. Danesi, E.D.G., et al., *Effect of reducing the light intensity on the growth and production of chlorophyll by Spirulina platensis.* Biomass and Bioenergy, 2004. **26**(4): p. 329-335.

8. Jacob-Lopes, E., et al., *Handbook of microalgae-based processes and products: Fundamentals and advances in energy, food, feed, fertilizer, and bioactive compounds*. 2020: Academic Press.

9. Osório, C., et al., *Pigments content (chlorophylls, fucoxanthin and phycobiliproteins) of different commercial dried algae.* Separations, 2020. **7**(2): p. 33.

10. Tavanandi, H.A., et al., *Simple and efficient method for extraction of C-Phycocyanin from dry biomass of Arthospira platensis.* Algal research, 2018. **31**: p. 239-251.

11. Kong, W., et al., *Optimization of ultrasound-assisted extraction parameters of chlorophyll from Chlorella vulgaris residue after lipid separation using response surface methodology.* Journal of food science and technology, 2014. **51**: p. 2006-2013.

12. Lu, J., et al., *Optimization of subcritical fluid extraction of carotenoids and chlorophyll a from Laminaria japonica Aresch by response surface methodology.* Journal of the Science of Food and Agriculture, 2014. **94**(1): p. 139-145.

13. Abugri, D.A. and W.H. McElhenney, *Extraction of total phenolic and flavonoids from edible wild and cultivated medicinal mushrooms as affected by different solvents.* J Nat Prod Plant Resour, 2013. **3**(3): p. 37-42.

14. Pan-utai, W. and S. Iamtham, *Extraction, purification and antioxidant activity of phycobiliprotein from Arthrospira platensis.* Process Biochemistry, 2019. **82**: p. 189-198.

15. Minchev, I., N. Petkova, and I. Milkova-Tomova, *Ultrasound-assisted extraction of chlorophylls and phycocyanin from Spirulina platensis.* Biointerface Res Appl Chem, 2020. **11**: p. 9296-9304.

16. Macías-Sánchez, M., et al., *Comparison of supercritical fluid and ultrasound-assisted extraction of carotenoids and chlorophyll a from Dunaliella salina.* Talanta, 2009. **77**(3): p. 948-952.

17. Majid, I., G.A. Nayik, and V. Nanda, *Ultrasonication and food technology: A review.* Cogent Food & Agriculture, 2015. **1**(1): p. 1071022.

18. Alotaiby, S., et al., *Sustainable approach towards isolation of photosynthetic pigments from Spirulina and the assessment of their prooxidant and antioxidant properties.* Food Chemistry, 2024. **436**: p. 137653.

19. Wellburn, A.R., *The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution.* Journal of plant physiology, 1994. **144**(3): p. 307-313.

20. Bennett, A. and L. Bogorad, *Complementary chromatic adaptation in a filamentous blue-green alga.* The Journal of cell biology, 1973. **58**(2): p. 419-435.

21. Lourenço-Lopes, C., et al., *A HPLC-DAD method for identifying and estimating the content of fucoxanthin, β-carotene and chlorophyll a in brown algal extracts.* Food Chemistry Advances, 2022. **1**: p. 100095.

22. Hsu, C.-Y., et al., *The antioxidant and free radical scavenging activities of chlorophylls and pheophytins.* 2013.

23. Fernando, G.S.N., et al., *Application of an Ultrasound-Assisted Extraction Method to Recover Betalains and Polyphenols from Red Beetroot Waste.* ACS Sustainable Chemistry & Engineering, 2021. **9**(26): p. 8736-8747.

24. Govindappa, C., M, T. Sadananda, and C. Chandrappa, *Phytochemical Analysis of Loranthus micranthus Extracts and Their in Vitro Antioxidant and Antibacterial Activities.* Journal of Biologically Active Products from Nature, 2014. **4**(4): p. 303-315.

25. Chandini, S.K., P. Ganesan, and N. Bhaskar, *In vitro antioxidant activities of three selected brown seaweeds of India.* Food chemistry, 2008. **107**(2): p. 707-713.

26. Zuorro, A., G. Maffei, and R. Lavecchia, *Optimization of enzyme-assisted lipid extraction from Nannochloropsis microalgae.* Journal of the Taiwan Institute of Chemical Engineers, 2016. **67**: p. 106-114.

27. Tsubaki, S., et al., *Microwave-assisted hydrothermal extraction of sulfated polysaccharides from Ulva spp. and Monostroma latissimum.* Food chemistry, 2016. **210**: p. 311-316.

28. Salinas-Salazar, C., et al., *Methods for extraction of valuable products from microalgae biomass.* Microalgae biotechnology for development of biofuel and wastewater treatment, 2019: p. 245-263.

29. Płotka-Wasylka, J., et al., *Extraction with environmentally friendly solvents.* TrAC Trends in Analytical Chemistry, 2017. **91**: p. 12-25.

30. Sartory, D. and J. Grobbelaar, *Extraction of chlorophyll a from freshwater phytoplankton for spectrophotometric analysis.* Hydrobiologia, 1984. **114**: p. 177-187.

31. Wright, S., S. Jeffrey, and R. Mantoura, *Phytoplankton pigments in oceanography: guidelines to modern methods*. 2005: Unesco Pub. Paris, France.

32. Li, Y., et al., *Brown algae carbohydrates: Structures, pharmaceutical properties, and research challenges.* Marine Drugs, 2021. **19**(11): p. 620.

33. Sartory, D. and J. Grobbelaar, *Extraction of chlorophyll a from freshwater phytoplankton for spectrophotometric analysis.* Hydrobiologia, 1984. **114**(3): p. 177-187.

34. Moraes, C.C., J.F. De Medeiros Burkert, and S.J. Kalil, *C-phycocyanin extraction process for large-scale use.* Journal of food biochemistry, 2010. **34**: p. 133-148.

35. Choi, W.Y. and H.Y. Lee, *Enhancement of chlorophyll a production from marine spirulina maxima by an optimized ultrasonic extraction process.* Applied Sciences, 2017. **8**(1): p. 26.

36. Zhu, Z., et al., *Multistage recovery process of seaweed pigments: Investigation of ultrasound assisted extraction and ultra-filtration performances.* Food and Bioproducts Processing, 2017. **104**: p. 40-47.

37. Acker, J.P. and L.E. McGann, *Protective effect of intracellular ice during freezing?* Cryobiology, 2003. **46**(2): p. 197-202.

38. Pesang, M.D., et al., *Komposisi Pigmen pada Ulva sp., Padina australis dan Hypnea sp. dari Pantai Tablolong Provinsi Nusa Tenggara Timur.* Jurnal Kelautan Tropis, 2020. **23**(2): p. 225-233.

39. Indriatmoko, M., R. Indrawati, and L. Limantara, *Composition of the Main Dominant Pigments from Potential Two Edible Sea-weeds.* Philipp. J. Sci, 2018. **147**: p. 47-55.

40. Lichtenthaler, H.K. and C. Buschmann, *Chlorophylls and carotenoids: Measurement and characterization by UV-VIS spectroscopy.* Current protocols in food analytical chemistry, 2001. **1**(1): p. F4. 3.1-F4. 3.8.

41. Krajewska, M., M. Szymczak-Żyła, and G. Kowalewska, *Carotenoid determination in recent marine sediments-practical problems during sample preparation and HPLC analysis.* Current Chemistry Letters, 2017. **6**(3): p. 91-104.

42. Ranga, R., et al., *Identification of carotenoids from green alga Haematococcus pluvialis by HPLC and LC-MS (APCI) and their antioxidant properties.* J. Microbiol. Biotechnol, 2009. **19**(11): p. 1333-1341.

43. Daigo, K., et al. *High-performance liquid chromatographic analysis of photosynthetic pigments in corals: An existence of a variety of epizoic, endozoic and endolithic algae*. in *Proceedings of the 11th International Coral Reef Symposium*. 2008.

44. Schmid, H. and H.B. Stich, *HPLC-analysis of algal pigments: comparison of columns, column properties and eluents.* Journal of applied phycology, 1995. **7**: p. 487-494.

45. Zeb, A., A. Haq, and M. Murkovic, *Effects of microwave cooking on carotenoids, phenolic compounds and antioxidant activity of Cichorium intybus L.(chicory) leaves.* European Food Research and Technology, 2019. **245**: p. 365-374.

46. Singh, A.K., H.K. Rana, and A.K. Pandey, *Analysis of chlorophylls*, in *Recent advances in natural products analysis*. 2020, Elsevier. p. 635-650.

47. Andersen, R.A., *Algal culturing techniques*. 2005: Elsevier.

48. Büchel, C., *Evolution and function of light harvesting proteins.* Journal of Plant Physiology, 2015. **172**: p. 62-75.

49. Porra, R.J., *The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b.* Photosynthesis research, 2002. **73**: p. 149-156.

50. Garcia-Perez, P., et al., *Pigment composition of nine brown algae from the Iberian Northwestern Coastline: Influence of the extraction solvent.* Marine Drugs, 2022. **20**(2): p. 113.

51. Yalçın, S., et al., *HPLC Detection and antioxidant capacity determination of brown, red and green algal pigments in seaweed extracts.* Journal of Chromatographic Science, 2021. **59**(4): p. 325-337.

52. Schubert, N., E. García-Mendoza, and I. Pacheco-Ruiz, *Carotenoid composition of marine red algae 1.* Journal of Phycology, 2006. **42**(6): p. 1208-1216.

53. Rajauria, G., B. Foley, and N. Abu-Ghannam, *Characterization of dietary fucoxanthin from Himanthalia elongata brown seaweed.* Food Research International, 2017. **99**: p. 995-1001.

54. Honda, M., et al., *Enhanced solubility and reduced crystallinity of carotenoids, β-carotene and astaxanthin, by Z-isomerization.* European Journal of Lipid Science and Technology, 2018. **120**(11): p. 1800191.

55. Davani, L., et al., *Integrated analytical approaches for the characterization of Spirulina and Chlorella microalgae.* Journal of Pharmaceutical and Biomedical Analysis, 2022. **219**: p. 114943.

56. Wright, S., et al., *Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton.* Marine ecology progress series, 1991: p. 183-196.

57. Milenković, S.M., et al., *The identification of chlorophyll and its derivatives in the pigment mixtures: HPLC-chromatography, visible and mass spectroscopy studies.* Adv. Technol, 2012. **1**(1): p. 16-24.

58. Li, W., et al., *Phycobiliproteins: Molecular structure, production, applications, and prospects.* Biotechnology Advances, 2019. **37**(2): p. 340-353.

59. Sueishi, Y., et al., *Antioxidant capacities in various animal sera as measured with multiple free-radical scavenging method.* Bioorganic & Medicinal Chemistry Letters, 2019. **29**(16): p. 2145-2149.

60. Gündüz, M., Ş.K. Çiçek, and S. Topuz, *Extraction and Optimization of Phenolic Compounds from Butterbur Plant (Petasites hybridus) by Ultrasound-Assisted Extraction and Determination of Antioxidant and Antimicrobial Activity of Butterbur Extracts.* Journal of Applied Research on Medicinal and Aromatic Plants, 2023: p. 100491.

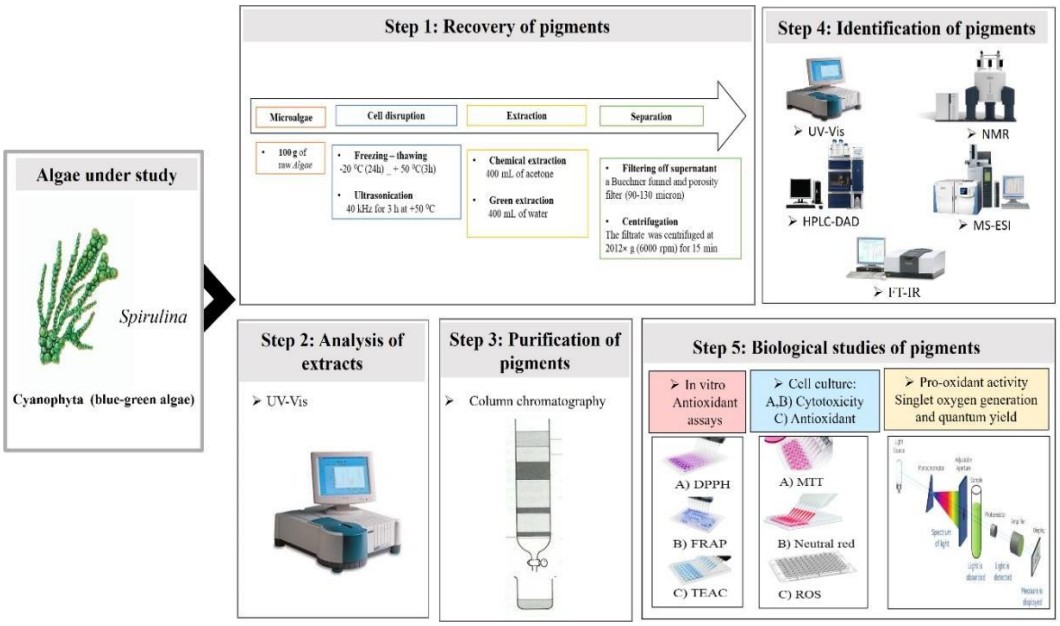
61. Mendonça, J.d.S., et al., *Natural antioxidant evaluation: A review of detection methods.* Molecules, 2022. **27**(11): p. 3563.

62. Lanfer-Marquez, U.M., R.M. Barros, and P. Sinnecker, *Antioxidant activity of chlorophylls and their derivatives.* Food research international, 2005. **38**(8-9): p. 885-891.

Chapter 4 Sustainable Approach Towards Isolation of Photosynthetic Pigments from Spirulina and the Assessment of their Prooxidant and Antioxidant Properties

Highlights

- Three key types of pigments from the same batch of *Spirulina* were extracted by water.
- The use of non-mechanical cell wall disruption methods boosts extraction in water.
- This method significantly improves the extraction of chlorophyll *a* and carotenoids.
- Chlorophyll extract is directly converted into its stable products.
- Antioxidant *in vitro* activity and singlet oxygen production are tested.



Abstract.

Spirulina contains three types of photosynthetic pigments, differing in colour composition, stability, solubility, and commercial importance. Such diversity of structures creates a challenge to extract these pigments simultaneously from the same batch of raw materials in an efficient, sustainable manner and minimize waste. In this study, we demonstrate that water can be successfully used as a single solvent together with combined (non)mechanical cell membrane disruption techniques to extract these photosynthetic pigments from the same batch. Acetone was tested as an alternative solvent; however, a water-based approach delivers a significant improvement in isolating green pigments, which are often overlooked during extraction due to a preference for blue and yellow pigments. Chlorophyll was quantitatively converted to its stable derivatives to carry out a comparative analysis of antioxidant properties (DPPH, TEAC, FRAP), singlet oxygen production and intracellular activities (MTT, ROS assays) using Caco-2 cells, revealing the superior performance of green pigments over phycocyanin.

4.1 Introduction

Natural or naturally derived bioactive compounds support many vital applications in the pharmaceutical, food, cosmetics, feed, and biofuel industries [1]. Among commercially viable marine algae, cyanobacteria represent a renewable source of bioactives as they are cultivated on a large scale due to their ability to survive in harsh environmental conditions [2]. *Spirulina* is a commercial name given to the *Arthrospira platensis* and *Arthrospira maxima* species of cyanobacteria. For centuries, *Spirulina* has been used in many parts of the world as a food source; the United Nations classified it as “one of the best foods of the future” and an important natural food source [3]. Although *Spirulina* is an excellent source of polysaccharides, lipids, and proteins, it is also an important source of edible natural colouring agents, which show health benefits and are often a part of nutraceuticals, cosmeceuticals, and drugs [4]. Although *Spirulina* is considered safe, raw extracts can be contaminated with cyanotoxins such as nodularins and microcystins, which are synthesized by a range of cyanobacteria, posing a hazard to human health [5]. Moreover, extracts may contain heavy metals, so the production of pure photosynthetic pigments has become even more important. However, the economics of any pure bioactive are driven by the production costs which can be as high as 60% of the total expenditure [1]. The commercial value of *Spirulina* is primarily directed by phycocyanin (the most common phycobiliprotein), 80% of which is utilized as a blue pigment by the food and cosmetic industry [1]. But *Spirulina* is also a source of other important photosynthetic pigments such as yellow xanthophylls and green chlorophylls; even more, the unique photosynthetic system of cyanobacteria produces primarily chlorophyll *a*. However, the developed practical strategies are mainly tailored to isolate blue phycocyanin, overlooking chlorophylls [6]. The limited number of extraction strategies is due to chlorophyll’s stability towards heat, light, and pH. Methanol was the first solvent used to extract chlorophyll *a* from *Spirulina*, but due to its toxicity, it has been replaced by other solvents such as ethanol and acetone [7]. Recent studies reported a successful attempt to extract chlorophyll using supercritical carbon dioxide [8] and ionic liquid [9].

Biorefineries are processing facilities that convert biomass into value-added products such as biofuels, biochemicals, bioenergy/biopower, and other biomaterials. Most of them are mainly defined based on the individual feedstock, such as corn-based biorefinery, wood-based biorefinery, forest-based biorefinery, palm-based biorefinery, algae-based biorefinery, etc [10]. A green biorefinery concept is a very attractive alternative that can potentially deliver a wide range of complex molecules as opposed to traditional extractions of value-added chemicals. But the pigment fraction, i.e., the total phytochemical content, tends to be very small and is the most complex one to separate due to the different solubilities and stabilities of the diverse structures [10]. Already developed practical strategies fail to deliver pigments such as phycobiliproteins, carotenoids, and chlorophylls from the same source simultaneously. For example, when isolating blue colorants, green and yellow fractions are usually discarded. This is especially true when the concentration of target colorants is relatively low, requiring less sustainable and labour-intense approaches [8].

The current study aims to develop a smart extraction method that avoids the loss of target molecules and minimizes waste to reduce production costs. To further decrease the detrimental impact (both economic and environmental), this study aims to improve the existing extraction and purification stages, which can help advance the overall purification process. This work also aims to demonstrate the potential of the green pigments by studying their antioxidant and prooxidant properties, in comparison to phycocyanin.

4.2 Materials and methods

4.2.1 Chemicals and reagents

Spirulina powder (*Arthrospira platensis*) was purchased from the Nukraft brand. All chemicals and solvents were obtained from Fisher Scientific (Loughborough, UK) and Sigma Aldrich (Dorset, UK). Caco-2 cells were purchased from EACC, the European Collection of Authenticated Cell Collection. Dulbecco's modified Eagle Medium (DMEM), foetal bovine serum (FBS), antibiotics (10,000 U/mL penicillin and 10 mg streptomycin/mL), and MEM non-essential amino acids were all purchased from Gibco Life Technologies (New York, USA). Thiazolyl Blue Tetrazolium Bromide (MTT), Neutral Red, hydrogen peroxide solution, 30% (w/w) in water, Sulforhodamine B (SRB), trichloroacetic acid (TCA), 2',7'-dichlorodihydrofluorescein diacetate (DCFH2-DA), acetic acid, and Trizma base were all obtained from Sigma-Aldrich.

4.2.2 Ultrasound assisted water extraction of photosynthetic pigments.

Approximately 100 g of *Spirulina* powder was dispersed in 400 mL of distilled water and allowed to freeze for 24 h in the freezer (-20 °C). Then the mixture was thawed at +50 °C and sonicated at 40 kHz for 3 h. The mixture reached +50 °C after 2 h of sonication. The supernatant was filtered through a Buechner funnel with the sealed-in coarse porosity filter (90-130 micron), and the solid residue was washed three times with 20 mL of distilled water. After that, the water filtrate was centrifuged at 1207 ×g for 15 min, yielding 10.8 g of dark green solid, which was dried in air for 72 h in the dark and stored at -20 °C under a nitrogen atmosphere. A blue aqueous solution (200 mL) containing phycocyanin was stored at +4 °C in the dark, prior to use. (**Figure 4.1**)

4.2.3 Ultrasound assisted acetone extraction of photosynthetic pigments.

Approximately 100 g of *Spirulina* powder was mixed with 400 mL of acetone and left in the freezer (-20 °C) for 24 h. Then the mixture was thawed at +50 °C and sonicated at 40 kHz for 3 h. The mixture reached +50 °C after 1 h of sonication. The supernatant was filtered through a Buechner funnel, and the solid residue was washed three times with 20 mL of acetone. The combined green acetone extract was evaporated under reduced pressure, yielding 7.4 g of dark green precipitate. This sample was stored at -20 °C under a nitrogen atmosphere. **(Figure 4.1)**

4.2.4 Relative efficiency of the extraction method: Extraction yield

The extraction yield (%) from powdered *Spirulina* algae was calculated as follows: [(the mass of extracted solid, g) / (the mass of initial dry material, g)] x 100% [11].

4.2.5 Isolation and purification of phycocyanin

Approximately 200 mL of crude blue aqueous extract (from sec. 2.2) was treated by gradually adding 10 g of solid ammonium sulphate to reach saturation. The mixture was stirred for 1 h and kept overnight at +4 °C in the dark. The mixture was centrifuged at 1207 ×g for 15 min, and then 18 g of a blue precipitate was obtained. The blue precipitate was dissolved in a sodium phosphate solution at a ratio of 1:5 (w/v) and kept at +4 °C in the dark for 24 h. The mixture was centrifuged at 1207 ×g for 15 min and filtered, yielding 15 g of phycocyanin [8]. The purity and grade were calculated using HPLC and UV-vis absorption methods.

4.2.6 Isolation and purification of chlorophyll derivatives

The thin layer chromatography (TLC) technique was used to pre-determine the separation of pigments in two different solvent systems: (a) hexane-acetone 70:30 (v/v) and (b) dichloromethane-tetrahydrofuran 95:5 (v/v). Briefly, the crude green extract was analyzed using TLC Silica Gel 60 F254

aluminium-coated plates (Merck). The solvent system of hexane-acetone 70:30 (v/v) showed the best separation (Appendix B, **Figure 4.2**) and was therefore used in flash chromatography to purify the pigments. Here, 6 g of crude green extract was submitted to a normal phase flash chromatography procedure by applying this extract on top of a glass column (5×150 cm) packed with 190 g silica gel 60 mesh, eluted using a hexane-acetone 90:10 (v/v) system (Appendix B, **Figure 4.3**). The polarity of the system was gradually raised until the ratio of hexane–acetone 60:40 (v/v) was reached. Four coloured fractions were collected: orange, green, grey, and yellow (ca. 200 mL each). These fractions were evaporated under reduced pressure, yielding β-carotene (0.9 g), pheophorbide *a* (1.4 g), pheophytin *a* (2.6 g), and lutein (0.5 g).

4.2.7 Conversion of chlorophyll *a* from green extract into methyl pheophorbide *a*

Approximately 1 g of crude green solid extract (from **sec. 2.2**) was treated following the modified method by Smith *et al* [12]. In brief, 110 mL of methanol containing 5% sulfuric acid was added to 1 g of the dark green solid. The mixture was degassed and stirred under a nitrogen atmosphere for 12 h in the dark at room temperature. The residue was diluted with dichloromethane; this solution was rinsed with water (50 mL), then with 40 mL of a saturated solution of aqueous sodium bicarbonate (10%) and dried over sodium sulphate. The solvent was removed under reduced pressure, leaving a green precipitate that was recrystallized from dichloromethane/methanol yielding 0.4 g of green methyl pheophorbide *a*.

4.2.8 Quantification of the individual pigments in extracts

For the quantitative determination of pigments, stock solutions were prepared from the green extracts at a concentration of 1 mg/mL in 80% acetone. Briefly, measurements were performed using a quartz cuvette containing 30 µL of stock solution for green extracts with 3 mL of 80% acetone and 200 µL blue (phycocyanin) extract with 3 mL of distilled water. The absorbances of the green extracts were measured at 663 nm, 645 nm, and 470 nm against a

blank (80% acetone). For the phycocyanin extract, the absorbance was measured at 652 nm and 620 nm against a blank (distilled water).

The concentration of chlorophylls, total carotenoids [13], and phycocyanin [14] was determined by the following equations:

(1) $C_{chlorophyll\,a}\left(\frac{\mu g}{mL}\right) = 12.25 \times A_{663} - 2.79 \times A_{645}$

(2) $C_{chlorophyll\,b}\left(\frac{\mu g}{mL}\right) = 21.5 \times A_{645} - 5.10 \times A_{663}$

(3) $C_{carotenoids}\left(\frac{\mu g}{mL}\right) = \frac{1000 \times A_{470} - 1.82C_{(chlorophyll\,a)} - 85.02C_{(chlorophyll\,b)}}{198}$

(4) $C_{phycocyanin}\left(\frac{mg}{mL}\right) = \frac{A_{620} - 0.474 \times A_{652}}{5.34}$

Where *A* is absorbance (a.u.) at the corresponding wavelength for the sample. The concentration of each pigment is expressed as mg of pigment per g of powdered *Spirulina* (mg/g).

4.2.9 Identification of pigments

In this study, NMR, MS, HPLC, UV-vis absorption, and IR spectroscopy were used to identify the isolated pigments.

NMR spectroscopy. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DRX 400, a Bruker AV3-400, or a Bruker Advanced 500 spectrometer. Chemical shifts (δ) were reported in parts per million (ppm) relative to the residual solvent peak, and peaks are described as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), broad singlet (br), and coupling constants (J) are quoted in Hertz (Hz). Spectra were recorded in deuterated acetone and were measured at room temperature unless otherwise stated. Where needed, two-dimensional correlation spectroscopy (2D-COSY) was used to aid assignment.

Ultraviolet Visible (UV- Vis) spectroscopy. The UV- Vis Spectrophotometer (Cary 50 UV-Vis) was used to determine the characteristic absorption bands of pure pigments in the range from 300 nm to 800 nm to detect the optical properties of the pigments associated with the structure.

Fourier transfer infrared analysis (FT-IR). FT-IR spectra of solid phycocyanin were recorded on a Bruker Alpha Platinum (ATR FTIR) spectrometer with vibrational frequencies given in cm^{-1} and 100 scans to investigate the chemical structure and molecular bonds of phycocyanin.

Accurate mass spectroscopy (MS). A Maxis Impact HD mass spectrometer (Bruker MaXis Impact, Bremen, Germany) was applied to prove the results of the NMR analytical characterization for the purified pigments by determining the exact molecular weight of the compounds. Samples were prepared by preparing a stock solution for each pigment in acetonitrile at a concentration of 1 mg/mL (w/v). From the stock solution, the measurement solution was prepared by taking 10 µL dissolved in 1 ml acetonitrile (v/v). Samples were sent to electrospray ionization (ESI) mass spectrometry, and ESI-positive ion mass spectrometry was recorded. The scan starts at 50 m/s and the scan ends at 1500 m/s. Spectra were analysed using Bruker Compass Data Analysis version 4.3. software.

4.2.10 Determination of purity by high performance liquid chromatography (HPLC/DAD).

The purity of the pigments was determined using the HPLC (Agilent 1290 Infinity II LC, USA) with the diode-array detector method (DAD) and Poroshell 120 EC reverse column C 18 (5 µm, 120 µ, 4.6 mm × 150 mm). The mobile phase consisted of 50-95% acetonitrile - trifluoroacetic acid (TFA). The UV wavelength was set at 430 nm and 620 nm. The injection volume was 20 µL, and a flow rate of 0.95 mL/min for 10 min was used. The purity of the isolated pigments was quantified using the peak area of the DAD chromatogram observed at 430 nm and 620 nm for pigments.

4.2.11 Assessment of antioxidant activities

The antioxidant capacity of pure pigments was evaluated using 2,2 -diphenyl-1-picryl (DPPH), ferric-reducing antioxidant power (FRAP), and Trolox equivalent antioxidant capacity (TEAC). Stock solutions (10 mg/mL) of each purified compound were prepared in an ethanol/DMSO mixture (60:40, v/v) and further diluted according to the assay requirements (0, 25, 50, 100, 150, 200, and 250 µg/mL concentrations). Trolox (10 mg/mL) was used as a standard to cover a range of 0-1000 µg/mL, diluted in an ethanol / DMSO mixture (60:40, v/v). Briefly, the antioxidant capacities of pigments were determined by DPPH according to the method described by Hsu *et al* [15], with modifications. A fresh ethanol solution of DPPH reagent (120 µM) was prepared. 10 µL of samples were mixed with 300 µL of DPPH reagent and incubated for 30 min at room temperature. Subsequently, absorbance was measured at 515 nm. Trolox equivalent antioxidant capacity (TEAC) was conducted based on the method described by Ganwarige Sumali *et al* [16]. 10 µL of samples were mixed with 300 µL of $ABTS^{+}$ solution and incubated for 10 min at room temperature in the dark. Then, the absorbance was measured at 734 nm. The method described in Ganwarige Sumali *et al* [17] was used to perform the FRAP assay; 10 µL of samples were mixed with 300 µL of FRAP reagent and incubated for 15 min at 37 °C. Subsequently, the absorbance of the colored product was taken at 593 nm. All assays were performed in 96-well plates, and absorbance measurements were recorded by a microplate reader (TECAN, Mannedorf, Switzerland). In addition, the results are expressed as mean ± standard deviation as µg Trolox Equivalent Antioxidant Capacity per mL of pigments (µg TE/mL).

4.2.12 Cell culture and cytotoxicity assays

Caco-2 cells were routinely cultivated in DMEM medium with 10% FBS, 1% antibiotics and 1% non-essential amino acids under standard conditions (5% CO_2, 37 °C). The cytotoxicity of the four pigment samples (pheophytin *a*, phycocyanin, pheophorbide *a* and methyl pheophorbide *a*) was determined by

MTT assay and neutral red assay as previously described [18]. Briefly, Caco-2 cells were seeded at 1.0×10^5 cells per well in 24-well plates. Upon reaching 90% confluence, the medium was removed, and cells were incubated in DMEM containing different concentrations of pigments (0, 1, 2.5, 5, 10, 25, 50 and 100 µg/mL). The samples were dissolved in DMSO/ethanol (80:20, v/v) or, in the case of phycocyanin, in distilled water, with a maximum solvent concentration of 0.2% (1%, 2%). After 24 h, the medium was replaced by DMEM containing MTT (1 mM). Following 2 h incubation at 37 ℃, the cells were washed with DPBS and DMSO added to each well followed by 10-15 min on a shaker. Then, 3 x 200 µL from each well were transferred on a 96 well pate, and absorbance was recorded at 570 nm using microplate reader (Tecan Spark 10M), against DMSO as the control. For the neutral red assay, cells were incubated for 2 h with neutral red dye (40 µg/mL) in FBS-free DMEM medium. Subsequently, a bleaching solution (50 % ethanol, 49 % deionized H_2O, 1 % acetic acid) was used to dissolve the dye. The absorbance was recorded at 540 nm. Cell viability in both assays was calculated as a percentage of treated cells in relation to the negative control.

4.2.13 Intracellular reactive oxygen species (ROS) assay

For the ROS assay, Caco-2 cells were seeded in 96-well plates at a density of 7.0×10^4 cells/well in a volume of 100 µL [18, 19]. After 24 h the medium was removed and replaced by 100 µL of medium containing 30 µM of DCFH2-DA. Following a 40 min incubation at 37 ℃, the cells were washed and incubated with pigment samples at different concentrations (0, 1, 5, 10, 25, 50, and 100 µg/mL) in phenol red-free and FBS-free DMEM. Curcumin (10 µM) was used as a positive control. After 1 h, H_2O_2 was added at a final concentration of 200 µM. To determine non-stimulated ROS levels, cells were incubated in parallel without hydrogen peroxide. The fluorescence intensity was read after 1 h with the TECAN reader in scan mode, at an excitation of 485 nm and an emission of 535 nm. Subsequently, protein content was determined by adding 50 µL of SRB-TCA (SRB in 10% TCA solution) to each well, incubating the plate for 15 min at room temperature, then removing the solution and washing with 200 µL of 1% acetic acid. Finally, the solution was removed, and 100 µL of trizma base (10 mM) was added for 5 min. The

fluorescence intensity was measured at excitation of 540 nm and emission of 590 nm. The levels of ROS were calculated as the ROS generation of sample treated cells in a percentage of the negative control and normalized according to protein content.

4.2.14 Assessment of pro-oxidant activity via singlet oxygen ($^1O_2$) generation assay

Photostability Analysis. The photostability of the individual compounds (pheophorbide *a*, pheophytin *a*, methyl pheophorbide *a*, phycocyanin, and 1,3-diphenylisobenzofuran) was determined according to Martins *et al* [9] with some modifications. In brief, a quartz cuvette containing a solution of an appropriate compound was irradiated in DMF during the 120 sec irradiation period with visible light (Xenon lamp, 50 W) using each of the following long-pass cut-off filters (420 nm, 455 nm, 475 nm, and 495 nm) **(**Appendix B, **Figure B.8)**. The absorption region was recorded, and the photostability was expressed as the ratio (%) of the absorbance of a characteristic band of the compound at a given time of irradiation (A_t) and its absorbance prior to the start of the irradiation process (A_0).

Singlet oxygen generation and quantum yield. A modified photochemical method was used to determine the ability of pigments (phycocyanin, pheophorbide *a*, pheophytin *a* and methyl pheophorbide *a*) to produce singlet oxygen using DPBF (1,3-diphenylisobenzofuran) as a singlet oxygen trapping agent and methylene blue as a reference compound [20, 21]. Briefly, the stock solutions of DPBF (1 mg/mL, 1.5 mM) and the pigments (1.5 mM) and phycocyanin (1 mg/mL) were prepared in DMF. The first sample contained 40 µL of DPBF stock solution in 3 mL of DMF. The remaining samples contained 10 µL of an appropriate pigment's stock solution, including methylene blue, and 40 µL of DPBF stock solution in 3 mL of DMF. The photooxidation rate was assessed by measuring DPBF absorbance at 410 nm every 6 sec for 2 min while illuminating the solution with visible light (Xenon lamp, 50 W) using a long-pass cut-off filter (495 nm).

Singlet oxygen ($^1O_2$) quantum yield (ΦΔ). (ΦΔ) of the isolated pigments was calculated in a relative manner using methylene blue (MB) as a reference. A linear plot of $\ln(A_0/A_t)$ vs. the irradiation time (t, sec) was obtained, where A_0 and A_t are the absorbances of DPBF at 410 nm before irradiation and at every 6 sec during illumination (120 sec), respectively. Singlet oxygen ($^1O_2$) quantum yield (ΦΔ) was calculated using the following equation:

$$\Phi = \Phi_R \left(\frac{k_S}{k_R}\right)\left(\frac{I_{aR}}{I_{aS}}\right)$$

where *R* and *S* are the reference (MB) and the sample, respectively; *k* is the slope of the linear plot of $\ln(A_0/A_t)$ vs. time; I_a is the total amount of light absorbed. The samples and reference were measured under the same conditions.

4.2.15 Data analysis

All experiments on antioxidant and prooxidant activities were carried out in triplicate. All cell culture experiments were performed in duplicate and repeated on three passages of cells. All results were expressed as the mean value ± standard deviation (SD). The graphs were plotted with Origin Pro 2020 for Windows. Topspin software was used to analyse the NMR data, and Chem Draw Prime 2019 for Windows was used to represent chemical structures.

4.3 Results and discussion

4.3.1 Development of sustainable extraction approach

In this study, we explore a water-based method to simultaneously isolate three types of photosynthetic pigments: carotenoids, chlorophylls, and phycocyanin, from the same batch of *Spirulina*. Two key steps in all extraction methodologies are the selection of an appropriate/ compatible solvent and the method for cell wall disruption. [6] Because the efficiency of solvent extraction strongly depends on the solubility and stability of these molecules in the selected medium, the current separation strategy is based on the relationship between the chemical composition of pigments and their water solubility.

Carotenoids are lipophilic pigments, and their solubility in aprotic organic solvents such as acetone is higher than that in water, whereas phycocyanin is hydrophilic. In contrast, chlorophylls are amphiphilic due to their hydrophilic and lipophilic nature. Thus, they can be dissolved in either polar or non-polar solvents to a varying degree [10].

The disruption of the cell wall is a key step in affecting the yield and purity of the extract; it can also facilitate the process of releasing pigments based on the type of bonding that holds them in place within the plant cell. For example, phycocyanin is covalently bound, and chlorophyll *a* is non-covalently bound to light-harvesting proteins. There are two principal pathways for cell wall disruption: (i) the non-mechanical pathway, e.g., thermal, chemical, and enzymatic treatments; and (ii) the mechanical pathway, e.g., ultrasonication, electric field, and microwaves [1]. Here, we employed mechanical separation techniques such as sonication and centrifugation with non-mechanical freezing/thawing. These steps can efficiently enhance the overall separation process as they exploit the properties of the material, such as its size, shape, and density, as well as its average viscosity and solubility in the chosen medium [22].

In this study, also compared this methodology to traditional acetone extractions, as the solvent choice determines the sustainability of the process, e.g., green (water, supercritical carbon dioxide) *vs*. traditional organic solvents [8]. Acetone is preferred in the scope of quantitative analysis of dyes because it provides the widest coverage of pigment solubility. In addition, several studies have shown that acetone can be the best extraction solvent for chlorophylls because the ethanol increases the activity of chlorophyllase, an enzyme that increases its activity mainly with cell wall disruption, leading to the degradation of chlorophyll during or after extraction [7].

In this study, the separation strategy using water and a comparative acetone extraction is outlined in **Figure 4.1**. A batch prepared by suspending 100 g of *Spirulina* in 400 mL of water was cooled down (-20 °C) for 24 h and then sonicated, allowing efficient breakdown of the cell wall and release of the pigments. The removal of cell debris at this stage provides a fast and efficient

separation of the supernatant, which is saturated with yellow, green, and blue pigments. This step is crucial for the successful separation of the pigments at the centrifugation stage, which can separate chlorophylls and carotenoids from phycocyanin due to the difference in their solubility in water as well as their molecular size. The top aqueous layer contained 100% phycocyanin, while the bottom dark green viscous fraction consisted of chlorophyll and its derivatives (79.9%) and carotenoids (20.1%). This key difference in the current approach to removing cell debris at an earlier stage enables efficient separation by centrifugation, while the published methods [23] wasted chlorophyll content when discarding the supernatant and cell debris at the same stage. To demonstrate this point, a water extraction was carried out under previously published conditions, skipping the first filtration step prior to centrifugation. Here, the supernatant contained mostly phycocyanin, with the chlorophyll fraction being negligible.

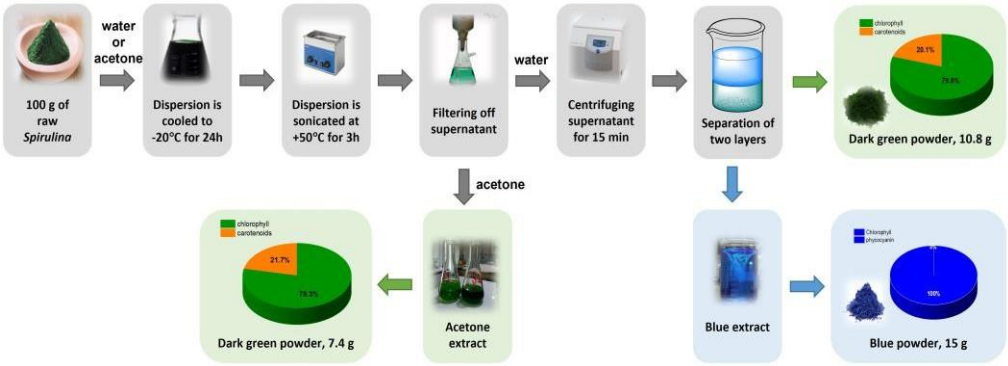


Figure 4.1 Separation strategy for photosynthetic pigments from Spirulina by water vs. acetone.

Additionally, we have tested a traditional acetone extraction combined with sonication and centrifugation; this method yielded only the extract containing carotenoids (21.7%) and chlorophylls (78.3%). But the total pigment content isolated from 100 g of *Spirulina* is only 7.4 g, which is significantly lower than the total pigment content isolated by our water method. The overall yield of green and yellow pigments from water extraction is 46% higher than that achieved by the acetone-based method and 69% higher for chlorophyll pigments.

The concentration range for the individual pigments in water and acetone green extracts showed that chlorophyll *a* was the dominant pigment. The content of chlorophyll *a* was 68.94 ± 0.04 mg g^{-1} for the water and 34.20 ± 0.04 mg g^{-1} for the acetone extract. The concentrations of carotenoids were 17.30 ± 0.03 mg g^{-1} for water and 8.35 ± 0.04 mg g^{-1} for the acetone method. Published studies to extract pigments from *Spirulina* with green solvents reported 5.7 mg g^{-1} for chlorophyll *a* in $scCO_2$ extraction [8], while the use of ionic liquids delivered 4.36 ± 0.78 mg g^{-1} of chlorophyll *a* [9]. Closer to our results is the study based on water extraction under rotary shaking conditions for cell wall disruption, reporting yields of 25.43 ± 0.02 mg g^{-1} for chlorophyll *a* and 4.25 ± 0.10 mg g^{-1} for carotenoids [24]. We believe this difference in the efficacy of extractions underlines the importance of the cell wall disruption method, where freezing and thawing increase extraction efficacy, as ice crystals can break the cell membrane during freezing [7]. The subsequent ultrasonication allows an effcient permeation of the plant cell by the solvent molecules, which also explains why acetone extaction is less efficient. The concentration of phycocyanin in the water extract was 7.55 ± 1.14 mg g^{-1}, which is similar to other studies; the yield can be further improved by increasing the number of freezing/thawing cycles and the duration of ultrasonication [4].

4.3.2 Purification and chemical functionalisation of raw green extract

A limited use of chlorophyll itself is mainly driven by its ease of degradation when exposed to light, moisture, or a harsh environment. [25]. The degradation is already an inevitable process, starting with the raw material itself. Analytical studies [25] showed *Spirulina* contains first-generation degradation products such as pheophytin *a* and pheophorbide *a* that are formed *via* the removal of the central magnesium ion followed by the hydrolysis of the phytyl chain from chlorophyll *a* (**Figure 4.4**). Chlorophyll derived pigments are known for their dose-dependent antioxidant properties, but under certain conditions, they can also be phototoxic, making them promising photosensitizers. Thus, we have incorporated isolation and purification steps to convert chlorophyll into more stable products as part of our overall strategy.

An initial phytochemical screening of green extract using thin layer chromatography analysis confirmed the presence of xanthophylls (yellow), chlorophyll *a* (green), and β-carotene (orange), along with pheophytin *a* and pheophorbide *a*. Optimal solvent mixtures were determined using the retention factor, which indicates the separation and elution efficiency of a particular compound in this solvent. The hexane-acetone 90:10 (v/v) solvent system proved to be practical to separate pigments with RF between 0.2 and 0.9 (Appendix B, **Figure 4.3**) and was adopted in the isolation of the compounds by flash chromatography.

Method	Acetone extraction	Water extraction
RF in system (Hexane – acetone) 70: 30 (v/v)	• Yellow(xanthophyll) 0.25 • Blue green (chlorophyll a) 0.33 • Gray(pheophorbide a) 0.47 • Gray (pheophytin a) 0.5 • Orange (β-carotene) 0.92	• Yellow (xanthophyll) 0.25 • Blue green (chlorophyll a) 0.33 • Gray (pheophorbide a) 0.47 • Gray (pheophytin a) 0.5 • Orange (β-carotene) 0.92
RF in system (Ethanol–water) 90: 10 (v/v)		• Phycocyanin 0.25

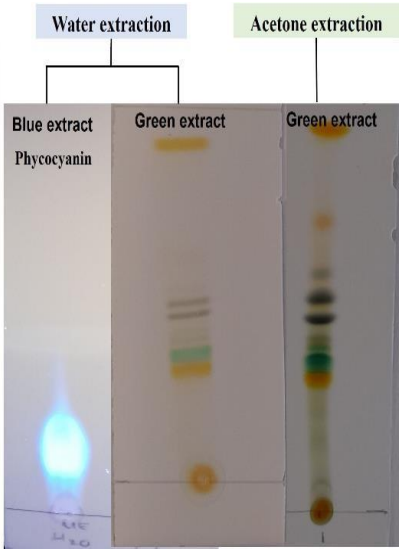


Figure 4.2 Comparison of solvent systems to separate extract contents and RF values (retention factor) for each system.

The method involved loading 190 g of silica (60-120 mesh) with 6 g of the crude dried green extract. The following pigments were isolated: β-carotene (0.9 g), pheophorbide *a* (1.4 g), pheophytin *a* (2.6 g), and lutein (0.5 g) (**Figure 4.4**).

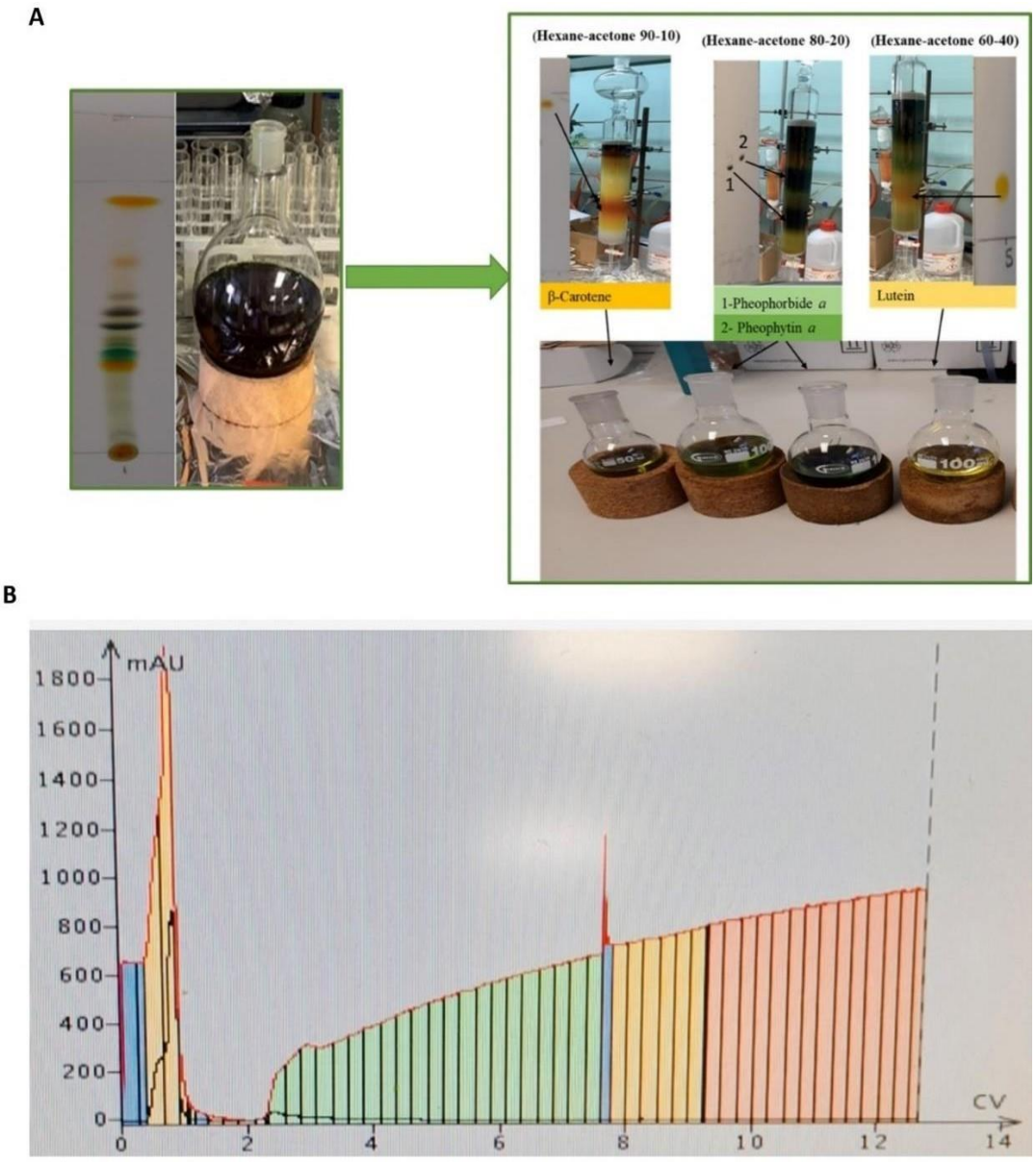


Figure 4.3 Isolation and purification using flash chromatography.

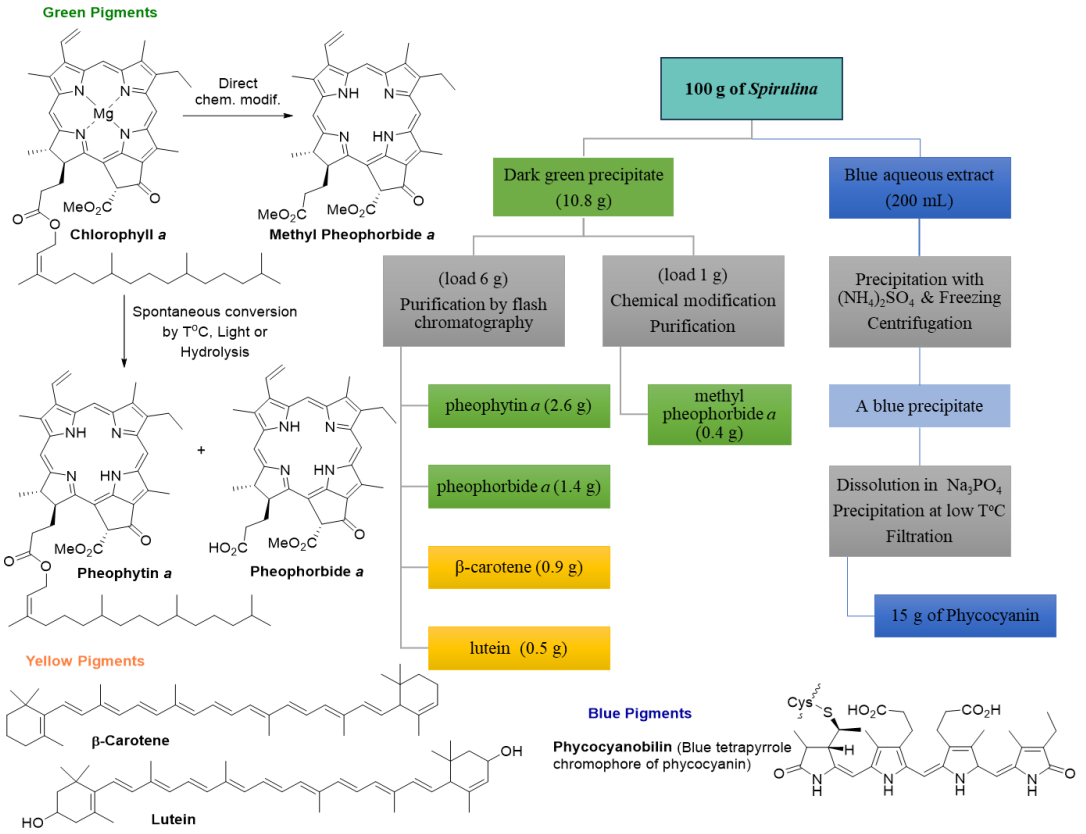


Figure 4.4 Isolation and purification of photosynthetic pigments from Spirulina. Chemical structures of the key isolated compounds: b-carotene, lutein, pheophytin a, pheophorbide a, methyl pheophorbide a, phycocyanin. Chemical transformations involving chlorophyll a.

To demonstrate the versatility of using untreated extracts, we have converted all chlorophyll- derived molecules into one single lipophilic methyl pheophorbide *a*. This can be achieved by treating 1 g of a raw green solid extract with acid in methanol, followed by re-crystallization, yielding 0.4 g of the compound with a purity of 94.5%. The alkyl chain is removed by hydrolysis, followed by esterification with methanol to obtain methyl pheophorbide *a* (**Figure 4.4**). This method can be used to prepare a variety of lipophilic derivatives using different alcohols for the esterification step.

The purity of the isolated pigments was confirmed by HPLC-DAD analysis using a reverse column, with the mobile phase containing trifluoroacetic acid (50-95%) and acetonitrile monitored at 430 nm (Soret band) and 620 nm (Q-band). A simultaneous detection at both wavelengths is very important, as it ensures the samples are not contaminated by carotenoids (430 nm) or phycocyanin (620 nm). The retention times at 430 nm and 620 nm were 8.60

min for pheophytin *a*, 8.73 min for pheophorbide *a*, and 8.65 min for methyl pheophorbide *a* (Appendix B, **Figure B.4)**. The purity of the isolated pigments was calculated using the DAD chromatogram peak area at 430 nm. A high purity (>95%) was confirmed for pheophorbide *a* and methyl pheophorbide *a*, while pheophytin *a* showed 92% under these conditions due to racemization facilitated by TFA [26].

4.3.2.1 Further identification of chlorophyll derivatives

Methyl pheophorbide *a*, pheophorbide *a* and pheophytin *a* are structurally very similar; their identities were confirmed by NMR, UV-Vis, and accurate MS. (Appendix B, **Table B.1, Figures B.1, B.2**). The UV-vis absorption spectra are similar, featuring an intense Soret band at ca. λ_{max} 410 nm and a series of so-called Q bands between 505 nm and 665 nm with an intensity several times lower than that of the Soret band. Despite the structural complexity, the ^{1}H NMR spectra of these pigments feature several characteristic regions: (1) the 7-10 ppm range contains three signals (singlets) for *β*- and *meso*-Hs; (2) the 3.3-4.9 ppm range contains four singlets of methyl groups at the pyrrole ring; (3) the 6-8 ppm range contains signals of the vinyl group (as ABX system) present in chlorophyll *a* and its derivatives; (4) the 0.8-5 ppm range contains protons of the phytyl chain; when de-pytylation occurs, these signals disappear [27].

The mass spectrometry (MS) analysis confirmed the structures of the chlorophyll derivatives. Pheophytin *a* gave a monoisotopic m/z 887.5697 value corresponding to [M + H_2O] [28], pheophorbide *a* and methyl pheophorbide *a* showed monoisotopic $[M + H]^+$ signals of m/z 593.2773 [29] and 607.2752 [12], respectively.

4.3.3 Purification of blue extract and identification of phycocyanin

Most of the studies in the literature on phycocyanin extractions focused on maximizing the extract yield, overlooking other phytochemicals [4]. In this study, an extract containing phycocyanin was obtained by separating the upper blue aqueous layer from the viscous green fraction **(Figure. 4.1).** This blue extract was treated with $(NH_4)_2SO_4$, frozen, and centrifuged to yield a blue (**Figure. 4.4)** precipitate, which was re-dispersed in K_3PO_4 and filtered, yielding 15 g of blue powder.

To confirm the structural features of phycocyanin, we have assessed its spectral properties, chemical purity, and protein content. Phycocyanin belongs to the phycobiliprotein (PBP) family, which can be broadly divided into three classes based on their spectral properties: phycoerythrin (λ_{max} 565 nm), phycocyanin (λ_{max} 620 nm), and allophycocyanin (λ_{max} 650 nm). In this study, the UV-vis absorption spectrum of the purified phycocyanin shows a prominent peak at λ_{max} 620, which corresponds to phycocyanin.

To further characterize the purified samples, infrared (IR) spectroscopy was employed. (Appendix B, **Table B.2, Figure. B.4**). IR analysis confirmed the presence of the key functional group regions: (1) centered at 3466 cm^{-1} denotes the stretching vibrations of N-H and O-H, (2) centered at 1638 cm^{-1} refers to the stretching vibrations of C=O (amide), (3) centered at 1460 cm^{-1} indicates C=C and O-C-O stretches, (4) centered at 1101 cm^{-1} refers to C-O stretching; this data is consistent with the literature [30].

The purity of phycocyanin was determined by HPLC/DAD. A single peak with a retention time of 1.88 min was recorded in the reverse phase (DAD 620 nm, 100% purity), confirming that phycocyanin did not contain chlorophyll and its derivatives. Furthermore, the structural study of phycocyanin [31] shows that it is a chromophore bound to two distinct types of polypeptides with a large molecular weight β-unit (MW: 14-21 kDa) and a low molecular weight α-unit (MW: 12-19 kDa), generally present in equal amounts. Thus, the purity of phycocyanin relative to the polypeptide content is an essential factor that determines its application [4]. The presence of protein content in phycocyanin

extracts can be assessed by calculating the ratio between the absorption of phycocyanin at 620 nm and the absorption of all proteins at 280 nm (A_{620}/A_{280}). Based on the classification, the A_{620}/A_{280} ratio greater than 4.0 is analytical grade, the A_{620}/A_{280} ratio between 0.7 and 3.9 is reagent grade, and the ratio below 0.7 is considered food grade [8]. In this study, the purity of phycocyanin was at the food grade level (0.6). When lyophilizing *Spirulina*, the purity of phycocyanin usually does not exceed 0.6; however, dialysis can be performed on the extracted samples to increase purity to 2.2 by removing the protein surrounding the chromophore (phycocyanin) [6].

4.3.4 Antioxidant and pro-oxidant evaluation

Unique structural features allow chlorophyll and its derivatives to exhibit both antioxidant and pro-oxidant properties. The antioxidant potential of chlorophyll derivatives and phycocyanin was highlighted by several studies [10]. However, due to challenging purification, very few have analysed methyl pheophorbide *a*, pheophorbide *a* and pheophytin *a* in pure form. Also, some studies report on the pro-oxidant activities (singlet oxygen production) of phycocyanin. Here, we carried out a comparative systematic analysis between chlorophyll derivatives and phycocyanin as biological molecules with dual activity.There is a plethora of studies assessing the antioxidant and radical scavenging activity of β-carotene and lutein; therefore, their analysis was not covered in this study.

4.3.4.1 General antioxidant capacity: DPPH, TEAC and FRAP assays

It is accepted that a single assay cannot provide a full picture of antioxidant activity due to the different mechanisms and limitations of individual assays [32]. Moreover, the analysis of impurified raw extracts is problematic due to the presence of multiple classes of antioxidants, e.g., small molecules and proteins.

Additionally, pigments can interfere with colorimetric analysis as they can give their own signals in the working region of the assay, affecting the results. Thus, we employed ferric-reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC), and free radical scavenging activity (DPPH) assays to provide a comprehensive rating of antioxidant capacity. The FRAP assay measures antioxidant capacity directly by reducing ferric (III) ions to ferrous (II) ions *via* a single electron transfer mechanism (SET), while DPPH and TEAC assess antioxidant capacity by scavenging free radicals through both SET and hydrogen atom transfer (HAT) [32].

All purified chlorophyll derivatives showed a dose-dependent response in DPPH, TEAC and FRAP assays. (**Figure. 4.5**) When compared at the highest concentration of 250 µg/mL, methyl pheophorbide *a* displayed the best radical scavenging activity, followed by pheophorbide *a* and pheophytin *a* (**Figure. 4.5** and Appendix B, **Figure. B.5**). This trend is confirmed by other studies using TEAC assay [10].

The results of FRAP and TEAC assays are very similar. As $ABTS^{\cdot+}$ radical in TEAC assay has a redox potential (+0.68V) close to the one in FRAP (Fe^{2+}/Fe^{3+}) with the latter being a purely SET process, it is possible to conclude that chlorophyll derivatives predominately act through electron transfer rather than hydrogen transfer (HET). This fact can help to explain the lower antioxidant activity in the DPPH assay, as DPPH is a stable radical that can act as a radical as well as an oxidant that can be quenched *via* HET and SET mechanisms. Additionally, a strong interference between chlorophyll derivatives and DPPH radicals at 515 nm, especially at higher concentrations, can obscure the results (Appendix B, **Figure. B.6**).

The difference in the radical scavenging ability of the chlorophyll derivatives is driven by the side chains: phytyl (in pheophytin *a*), methoxy (-OMe) (in methyl pheophorbide *a*), and hydroxy (-OH) (in pheophorbide *a*) (**Figure. 4.4**). The methoxy group is electron donating, which increases the stability of the resonance system in the macrocyclic tetrapyrroles and may lead to increased radical scavenging activity, while a lesser stability is associated with an electron withdrawing group such as -OH. This can result in a decrease in radical scavenging activity [33]. Moreover, the presence of a phytyl chain can provide a steric hinderance, delaying the interaction and, consequently, decrease the quenching of radicals.

Several studies have claimed that phycocyanin has high radical scavenging activity *in vitro*. [34] Although phycocyanin demonstrated a dose-dependent response in TEAC, FRAP, and DPPH assays, its activity was significantly lower than that of green pigments in this concentration range. A low response in the DPPH assay can be explained by the steric bulkiness of phycocyanin. It is known that antioxidant activity measured by DPPH is higher for small molecules, where unhindered and rapid access to DPPH radicals is favored. Lower antioxidant activity indicates that the mechanism of action by which phycocyanin quenches these radicals is limited. Similar to green pigments, the FRAP assay delivers the best results, indicating its potential to act as an electron donor, hence demonstrating that phycocyanin acts preferentially through the SET electron transfer mechanism [32].

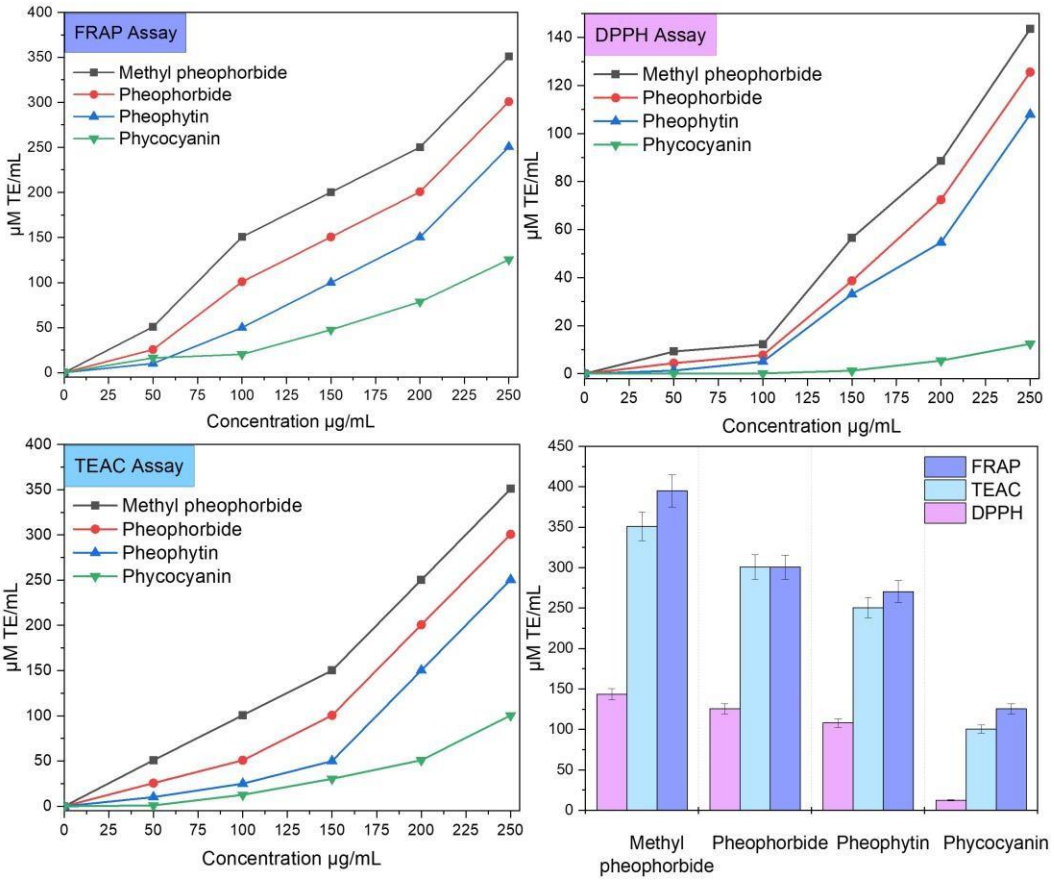


Figure 4.5 Dose-dependent antioxidant activity of purified pigments assessed by FRAP, DPPH and TEAC assays. Comparative analysis of individual pigments at the highest concentration (250 µg/mL).

The activity of purified pigments decreases in the following order: methyl pheophorbide a > pheophorbide a > pheophytin a > phycocyanin. This difference in radical scavenging activity is due to the chemical structure of the chromophore in the pigments. Radical scavenging activity of antioxidants is often associated with an increased number of hydrogen donors, such as hydroxy (-OH) and amino (-NHR) groups. Although chlorophyll derivatives and phycocyanin have some structural similarities, such as tetrapyrrole chromophore with hydroxy and NHR groups, the hydrogen transfer ability of these groups in phycocyanin is rather reduced due to being a part of carboxylic and pyrrole units, respectively **(Figure. 4.4).** However, chlorophyll derivatives are macrocyclic tetrapyrroles, which are aromatic compounds (18π-electrons), while the phycocyanin chromophore is an open chain tetrapyrrole. Noteworthy chlorophyll derivatives are chlorins, which are porphyrins with one reduced □C-□C single bond, which can be easily oxidized to a □C=□C double bond. This facile

oxidation improves the stability of the macrocycle and hence is a favored process, which can also explain their readiness to act through the SET mechanism in the antioxidant assays. And finally, phycocyanin is a protein-based pigment with limited accessibility to the chromophore unit; the protein content can impact the activity, i.e., analytical grade has a higher activity than food grade. This may indeed be a factor in the lower antioxidant capacity when compared to the chlorophyll derivatives.

4.3.4.2 Effect of algal pigments on viability of Caco-2 cells

Caco-2 cells are used to measure the reaction, absorption and cellular transport of food components and drugs. Obviously, analysis of experimental data in the Caco-2 cell model cannot be directly compared with in vivo data. However, intestinal epithelial cell models, such as the Caco-2 model, hold several advantages due to their simplicity and reproducibility, allowing results to be compared between laboratories. Furthermore, pursuing the effect of a bioactive food in a cell line model opens the door to studies of molecular mechanisms that may be difficult to address in vivo. In this study, MTT and neutral red (NR) assays were employed to determine the impact of individual algal pigments on cell viability. All pigments exerted dose-dependent cytotoxicity towards Caco-2 cells except phycocyanin, which did not reduce cell viability, even at the highest concentration of 100 µg/mL. Pheophorbide *a* and pheophytin *a* showed a noticeable reduction in cell viability when the concentrations reached 25 µg/mL ($p < 0.05$). Methyl pheophorbide *a* only demonstrated decreased cell viability at 100 µg/mL. In contrast, NR assay results for all samples indicated no significant cytotoxicity in the target concentration range (0 - 100 µg/mL) (**Figure. 4.6B**). This different outcome in the cytotoxicity for these assays is likely due to the different mechanisms of action. The MTT assay is based on the enzymatic conversion of MTT to its insoluble formazan in the mitochondria, while the NR assay depends on the uptake and lysosomal accumulation of the supravital dye [35]. Moreover, it is known that pheophorbide *a* can accumulate in mitochondria, leading to a targeted phototoxicity when activated by light [36]. Thus, a high concentration of green pigments could have some effect on mitochondrial respiratory activity, leading to a decreased viability of Caco-2 cells. Previous studies on

the cytotoxicity of green pigments using the MTT assay reported differing observations. For example, pheophorbide *a* (0.5-1.0 µM) and pheophytin *a* (5-30 µM) fractions from the algae *Saccharina japonica* did not impact the cell viability of LPS-stimulated RAW 264.7 macrophage cells when analysed by the MTT assay [37]. Similarly, using the MTT assay, C-phycocyanin from blue green algae did not show toxicity towards RAW 264.7 cells, even at concentrations up to 250 µg/mL [38]. The differing sensitivity of individual cell lines to algal pigments could in part explain the variable findings in the literature regarding their cytotoxicity.

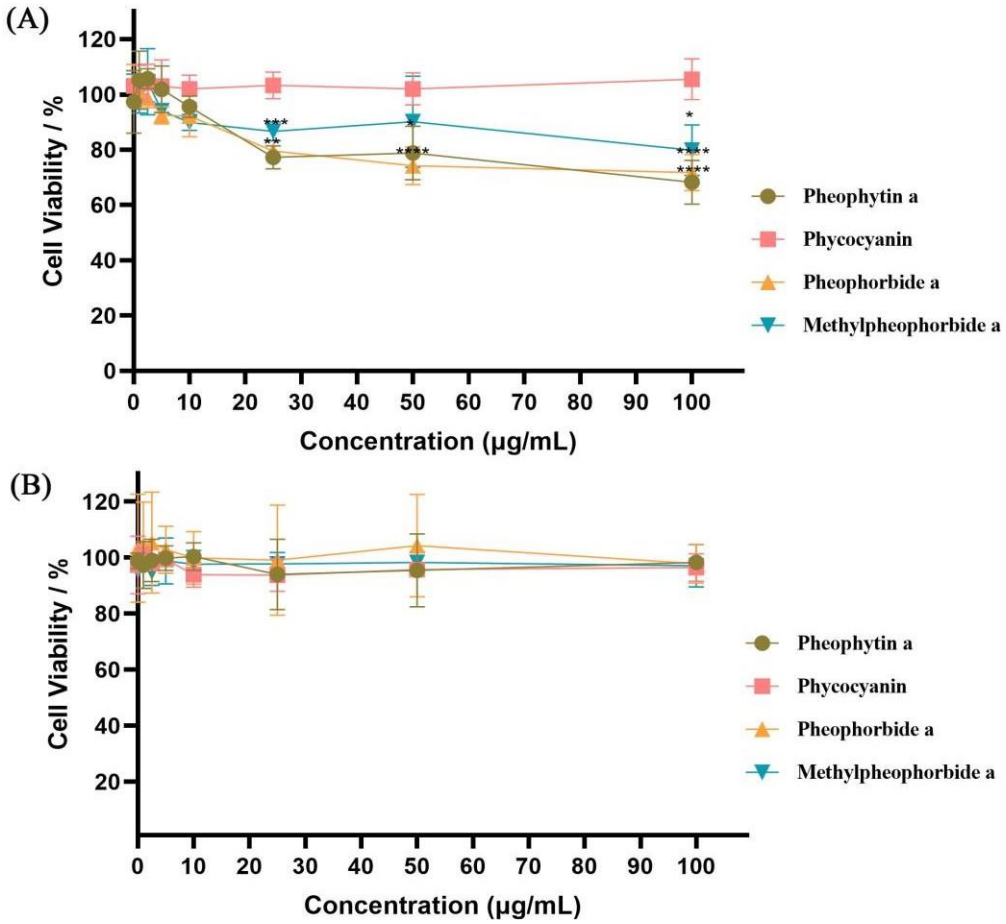


Figure 4.6 Effect of purified algal pigments on viability of Caco-2 cells determined by MTT (A) and Neutral Red assay (B). The results are presented as mean value ± SD, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

4.3.4.3 Effect of algal pigments on intracellular generation of ROS

The intracellular antioxidant capacity of algal samples against H_2O_2-induced ROS generation in Caco-2 cells is shown in **Figure. 4.7**. Under H_2O_2-induced conditions, pheophytin *a*, pheophorbide *a* and methyl pheophorbide *a* demonstrated a dose-dependent reduction in ROS levels, starting at 25 µg/mL, 25 µg/mL, and 5 µg/mL, respectively. In contrast, phycocyanin exerted significant intracellular antioxidant capacity at 1 µg/mL, which did not display a clear dose-dependent pattern across the range of concentrations tested (**Figure. 4.7B**). Indeed, the protective effects of marine algae on cancer, neurodegenerative, inflammatory, and other ROS-related diseases have been linked to the presence of bioactives such as photosynthetic pigments [39]. Previous research has shown that phycocyanin has antiviral effects by reducing the level of cellular ROS [40]. Pheophorbide *a* was reported to significantly reduce UVB-induced ROS accumulation by 36.5% and 46.2% at concentrations of 0.1 and 1 µM in CCD-986sk cells [19]. In this study, comparative analysis showed that methyl pheophorbide *a* seemed to reduce ROS generation more effectively than pheophorbide *a*, which might be due to the structural difference discussed under general antioxidant activity. Overall, the intracellular results are in line with the findings observed for DPPH, TEAC, and FRAP assays.

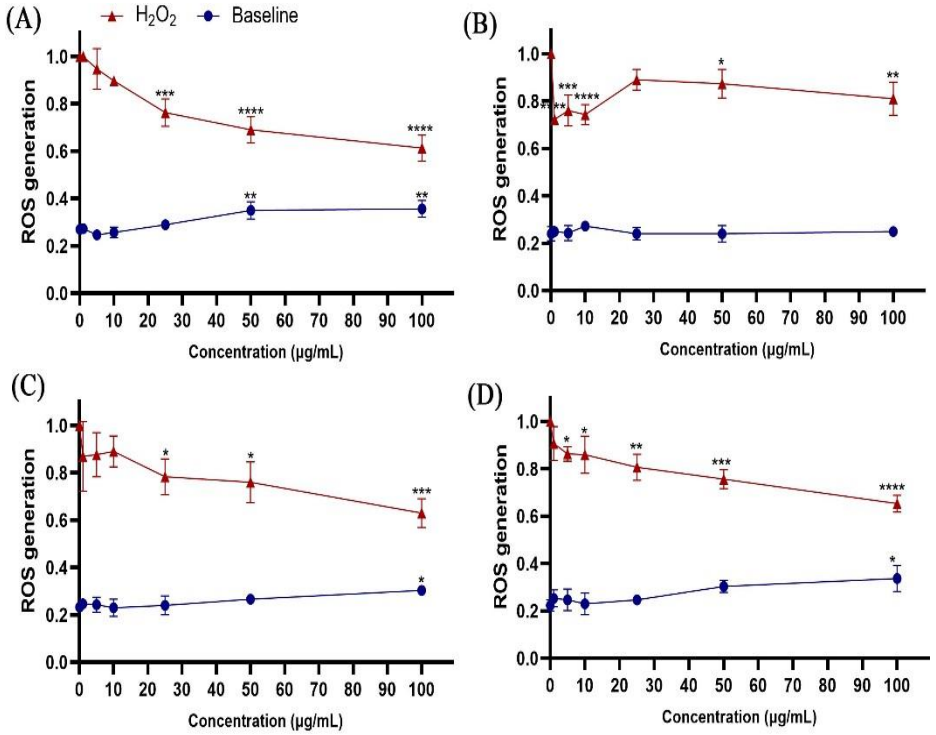


Figure 4.7 Effect of pheophytin a (A), phycocyanin (B), pheophorbide a (C), methyl pheophorbide a (D) against H2O2-stimulated ROS generation in Caco-2 cells. The results are presented as mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

As well, the baseline (cells without being exposed to H_2O_2, blue line) for pheophytin *a*, pheophorbide *a,* and methyl pheophorbide *a* slightly increased at concentrations of 50 µg/mL, 100 µg/mL and 100 µg/mL, respectively. Phycocyanin showed no effect on ROS generation in cells without H_2O_2 induction. An increase of the baseline in the samples containing green pigments could be due to color interference between the samples and assay, as pheophytin *a*, pheophorbide *a,* and methyl pheophorbide *a* were observed to have their own signals under the same measurement conditions for ROS detection (Appendix B, **Figure. B.7**).

4.3.4.4 Pro-oxidant activities

It is well known that porphyrins are excellent Type II photosensitizers (PS) with good capabilities to generate singlet oxygen ($^1O_2$) upon irradiation with visible light. This feature is widely explored in photomedical applications such as photodynamic therapy (PDT). Although chlorophyll derivatives exhibit $^1O_2$ production, comparative analysis between the derivatives is lacking. In this study, we have compared the ability of pheophorbide *a*, pheophytin *a*, methyl pheophorbide *a* and phycocyanin to produce singlet oxygen using methylene blue as a reference. This $^1O_2$ method is based on measuring a decrease in the absorption at λ_{max} 410 nm of a trapping agent (1,3-diphenylisobenzofuran, DPBF) in DMF in the presence of PS and light. The decrease in absorption occurs due to the photooxidation of DPBF by $^1O_2$ generated by PS, which leads to the DBB product of the ring-opening reaction [20] and is shown in **Figure. 4.8**.

Initial experiments were aimed at establishing the photostability of pigments and DPBF to visible light using long-pass cut-off filters at 420 nm, 455 nm, 475 nm, and 495 nm during the 120 s irradiation period with a Xenon lamp (50 W). On its own, DPBF shows photodegradation of up to 50% by visible light when a 420 nm filter is used. The degree of degradation falls to 20% and 5% for the 455 nm and 475 nm filters, respectively. Finally, 100% of photostability is achieved when using the 495 nm long-pass cut-off filter (Appendix B, **Figure. B.9**). In contrast, all pigments under investigation exhibit remarkable photostability, regardless of the filters used. The effectiveness of pigments to generate singlet oxygen was probed using a 495 nm filter, and the results are shown in **Figure. 4.8**. Pheophorbide *a*, pheophytin *a,* and methyl pheophorbide *a* are effective in $^1O_2$ generation, while phycocyanin shows only marginal activity when compared to chlorophyll derivatives. The results also indicate that the efficacy of methyl pheophorbide *a* to generate singlet oxygen is slightly higher than that of pheophorbide *a* and pheophytin *a*.

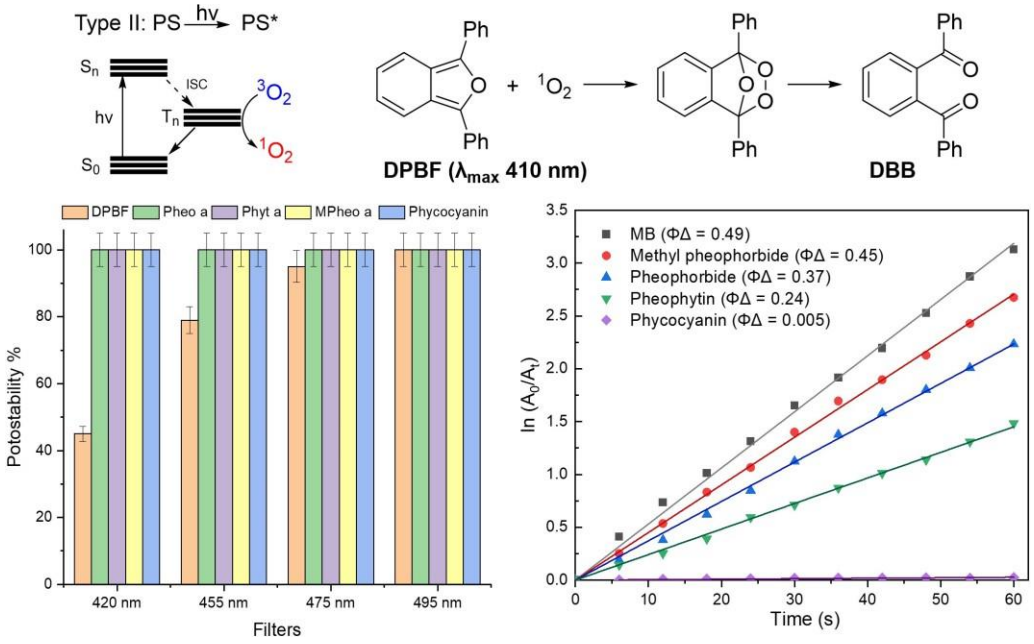


Figure 4.8 The photostability ratio of DPBF and pigments at 410 nm when illuminated by visible light (Xenon lamp - 50 W) using four cut-off filters at 120 sec. Photooxidation reaction between DPBF and 1O2 generated by PS. The linear fitting curve comparison of pigments and methylene blue.

The $^1O_2$ quantum yield (ΦΔ) for the pigments was obtained from the DPBF reaction using methylene blue (MB) as a standard. The linear plots of y(lnA) = f(t) were obtained by plotting the $\ln(A_0/A_t)$ values of the DPBF absorbance at 410 nm against the reaction time (t) under irradiation **(Figure. 4.8).** The $^1O_2$ quantum yield of MB measured in DMF is 0.49, which marks an efficient and powerful photosensitizer. Remarkably similar to MB, methyl pheophorbide *a* has a (ΦΔ) of 0.45, which is the highest value among chlorophyll derivatives. This was followed by pheophorbide *a* (ΦΔ 0.37) and pheophytin *a* (ΦΔ 0.24). In contrast, the singlet oxygen quantum yield of phycocyanin is only about 0.005 under these conditions. It was speculated that high FRAP values may correlate with the tendency to become pro-oxidants under certain conditions, which had been demonstrated for some flavones and flavanones [33-34]. In this study, this trend shows that the high values in FRAP indeed correspond to a high $^1O_2$ quantum yield.

4.4 Conclusion

To summarize, a sustainable approach was developed to extract photosynthetic pigments from *Spirulina* by freezing, thawing, and ultrasound assisted extraction using water as the sole solvent. This approach succeeded in producing three main types of photosynthetic pigments: carotenoids, chlorophylls, and phycocyanin from the same batch of *Spirulina*. We have also incorporated steps to convert chlorophyll into stable products such as pheophorbide *a* and pheophytin *a* with a high yield and purity. Additionally, the treatment of chlorophyll extract with acid followed by methylation led to the production of methyl pheophorbide *a*. Overall, the current results demonstrate that the combination of environmentally friendly solvents with a range of cell membrane disruption techniques can successfully recover photosynthetic pigments in a highly efficient manner and thereby contribute to reducing waste. A comparative analysis of green and blue pigments revealed that green pigments have significantly higher activity as antioxidants and prooxidants compared to phycocyanin. The superior antioxidant activity of green pigments can be attributed to their unique structure and ability to undergo SET; the presence of electron rich functional groups also enhances this ability. Equally, the pro-oxidant properties to generate singlet oxygen are excellent and on par with methylene blue, which is a well-known PS. In contrast, phycocyanin showed exceptionally low activity to generate singlet oxygen. The demonstration and comparison of these activities and the availability of the method may change the outlook for a wider utilization of these green pigments as food additives, in the cosmetic industries, or in the pharmaceutical industries in terms of protection and treatment.

4.5 References

1. Pagels, F., et al., *Extraction of pigments from microalgae and cyanobacteria—A review on current methodologies.* Applied Sciences, 2021. **11**(11): p. 5187.

2. Morocho-Jácome, A.L., et al., *(Bio) Technological aspects of microalgae pigments for cosmetics.* Applied Microbiology and Biotechnology, 2020. **104**(22): p. 9513-9522.

3. Moorhead, K., B. Capelli, and G. Cysewski, *SPIRULINA nature's superfood.* Hawaii: Cyanotech Corporation, 1993: p. 1-63.

4. Jaeschke, D.P., et al., *Phycocyanin from Spirulina: A review of extraction methods and stability.* Food Research International, 2021. **143**: p. 110314.

5. Schreidah, C.M., et al., *Microcystins: Biogenesis, toxicity, analysis, and control.* Chemical Research in Toxicology, 2020. **33**(9): p. 2225-2246.

6. Minchev, I., N. Petkova, and I. Milkova-Tomova, *Ultrasound-assisted extraction of chlorophylls and phycocyanin from Spirulina platensis.* Biointerface Res Appl Chem, 2020. **11**: p. 9296-9304.

7. Andersen, R.A., *Algal culturing techniques*. 2005: Elsevier.

8. Marzorati, S., et al., *Supercritical CO2 and Green Extraction methods for Added Value Products: Carotenoids, Chlorophylls and Phycocyanin from Spirulina Microalgae.* Green Chemistry, 2020.

9. Martins, M., et al., *Recovery of chlorophyll a derivative from Spirulina maxima: Its purification and photosensitizing potential.* ACS Sustainable Chemistry & Engineering, 2021. **9**(4): p. 1772-1780.

10. Pérez-Gálvez, A., I. Viera, and M. Roca, *Carotenoids and chlorophylls as antioxidants.* Antioxidants, 2020. **9**(6): p. 505.

11. Ferreira-Santos, P., et al., *Sequential multi-stage extraction of biocompounds from Spirulina platensis: Combined effect of ohmic*

heating and enzymatic treatment. Innovative Food Science & Emerging Technologies, 2021. **71**: p. 102707.

12. Smith, K.M., D.A. Goff, and D.J. Simpson, *The meso substitution of chlorophyll derivatives: direct route for transformation of bacteriopheophorbides d into bacteriopheophorbides c.* Journal of the American Chemical Society, 1985. **107**(17): p. 4946-4954.

13. Wellburn, A.R., *The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution.* Journal of plant physiology, 1994. **144**(3): p. 307-313.

14. Bennett, A. and L. Bogorad, *Complementary chromatic adaptation in a filamentous blue-green alga.* The Journal of cell biology, 1973. **58**(2): p. 419-435.

15. Hsu, C.-Y., et al., *The antioxidant and free radical scavenging activities of chlorophylls and pheophytins.* 2013.

16. Fernando, G.S.N., et al., *Application of an Ultrasound-Assisted Extraction Method to Recover Betalains and Polyphenols from Red Beetroot Waste.* ACS Sustainable Chemistry & Engineering, 2021. **9**(26): p. 8736-8747.

17. Fernando, G.S.N., et al., *Novel approach for purification of major betalains using flash chromatography and comparison of radical scavenging and antioxidant activities.* Food Chemistry, 2022. **385**: p. 132632.

18. Wang, Y., et al., *Uptake and Immunomodulatory Properties of Betanin, Vulgaxanthin I and Indicaxanthin towards Caco-2 Intestinal Cells.* Antioxidants, 2022. **11**(8): p. 1627.

19. Lee, H., H.-Y. Park, and T.-S. Jeong, *Pheophorbide a derivatives exert antiwrinkle effects on UVB-induced skin aging in human fibroblasts.* Life, 2021. **11**(2): p. 147.

20. Rogers, L., et al., *Lead structures for applications in photodynamic therapy. 6. Temoporfin anti-inflammatory conjugates to target the*

tumor microenvironment for in vitro PDT. PLoS One, 2015. **10**(5): p. e0125372.

21. Tan, G., et al., *The in vitro photodynamic activity, photophysical and photochemical research of a novel chlorophyll-derived photosensitizer.* Medicinal Chemistry Research, 2017. **26**(10): p. 2639-2652.

22. Anlauf, H., *Recent developments in centrifuge technology.* Separation and Purification Technology, 2007. **58**(2): p. 242-246.

23. Tavanandi, H.A., et al., *Simple and efficient method for extraction of C-Phycocyanin from dry biomass of Arthospira platensis.* Algal research, 2018. **31**: p. 239-251.

24. Gabr, G.A., S.M. El-Sayed, and M.S. Hikal, *Antioxidant activities of phycocyanin: A bioactive compound from Spirulina platensis.* J. Pharm. Res. Int, 2020. **32**: p. 73-85.

25. Činčárová, D., et al., *Recommendations on the quantitative analysis of pheophorbides, photosensitizers present in algal biomass intended as food supplement.* Algal Research, 2021. **56**: p. 102298.

26. Davani, L., et al., *Integrated analytical approaches for the characterization of Spirulina and Chlorella microalgae.* Journal of Pharmaceutical and Biomedical Analysis, 2022. **219**: p. 114943.

27. Katz, J.J. and C.E. Brown, *Nuclear magnetic resonance spectroscopy of chlorophylls and corrins.* Bull. Magn. Reson, 1983. **5**: p. 3-49.

28. Freitas, S., et al., *Chlorophyll derivatives from marine cyanobacteria with lipid-reducing activities.* Marine drugs, 2019. **17**(4): p. 229.

29. Miranda, N., et al., *Pheophorbide a, a compound isolated from the leaves of Arrabidaea chica, induces photodynamic inactivation of Trypanosoma cruzi.* Photodiagnosis and Photodynamic Therapy, 2017. **19**: p. 256-265.

30. Izadi, M. and M. Fazilati, *Extraction and purification of phycocyanin from spirulina platensis and evaluating its antioxidant and anti-inflammatory activity.* Asian Journal of Green Chemistry, 2018. **2**(4): p. 364-379.

31. Dagnino-Leone, J., et al., *Phycobiliproteins: Structural aspects, functional characteristics, and biotechnological perspectives.* Computational and Structural Biotechnology Journal, 2022.

32. Prior, R.L., X. Wu, and K. Schaich, *Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements.* Journal of agricultural and food chemistry, 2005. **53**(10): p. 4290-4302.

33. Hoshina, C., K. Tomita, and Y. Shioi, *Antioxidant activity of chlorophylls: Its structure-activity relationship*, in *Photosynthesis: mechanisms and effects*. 1998, Springer. p. 3281-3284.

34. Renugadevi, K., et al., *Antioxidant activity of phycocyanin pigment extracted from marine filamentous cyanobacteria Geitlerinema sp TRV57.* Biocatalysis and agricultural biotechnology, 2018. **16**: p. 237-242.

35. Fotakis, G. and J.A. Timbrell, *In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride.* Toxicology letters, 2006. **160**(2): p. 171-177.

36. Tang, P.M.-K., et al., *Pheophorbide a based photodynamic therapy induces apoptosis via mitochondrial-mediated pathway in human uterine carcinosarcoma.* Cancer biology & therapy, 2009. **8**(6): p. 533-539.

37. Islam, M.N., et al., *Anti-inflammatory activity of edible brown alga Saccharina japonica and its constituents pheophorbide a and pheophytin a in LPS-stimulated RAW 264.7 macrophage cells.* Food and chemical toxicology, 2013. **55**: p. 541-548.

38. Cherng, S.-C., et al., *Anti-inflammatory activity of c-phycocyanin in lipopolysaccharide-stimulated RAW 264.7 macrophages.* Life sciences, 2007. **81**(19-20): p. 1431-1435.

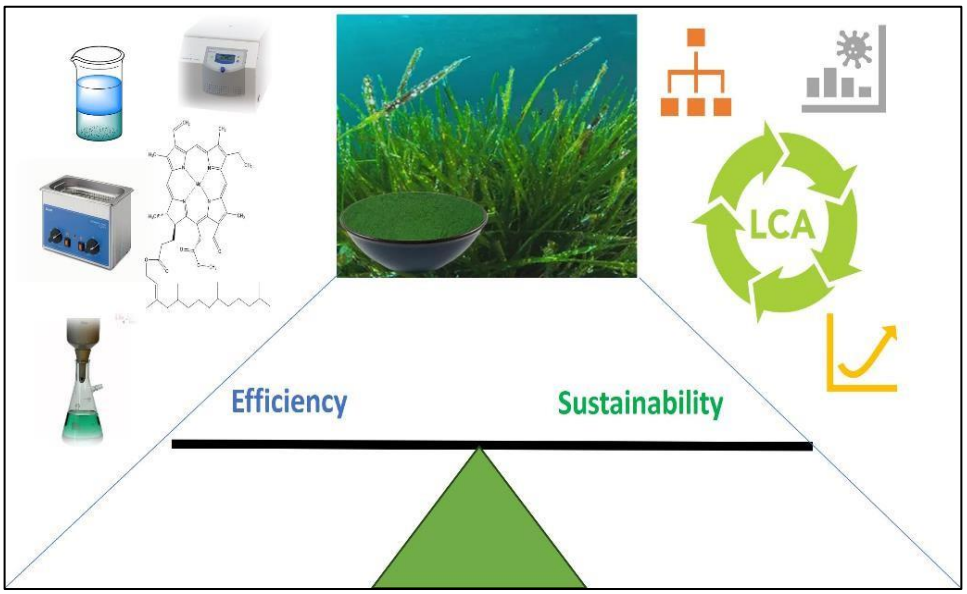
39. Pradhan, B., et al., *Bioactive metabolites from marine algae as potent pharmacophores against oxidative stress-associated human diseases: A comprehensive review.* Molecules, 2020. **26**(1): p. 37.

40. Jadaun, P., et al., *Elucidation of Antiviral and Antioxidant Potential of C-Phycocyanin against HIV-1 Infection through In Silico and In Vitro Approaches.* Antioxidants, 2022. **11**(10): p. 1942.

Chapter 5 Life-Cycle-Assessment of Spirulina as a Biomass Source for Photosynthetic Pigments: Trade-Off between Efficiency and Sustainability of the Extraction Methods

Highlights

- The impact of conventional solvent extraction is higher than that of innovative green extraction.
- Energy is crucial for the environmental footprint of the total process for matrices.
- Simultaneous use of ultrasound (UAE) and freezing-thawing cycles is recommended for the extraction of chlorophyll from *Spirulina*.
- Food-grade solvents influence eutrophication, freshwater, and terrestrial ecotoxicity.



Abstract

Spirulina platensis is used as a food additive, in the cosmetic or pharmaceutical industries because of its rich concentration of functional components, particularly pigments such as chlorophyll, carotenoids, and phycocyanin. This study aimed to investigate the environmental impact of laboratory techniques used to extract these materials from *Spirulina* through life cycle assessment (LCA), evaluating the efficiency of the processes based on the total pigment amount extracted. The examined extraction processes included conventional maceration (SLE), ultrasound-assisted (AUE), and freeze-thaw (FT) methods. The functional unit was 1 mg/g of *spirulina* pigments extracted, and the life cycle inventory contained the extraction methods. The life cycle impact assessment was carried out utilizing ReCipe 2016 at the midpoint level. Electricity had the greatest environmental impact in most categories during extraction procedures, followed by solvents such as acetone and ethanol. Even the decision to use a "greener" solvent like ethanol instead of acetone was disputable, as the extraction yield has an impact greater than the “greenness” of the raw materials/ solvents. For the scenario of combining FT with AUE and using water as a solvent, the lowest environmental impact was achieved in all categories with high yields of the three pigments: chlorophyll, carotenoid, and phycocyanin.

5.1 Introduction

Nowadays, there is an increasing demand for natural products from microalgae with applications in the food industry (additives, pigments, vitamins, and colourants) as well as in the pharmaceutical, nutritional, and cosmetics sectors [1]. Blue-green microalgae (*Spirulina*) is the richest source of natural chlorophyll *a*, and phycocyanin. Chlorophyll *a* is a green pigment that dissolves in water and organic solvents to varying degrees, while phycocyanin is a blue pigment that is soluble in water. Both are highly valuable and commonly used in many industries due to their antioxidant, anti-inflammatory, and anti-tumour properties [2]. There is a great deal of interest in determining the optimal extraction processes in terms of sustainability (environmental impact) and yield of these pigments from their natural sources [3]. “Sustainability” has received increasing attention from researchers in a wide range of disciplines [4]. A significant amount of research has been devoted to sustainable packaging, eco-labelling, product life cycles, eco-products, “green” consumption, and sustainable manufacturing and recycling systems [4].

Sustainability is an essential principle in the management of natural resources, including the optimization of operations, minimization of environmental harm, and integration of social and economic factors [5]. Therefore, the life cycle assessment allows an understanding of the most critical phases, such as the sustainability of operations, to pursue closed-loop strategies and related objectives [6]. From these considerations emerged the need for a multidisciplinary approach capable of analysing the life cycle of pigments extracted from natural products and identifying environmental priorities that accelerate the development of sustainable practices and actions [7]. Currently, there is a limited literature available in this interdisciplinary field quantifying the impact of sustainable improvements. However, the alignment between industry and scientific research can enhance the current basic production cycle by implementing appropriate proposals in the short to medium term [8].

In recent years, the extraction of pigments from natural products has addressed the development of technologies and the use of different solvents to create conditions for improved environmental performance in accordance with

sustainability goals [9]. The utilization of conventional solvent extractions has been extensively employed for pigments from natural sources. These extraction methods are regarded as simple and reasonably cost-effective, but they require a significant amount of time to perform, which can result in the degradation of active components. The overutilization of toxic solvents is likewise seen as a highly exacerbating element for both human well-being and the ecosystem. Novel and eco-friendly methods for extracting pigments have been developed, employing microwave and ultrasonic treatments to get rapid results. The use of ultrasound to extract pigments is commonly employed because of its effectiveness and high extraction rate. Microwave-assisted extraction, on the other hand, is seen as an alternative method to quickly release bioactive compounds from the sample matrix into the solvent [5, 10, 11]. As far as this report is aware, no life cycle evaluation has been conducted utilizing green technologies to examine the connection between sustainability and the extraction efficiency of pigments derived from algae. The novel aspect of this work is addressing the gap between existing literature that only suggests a few sustainable choices, often analyzed from a chemical and technical perspective, and the experimental data is required to design sustainable measures in pigment extraction.

To enhance understanding of the relationship between the effectiveness of extracting pigments from algae and sustainability, this study conducted a comparative analysis of different extraction methods, including traditional solvent extraction (SLE) and innovative green extraction methods such as ultrasound-assisted extraction (UAE) combined with freeze-thaw cycles. Solvent selection, environmental impact assessment, and extraction method were factors taken into consideration when evaluating the processes. A comparative life cycle analysis (LCA) was performed using appropriate databases and software to evaluate the sustainability of the extraction techniques aimed at these pigments.

5.2 Experimental section

5.2.1 Materials.

Spirulina powder (*Arthrospira platensis*) was purchased from the Nukraft brand. All chemicals and solvents were obtained from Fisher Scientific (Loughborough, UK) and Sigma Aldrich (Dorset, UK).

5.2.2 Pigments extraction

Extraction experiments. In this study, four methods were used to extract photosynthetic pigments: solid-liquid extraction (SLE), ultrasound-assisted extraction (UAE), freezing-thawing (FTE), and ultrasound-assisted extraction combined with freezing-thawing (FT+UAE). Extraction was performed using three types of solvents: water, ethanol, and acetone. The solid-to-liquid ratio (1:4 w/v) was selected for all experiments. In brief, 100 g of dry *Spirulina* was added to 400 mL of solvent in 500 mL capped round flasks. For the SLE, the round flasks were placed in a thermal bath at +50 °C under magnetic stirring for 24 h under reflux. For the UAE, the capped round flasks were placed inside the ultrasound bath at +50 °C and 40 kHz for 3 h. In the FTE method, the capped round flasks were frozen for 24 hours in the freezer (-20 °C). Then the mixture was thawed at +50 °C using a water bath. In FT+UAE experiments, the capped round flasks were frozen for 24 hours in the freezer (-20 °C). Then the mixture was thawed at +50 °C and sonicated at 40 kHz for 3 h. The mixture reached +50 °C after 2 hours of sonication. At the end of the extraction process, the solids were filtered through a Buechner funnel with a sealed-in coarse porosity filter (90–130 microns), and the solid residue was washed three times with 20 mL of solvent. After that, the solvent filtrate was centrifuged at 1207 ×g to yield solid residues. Using a reduced pressure the extracts obtained from all methods were evaporated at a temperature of 40 °C. The extracts were vacuum-dried at 40 °C overnight, and the obtained powders were stored in vacuum glass vials filled with nitrogen.

Extract characterization. The extraction yield was calculated from the weight ratio between the powdered extract obtained after the vacuum-drying process and the initial amount of dry Spirulina. The total pigment content in the extracts

was determined spectrophotometrically by UV-vis absorption spectroscopy using the Wellburn [12] and Bennett [13] equations, which were explained in detail in **Chapter 4, Section 2.8**.

5.2.3 Life cycle assessment (LCA).

In this study, the methodology was conducted in accordance with ISO 14040 [14] and 14044 [15]. The steps followed were definition of goal and scope, life cycle inventory, life cycle impact assessment, and interpretation.

Goal and scope definition. The LCA study was carried out with a twofold goal: (i) identifying extraction techniques and solvent extraction that provide the optimal trade-off between yield and environmental sustainability, and (ii) comparing the environmental benefits and drawbacks of different extraction processes in a laboratory setting. The LCA study's functional unit was set by looking at the extraction yield in terms of the amount of recovered pigment species. This means producing a powered extract with 1 mg of pigment content. The concentration of pigments in the extracts is measured spectrophotometrically using the Wellburn equation, in which the concentration of pigments in the extracts is expressed as mg of pigment per gram of spirulina powder. (mg/g). The choice of this functional unit enables an effective comparison of the extraction methods by considering their technical efficiency.

The type of LCA considered in this study was selected to be gate-to-gate [16], which is a partial LCA with the system boundaries as shown in **Figure 5.1**. Since upstream (agriculture and harvest) and downstream (use of the product and disposal of the product) processes are the same for all extraction approaches, this analysis is limited to the core process (the extraction step). This follows the same strategy already proposed by Priyadarshini *et al* [17] for recovering functional food ingredients from seaweed by different extraction techniques. The solid wastes, specifically the biomass obtained from the filtration step after the extraction process, were excluded from the LCA study as they were set outside the system boundary. These wastes are non-toxic and can be effectively utilized, e.g., for animal feed [18-20], eventually resulting in a potential environmental credit. Also, ‘wastewater’ was kept outside the system

boundary and excluded from the LCA because this step represents further extraction and purification of blue phycocyanin (**Chapter 4**).

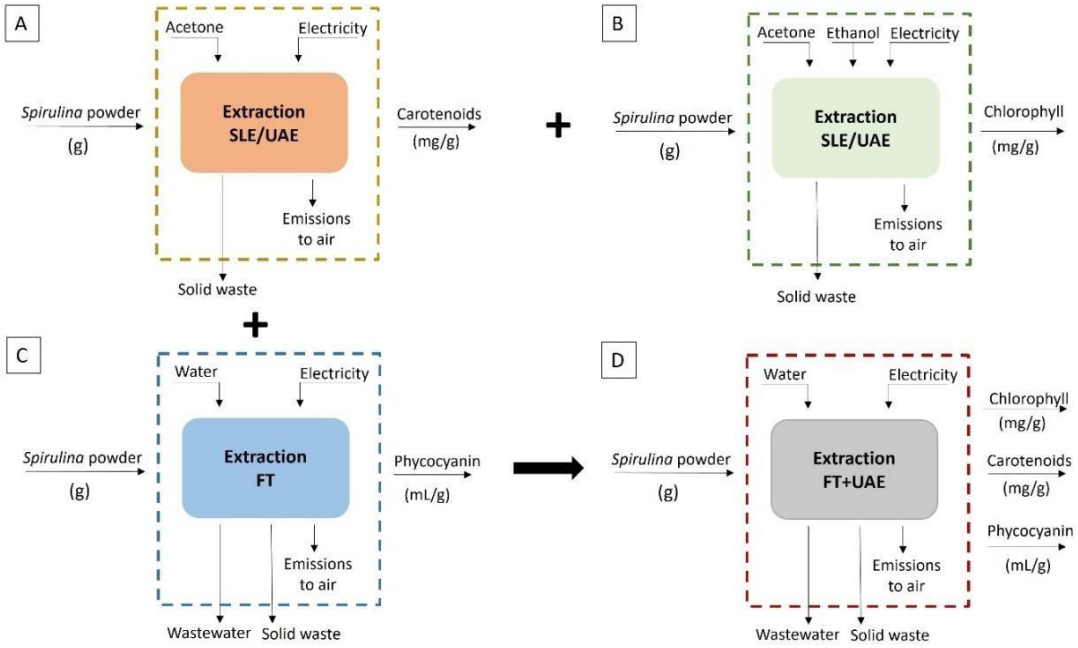


Figure 5.1 Simplified description of the system boundary for the LCA of the extraction processes of photosynthetic pigments from *Spirulina*.

Life cycle inventory. The life cycle inventory was modeled using primary data directly collected in the laboratory during the experimental activities and generic secondary data from databases. Experimental data was used for raw material and solvent inventories. Moreover, the electricity consumption of the used equipment was ascertained by recording their operation time and power ratings, as presented in **Table** 5.1. Generic data, as well as associated impacts, were sourced from the Environment Footprint (EF) database (version 3) using the openLCA 1.10.3 software (GreenDelta GmbH, Berlin, Germany).

Table 5.1. Inventory data used to model the extraction processes

Inventory	**Unit**	**SLE**	**UAE**	**FT**	**FT+UAE**	**Data Source/Remark**
Inputs						
Spirulina	g	100	100	100	100	outside system boundary
Acetone	g	326.4	326.4	-	-	measured
Ethanol	g	315.6	315.6	-	-	
Water	g	315.6	315.6	500	500	measured
Electricity for extraction	kWh	5.28	0.66	5.94	6.60	measured
Outputs						
Pigment	mg	1	1	1	1	outside system boundary
Solid waste (biomass)	g	92	92	90	75	outside system boundary
Evaporated acetone	g	32.1	32.1	-	-	Calculated from experiments using mass balance.
Evaporated Ethanol		28.5	28.5	-	-	Calculated from experiments using mass balance.
Recovered acetone	g	294,3	294,3	-	-	Considers a 90.16% acetone recovery rate
Recovered Ethanol		287.1	287.1	-	-	Considers a 90.69% acetone recovery rate
Wastewater	g	-	-	490	490	outside system boundary

Life cycle impact assessment (LCIA). The life cycle impact assessment was performed using the **ReCiPe 2016** method at the midpoint level. The impact categories evaluated were fine particle formation; freshwater ecotoxicity; and carcinogenic toxicity to humans. Non-carcinogenic human toxicity; marine ecotoxicity; marine eutrophication; scarcity of mineral resources; ozone for human health; ozone formation; terrestrial ecosystems; earth acidification. Terrestrial ecotoxicity and water consumption.

5.2.4 Data analysis

All experiments were carried out in triplicate. All results were expressed as the mean value ± standard deviation (SD). The graphs were plotted with Origin Pro 2020 for Windows. OpenLCA software was used to conduct the life cycle assessment.

5.3 Results and Discussion

In this study, extraction methodologies were analyzed in terms of efficiency and environmental impact. The extraction efficiency was determined by referring to the content of the pigment in the extracts after each process. The environmental effect of each method was modelled through LCA gate-to-gate, limited to the extraction step as only one value-added process. The results of these two complementary analyses were discussed in the following subsections:

5.3.1 Extraction Efficiency

Figure 5.2 shows the extraction yield and content of the three pigments: chlorophyll, carotenoids, and phycocyanin, from the extraction methods examined. Using the three methods (SLE, UAE, and FT+UAE) and the solvents (acetone, ethanol, and water), there was a difference in the extraction efficiency, as shown in **Figure 5.2A**, where the extraction production ranged between 30 and 100 mg/g, the chlorophyll content in the extracts ranged

between 9 and 68 mg/g, and the carotenoids content ranged from 1.25 mg/g to 17.3 mg/g. These ranges are close to the average values found in the literature for different *Spirulina* species and extraction conditions [21-23].

The extraction technique of combining freezing, thawing, and ultrasonic treatment in water as the solvent (a new approach) delivered the highest yield and the concentration of total chlorophyll and carotenoids. The process yield was 100 mg/g, with a total chlorophyll content of 68.94 mg/g and a carotenoids content of 17.3 mg/g. The SLE method using ethanol as a solvent had the lowest content of total chlorophyll, 3.5 mg/g, and carotenoids, 1.25 mg/g, with an extraction yield of 45 mg/g. Although SLE methods using acetone as a solvent yielded 30 mg/g, which is lower than in SLE (water) or SLE (EtOH), the concentrations of total chlorophyll (9.5 mg/g) and carotenoids (3.25 mg/g) were higher than in SLE (EtOH). The ultrasonic-assisted extraction (UAE) method yielded similar results, with the ethanol extract showing the highest yield and the lowest content of total chlorophyll and carotenoids compared to the acetone extract. However, the yield was higher compared to the SLE procedures for both solvents.

Obviously, the yield only provides partial information about the effectiveness of the extraction method and does not necessarily correlate with the quality of the extract in terms of target pigment content. For this reason, in this study, the extracts were characterized by measuring their pigment contents. The disparities among the three procedures became evident when examining the pigment concentrations in the extracts per gram of dry spirulina. In this regard, the most effective method was FT+UAE (new approach, solvent: water), in which the content of total chlorophyll and carotenoids increased by sevenfold compared to SLE (acetone and ethanol), while the concentration of total chlorophyll and carotenoids increased threefold when only employing UAE treatment. This difference can be explained by considering the mechanisms behind these approaches, as discussed in **Chapters 3 and 4**.

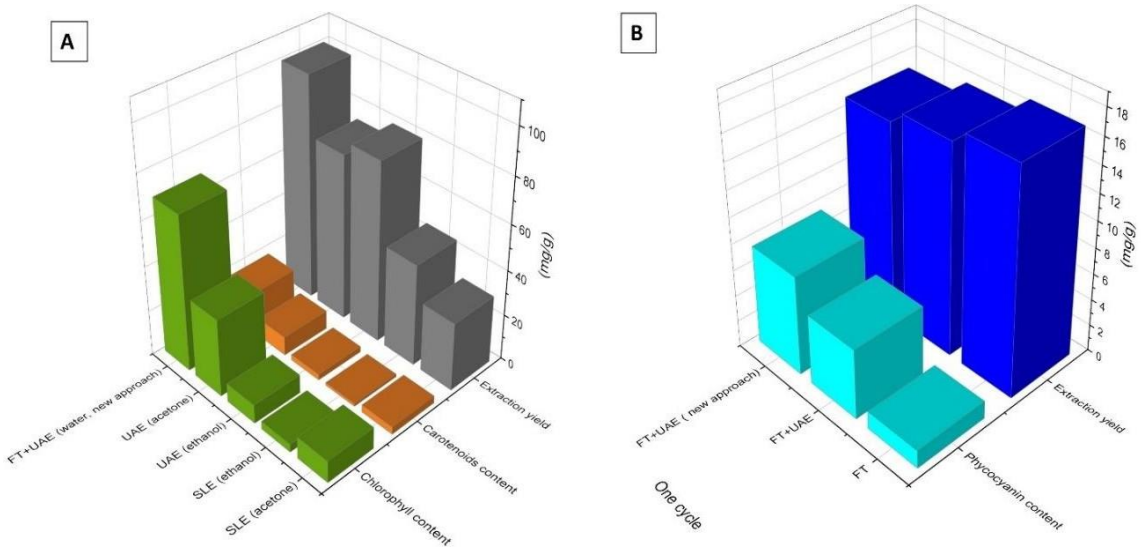


Figure 5.2 A) The extraction yield and chlorophyll content for the three extraction methods: conventional solid-liquid extraction (SLE, acetone or ethanol); ultrasound-assisted extraction (UAE, acetone, or ethanol) and combined freezing, thawing and ultrasonic extraction (FT+UAE, water). B) The extraction yield and phycocyanin content for the three extraction methods: FT, FT+UAE and FT+UAE (new approach).

It is important to note that employing the FT+UAE method resulted in a fivefold increase in the concentration of phycocyanin compared to a single cycle of freezing and thawing, as depicted in **Figure 5.2B**. The effectiveness of this method is highlighted by a significant increase in the total pigment yield and a high concentration of three types of pigments: carotenoids, chlorophyll, and phycocyanin, from a single batch of *Spirulina*.

5.3.2 Environmental profiles of extraction processes and comparison

In this study, the environmental profile of the processes applied to extract photosynthetic pigments from *Spirulina* using the ReCiPe 2016 method is described at the midpoint level, which contains 18 impact categories. This method was chosen among other well-known LCIA models such as CML, Eco-Indicator, and EcoScarcity due to its up-to-date nature in terms of time, location, and completeness. **Figure 5.3** (Appendix C. **Tables. C.1**, **C.2)** show the general environmental impacts of photosynthetic pigment extraction.

processes: SLE and AUE for carotenoids and chlorophyll extraction, and FT for phycocyanin extraction. When FT and AUE are combined using our new approach described in **Chapter 4**, these three pigments are isolated from the same batch of *Spirulina*.

The data indicates that extraction of SLE using different solvents (acetone or ethanol) produces the greatest impact in all categories compared to extraction by UAE. The different impact categories are related to the time of the extraction process and the type of solvent used in the extraction process, except for ozone formation, which depends mainly on the use and emission of chemicals [24] (in this study, acetone or ethanol). Accordingly, shorter extraction times in the UAE method generally correspond to lower environmental impacts due to the pigment extraction efficiency of 3 hours, compared to 24 hours for SLE, with the lowest impacts for 12 out of 18 indicators. When considering the impact on global warming, UAE-acetone extraction produces 0.62 kg of CO_2 equivalent per kg of pigment produced, while SLE (acetone) produces CO_2 equivalent to seven times this amount (Appendix C. **Table. C.1**). The highest environmental impact among the selected categories for SLE and AUE (acetone and ethanol) methods was eutrophication and the potential for aquatic and terrestrial ecotoxicity in freshwater. In the case of FT+AUE, the highest environmental impact was water consumption (**see Figure 5.3 A, B**)

Comparing these results with other studies is challenging due to differences in various elements of LCA, such as functional modules, system boundaries, product customization, and impact assessment methods. The citations provided are for benchmarking purposes only, and further comparison may not be appropriate. Ding *et al* [25] found approximately 4 kg CO_2 equivalent per kg of tannins extracted on a laboratory scale, while Modahl *et al* [26] found approximately 1 kg CO_2 equivalent per kg of vanillin extracted on a fully industrial scale.

For the FT extraction of phycocyanin, the extraction time was 27 hours, close to the time in SLE of 24 hours, but the environmental effects resulting from FT were much less than in SLE, with the lowest impacts for 17 effects out of 18 indicators (**Figure 5.3B**). This may be due to water as the extraction solvent.

A study conducted on the environmental impact of phycocyanin recovery from *Spirulina* by Papadaki *et al* [27] found that the use of aqueous solvents can lead to an environmentally friendly and effective extraction of phycocyanin compared to organic solvents. When combining FT and AUE, there was no noticeable change in the environmental effects, and they remained at levels close to FT. Here, the FT+AUE (water, new approach) method provides the optimal balance between extraction efficiency of chlorophyll pigments, carotenoids, and phycocyanin and environmental sustainability and is preferred over UAE and SLE due to the gains such as increased overall productivity and reduced environmental impact.

These results prompted further analysis of the main contributors in the impact categories (Appendix C **Tables C.3, C.4, and C.5**). It appears that electricity consumption is the dominant consumption for all the extraction techniques examined, along with the type of solvent used, with water making the least contribution to the effect categories compared to acetone or ethanol. The specific energy demand of each technology is less clear and is overlooked in the literature. Here, the energy consumption measured at operating conditions is 5.28 kWh, 0.66 kWh, 5.94 kWh, and 6.60 kWh for SLE, UAE, FT, and FT+UAE, respectively (the energy consumption during the evaporation, drying, and centrifugation processes was non-temporary), so these energy data play a key role in determining the environmental impacts of the three processes. This is in line with the LCA results reported by Barjoveanu *et al* [24] to recover polyphenols from spruce bark and the study by Salzano *et al* [3] to recover polyphenols from pine needles. Both studies used lab-scaled techniques.

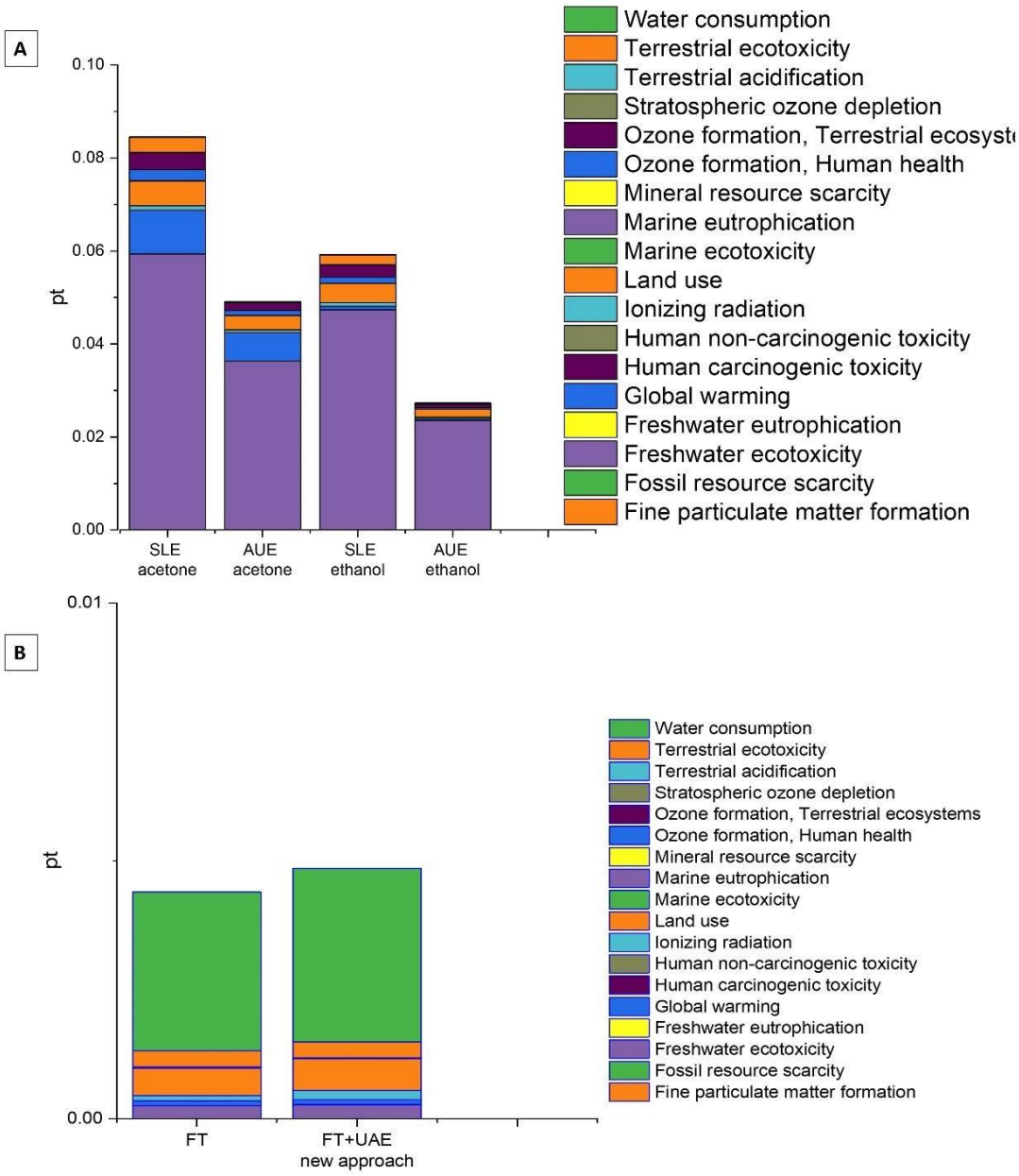


Figure 5.3 Impact assessment of 1 mg of photosynthesis pigment recovery from Spirulina under different extraction techniques.

Overall, these results indicated that a return to energy-intensive extraction methods would only be environmentally appropriate if it guaranteed higher yields at much shorter processing times compared to conventional methods. Energy consumption is the main factor in determining the environmental profile of different processes. From this perspective, choosing energy suppliers with a lower share of electricity derived from fossil fuels is expected to reduce

the environmental footprint of extraction products. Some literature indicates that when electricity is produced from renewable resources, the environmental impact of the process is significantly reduced across all impact categories. This is in line with the LCA study by Carlqvist *et al* [28]. who reported a significant influence of the power grid on the potential impact of different extraction processes.

In addition to addressing energy choices, substituting solvents with greener options can lead to greater environmental benefits, as observed in this study when using water and ethanol, which are often viewed as green options among traditional solvents [29, 30]. To give an idea of the extent of potential improvements, a re-evaluation of the environmental profiles for SLE and AUE was performed by replacing acetone with ethanol. This simple change can provide significant gains in the overall sustainability of these processes by reducing impacts in 11 out of 18 categories. However, this replacement led to significant reductions in chlorophyll and carotene pigment content (see **Figure 5.2**). Therefore, the use of green solvents is not always the most sustainable option, especially when their extraction efficiency is reduced, as previously reported [31]. Therefore, significant efforts are directed towards identifying low-impact but effective extraction media [31-33].

5.4 Conclusion

Spirulina is a natural, rich source of bioactive chemicals that can be extracted under different conditions and procedures. This study investigated the lab-scale processes for extracting photosynthetic pigments from dried *Spirulina*. This chapter compares three extraction methods: standard maceration (SLE), ultrasound-assisted methods (AUE), and freeze-thaw (FT), which are commonly viewed as sustainable approaches and offers a comprehensive overview of each method, considering efficiency and sustainability.

An LCA gate-to-gate analysis was conducted to evaluate the environmental impact of several extraction processes. The LCA methodology considered the specific bondaries needed to collect LCA data for the functional unit (1 mg/g of extracted pigment). The results showed that the greatest contributor in most

impact categories was the electricity consumed by extraction equipment, followed closely by solvent production and emissions.

The transition from fossil fuels to renewable energy sources can greatly enhance the utilization of biomass for producing high value products. Meanwhile, this study showed that selecting green solvents for extraction may not always be advantageous. Replacing acetone with ethanol improved the sustainability of the extraction processes but caused a decrease in pigment production. Therefore, studies focusing on identifying sustainable and effective solvents are urgently needed.

5.5 References

1. Pérez-López, P., et al., *Life cycle assessment of the production of bioactive compounds from Tetraselmis suecica at pilot scale.* Journal of Cleaner Production, 2014. **64**: p. 323-331.

2. Shahapurkar, A.A., M.A. Shahapurkar, and R.A. Shastry, *A REVIEW ON THE NUTRITIONAL VALUE AND POTENTIAL HEALTH BENEFITS OF SPIRULINA.* 2022.

3. Salzano de Luna, M., et al., *Pine Needles as a Biomass Resource for Phenolic Compounds: Trade-Off between Efficiency and Sustainability of the Extraction Methods by Life Cycle Assessment.* ACS Sustainable Chemistry & Engineering, 2023. **11**(12): p. 4670-4677.

4. Chu, A. and O. Rahman. *What color is sustainable? Examining the Eco-Friendliness of Color*. in *International foundation of fashion technology institutes conference: Conference Paper, Taipei*. 2010.

5. Brennan, L. and P. Owende, *Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products.* Renewable and sustainable energy reviews, 2010. **14**(2): p. 557-577.

6. Mazzi, A., *Introduction. Life cycle thinking*, in *Life cycle sustainability assessment for decision-making*. 2020, Elsevier. p. 1-19.

7. Ingrao, C., et al., *How can life cycle thinking support sustainability of buildings? Investigating life cycle assessment applications for energy efficiency and environmental performance.* Journal of cleaner production, 2018. **201**: p. 556-569.

8. Vink, K., *Sustainable life cycle design aspects: how aware are material scientists?* SN Applied Sciences, 2020. **2**(8): p. 1364.

9. Kusmayadi, A., et al., *Microalgae as sustainable food and feed sources for animals and humans–biotechnological and environmental aspects.* Chemosphere, 2021. **271**: p. 129800.

10. Tavanandi, H.A. and K. Raghavarao, *Ultrasound-assisted enzymatic extraction of natural food colorant C-Phycocyanin from dry biomass of Arthrospira platensis.* Lwt, 2020. **118**: p. 108802.

11. Płotka-Wasylka, J., et al., *Extraction with environmentally friendly solvents.* TrAC Trends in Analytical Chemistry, 2017. **91**: p. 12-25.

12. Wellburn, A.R., *The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution.* Journal of plant physiology, 1994. **144**(3): p. 307-313.

13. Bennett, A. and L. Bogorad, *Complementary chromatic adaptation in a filamentous blue-green alga.* The Journal of cell biology, 1973. **58**(2): p. 419-435.

14. ISO, I., *ISO 14040. Environmental management–Life cycle assessment–Principles and framework (ISO 14040: 2006).* 2006.

15. ISO, *ISO 14044. Environmental management—life cycle assessment—requirements and management*. 2006, International Standards Organization Geneva, Switzerland.

16. Jiménez-González, C., S. Kim, and M.R. Overcash, *Methodology for developing gate-to-gate life cycle inventory information.* The International Journal of Life Cycle Assessment, 2000. **5**: p. 153-159.

17. Priyadarshini, A., B.K. Tiwari, and G. Rajauria, *Assessing the Environmental and Economic Sustainability of Functional Food Ingredient Production Process.* Processes, 2022. **10**(3): p. 445.

18. Garcia-Garcia, G., et al., *Life-cycle assessment of microwave-assisted pectin extraction at pilot scale.* ACS Sustainable Chemistry & Engineering, 2019. **7**(5): p. 5167-5175.

19. Viretto, A., N. Gontard, and H. Angellier-Coussy, *Urban parks and gardens green waste: A valuable resource for the production of fillers for biocomposites applications.* Waste management, 2021. **120**: p. 538-548.

20. Kyriakopoulou, K., S. Papadaki, and M. Krokida, *Life cycle analysis of β-carotene extraction techniques.* Journal of Food Engineering, 2015. **167**: p. 51-58.

21. Sartory, D. and J. Grobbelaar, *Extraction of chlorophyll a from freshwater phytoplankton for spectrophotometric analysis.* Hydrobiologia, 1984. **114**: p. 177-187.

22. Minchev, I., N. Petkova, and I. Milkova-Tomova, *Ultrasound-assisted extraction of chlorophylls and phycocyanin from Spirulina platensis.* Biointerface Res. Appl. Chem, 2020. **11**: p. 9296-9304.

23. Guedes, A.C., et al., *Supercritical fluid extraction of carotenoids and chlorophylls a, b and c, from a wild strain of Scenedesmus obliquus for use in food processing.* Journal of Food Engineering, 2013. **116**(2): p. 478-482.

24. Barjoveanu, G., et al., *Life cycle assessment of polyphenols extraction processes from waste biomass.* Scientific reports, 2020. **10**(1): p. 13632.

25. Ding, T., et al., *Life cycle assessment of tannin extraction from spruce bark.* iForest: Biogeosciences and Forestry, 2017. **10**(5): p. 807-814.

26. Modahl, I.S., A. Brekke, and C. Valente, *Environmental assessment of chemical products from a Norwegian biorefinery.* Journal of Cleaner Production, 2015. **94**: p. 247-259.

27. Papadaki, S., et al., *Environmental impact of phycocyanin recovery from Spirulina platensis cyanobacterium.* Innovative Food Science & Emerging Technologies, 2017. **44**: p. 217-223.

28. Carlqvist, K., et al., *Life cycle assessment for identification of critical aspects in emerging technologies for the extraction of phenolic compounds from spruce bark.* Journal of Cleaner Production, 2022. **333**: p. 130093.

29. González-González, G.M., et al., *Coffee pulp as a source for polyphenols extraction using ultrasound, microwave, and green solvents.* Environmental Quality Management, 2022. **32**(1): p. 451-461.

30. Torres-Valenzuela, L.S., A. Ballesteros-Gómez, and S. Rubio, *Green solvents for the extraction of high added-value compounds from agri-food waste.* Food Engineering Reviews, 2020. **12**: p. 83-100.

31. Makris, D.P. and S. Lalas, *Glycerol and glycerol-based deep eutectic mixtures as emerging green solvents for polyphenol extraction: The evidence so far.* Molecules, 2020. **25**(24): p. 5842.

32. Hashemi, B., et al., *Green solvents and approaches recently applied for extraction of natural bioactive compounds.* TrAC Trends in Analytical Chemistry, 2022: p. 116732.

33. Balaraman, H., et al., *Sustainable and green engineering insights on deep eutectic solvents toward the extraction of nutraceuticals.* ACS Sustainable Chemistry & Engineering, 2021. **9**(34): p. 11290-11313.

Chapter 6 General Discussion, Conclusions and Future Directions

Highlights

- Ultrasound-assisted aqueous extraction, combined with freezing and thawing, is a sustainable method for accessing photosynthetic pigments.
- Flash chromatography is a direct approach to purifying pigments and converting chlorophyll extract into stable products.
- Green pigments have higher antioxidant and prooxidant activity than blue pigments.
- Not every effective method using green techniques or solvents for pigment extraction means it is sustainable.

6.1 General discussion

Marine algae-derived photosynthetic pigments such as chlorophyll, carotene, and phycobiliprotein have become a focus of interest as a novel group of antioxidants in recent times. Photosynthetic pigments are mostly utilized as natural extracts in food, medicine, and cosmetic goods because of their potent pigment characteristics [1, 2]. Phycobiliprotein is water-soluble and stable at pH 5–7, making it ideal for usage as food additives [3, 4]. In addition, emerging research has revealed various biological activities of chlorophyll and phycobiliprotein extracts, including antioxidant, anticancer, antilipidemic, antidiabetic, antimicrobial, and antiviral properties. These properties are linked to chronic diseases, suggesting that photosynthetic pigments could be considered in preventive and therapeutic approaches [5-7].

For example, *Spirulina* is a food source abundant in photosynthetic pigments, including chlorophyll and phycocyanin, along with many bioactive phytochemicals such as flavonoids, polyphenols, essential and non-essential amino acids, as well as high concentrations of iron, calcium, gamma-linolenic acid, linoleic acid, arachidonic acid, and phosphorus [8, 9]. Research has reported that *Spirulina* supplements can reduce both systolic and diastolic blood pressure, prevent platelet aggregation, enhance vascular and endothelial function, decrease blood sugar levels, improve insulin sensitivity, offer protective effects for the liver and kidneys, aid in toxin elimination, and more. Enhance hemoglobin levels to aid in the treatment of anemia [8].

However, more research is needed to understand the significance and impact of individual pigments in algal extracts on health and disease outcomes. The current understanding of the functional aspects of photosynthetic pigments derived from marine algae is lacking in comparison to other natural pigments like anthocyanins and flavonoids [10, 11]. Therefore, it is crucial to comprehend the specific role of each photosynthetic pigment in cellular and molecular processes to grasp the foundation of their prospective health advantages.

This thesis addressed four main areas of photosynthetic pigments derived from marine algae: (i) extraction and purification; (ii) characterization of

pigment contents in extracts and pure forms; (iii) evaluation of the biological activities of individual pigments via assessment of their antioxidant and pro-oxidant properties; (iv) life cycle assessment of the developed approaches for producing photosynthetic pigments from marine algae.

6.1.1 Conclusion: extraction and characterization of photosynthesis pigments

Photosynthetic pigments are usually extracted using solid and liquid extraction methods. Combining solid-liquid extraction with new techniques such as ultrasound and pulsed electric field extraction might improve the effectiveness of pigment extraction, as explained in **Chapter 2**. Studies conducted by multiple researchers [12-15] have demonstrated that ultrasound-assisted extraction can result in higher productivity of photosynthetic pigments compared to conventional extraction procedures such as soaking and shaking [13, 14]. In this study, ultrasound-assisted extraction was applied, and the yields of photosynthetic pigments were comparable to the studies mentioned above.

The extraction solvent is crucial for obtaining increased pigment yields and stability. There are no standardized extraction procedures to obtain photosynthetic pigments from various marine algae sources. Each source may contain a distinct array of chemicals with varying polarity and a complicated sample matrix. Therefore, the current study examined the extraction efficiency by employing various solvents such as water, ethanol, and acetone. Water was used as a solvent for extracting phycobiliproteins and chlorophylls. As phycobiliproteins are polar compounds and chlorophylls are amphiphilic, it is also possible to extract chlorophyll in an aqueous medium efficiently under the conditions of combining mechanical and non-mechanical treatments. The use of ethanol led to the extraction of chlorophyll pigments. However, the pigment was not very stable, and other polar compounds like sugars and polyphenols were extracted at the same time. This led to the study of other solvents, such as acetone, for the selective extraction of chlorophyll pigments. Based on the results of the present study, acetone can be highlighted as a selective solvent to efficiently extract chlorophyll pigments from marine algae samples. **Chapter 2** provides a summary of several works on extracting and characterizing diverse

sources of photosynthetic pigments. The current study presents new findings regarding the chemical composition of photosynthetic pigments and the biological characteristics of several photosynthetic pigments (**Chapter 3**). The methods and results of this work will be beneficial to other researchers analysing complicated matrices of photosynthetic pigments, based on their objectives and resources.

Overall, common limitations emerge when comparing data from extraction and characterization studies. One great limitation is the inconsistency in re- porting results. Often, different units (mg/g, mg/mL or mg/L) are reported for total chlorophyll and carotenoids content per extraction volume or sample weight. The cross-comparison of the results is only possible when the weight and substance information are calculated based on fresh/dry matter added. Another constraint is the inconsistency of raw materials. The concentration of photosynthetic pigments in various marine algae species varies significantly due to many factors such as growth conditions, seasons, nutrients, and geography [16]. However, information regarding this diversity is not always available when dealing with industrial or commercial samples. The current study utilized commercially sourced algae samples that were purchased online. Commercial samples frequently lack details regarding sample treatment, transportation, and storage conditions. The unknown processing, transportation, and storage conditions of the materials prior to analysis could impact the stability of photosynthetic pigments and other chemicals in the sample. Stramarkou *et al* [17] conducted a study on *Spirulina* comparing four drying methods: air drying (AD), freeze drying (FD), vacuum drying (VD), and accelerated solar drying (ASD), considering significant factors like decreasing moisture levels and adjusting temperature, composition, color change, pigment concentration, and antioxidant capacity. Vacuum drying results in quicker moisture reduction and a carotenoid content of 0.58 + 0.01 mg (g dry solids)$^{-1}$. Air drying effectively preserves phycocyanin, with a content of 9.65 + 0.19 mg (g dry solids)$^{-1}$of dry weight, but is not suitable for commercial use due to the lengthy drying time. The accelerated solar dried biomass exhibited the maximum antioxidant activity, primarily attributed to breakdown products formed under high-temperature and stress conditions.

Due to these limitations, it is challenging to compare this study's findings with most existing literature. When reporting the content of photosynthetic pigments, it is important to include precise information on raw materials, extraction processes (full or partial pigment extraction), and processing conditions.

6.1.2 Conclusion: purification of photosynthesis pigments

Purifying photosynthetic pigments (chlorophyll, carotenoids, and phycobiliproteins) derived from marine algae is a challenging task. The chlorophyll and carotenoids have been purified using a variety of processes, such as column chromatography, preparative HPLC, and supercritical fluid chromatography. Each method has advantages and disadvantages, so the choice of a purification process should be based on the needs and objectives of the research. Silica gel column chromatography is cost-effective and can be used with a broader range of mobile phases. The limitations of this technique are that it is time-consuming and requires higher quantities of solvent [18]. The benefits of high-performance liquid chromatography employing MS or UV include a single injection, a well-defined peak shape, high accuracy, and great sensitivity. Despite numerous disadvantages, the sample could be lost because of costly and intricate peak broadening [19]. Supercritical fluid chromatography offers benefits such as environmentally friendly extraction, solvent-free operation, and low operating temperatures. The drawbacks of this technique include expensive and complex equipment, high power consumption, and the inability to remove polar substances [20].

Common methods for isolating phycobiliproteins often involve a variety of standard procedures for cell wall distraction. These procedures utilize different conditions, such as pressure, abrasives, chemical treatment, freezing and thawing, and sonication. Additionally, efforts have been made to achieve high purity of the extracted phycocyanin (C-PC) from cyanobacteria. A combination of ammonium sulfate precipitation and various chromatographic methods, such as ion exchange, has been employed for C-PC purification [21, 22]. A study highlighted the application of high-speed countercurrent chromatography with an orthogonal test design to attain high purity of C-PC [23, 24]. A two-stage aqueous extraction method has been demonstrated as a viable and

sophisticated methodology for getting extremely pure C-PC [25]. Many existing approaches for extracting C-PC have relied on harsh and energy-intensive procedures, along with complex purification stages, to reach higher C-PC purity.

Phycocyanin was purified using two concurrent methods: six cycles of freezing-thawing and precipitation with salts. The advantages of purification using a silica gel column were tested for the purification of chlorophyll and carotenoid extracts. Column chromatography (**Chapter 4**) successfully separated carotenoid pigments, specifically beta-carotene and xanthophyll, and converted chlorophyll into more stable derivatives known as pheophytin *a* and pheophorbide *a*. The purification process yielded 0.9 g of β-carotene, 1.4 g of pheophorbide a, 2.6 g of pheophytin a, and 0.5 g of lutein from 100 g of spirulina powder.

The purity of β-carotene, lutein, and pheophorbide *a* was > 95%, while the purity of pheophytin *a* was 92%. The reason for the lower purity of pheophytin was due to isomerization facilitated by TFA. The purity of phycocyanin was initially 23% after one freezing and thawing cycle. The purity rose with each subsequent cycle until the fourth cycle, where it stabilized at 45%. Combining freezing, thawing, and ultrasound improved purity to 60% in the first cycle and to 89% in the fourth cycle (**Chapter 3**). After precipitating with ammonium sulphate, the purity of phycocyanin was 70% following one cycle of freezing and thawing combined with ultrasound (**Chapter 4**).

It is necessary to obtain photosynthetic pigments of high purity and yield to study their biological activities. Removing other bioactive compounds, like polyphenols, is extremely important as they can interact with the target pigments in synergistic or antagonistic ways. Also, sugars in extracts can cause cell stress, hyperglycemia, and alterations in osmotic pressure, which can also obscure the results of antioxidant assays [26].

6.1.3 Conclusion: biological studies of pigment extracts and pure pigments

Free radicals play important roles in oxidative stress and the pathogenesis of numerous diseases, such as cancer, neurological diseases, and cardiovascular diseases [27]. Various natural antioxidants, such as polyphenols, carotenoids, chlorophyll, and phycobiliprotein, can prevent numerous diseases by decreasing oxidative stress [28, 29]. In recent years, studies have reported that antioxidants such as polyphenols and carotenoids can have prooxidant effects directly when exposed to metal ions [30] or indirectly when exposed to light irradiation, as in the case of chlorophylls and phycocyanin [31]. However, most of these studies revealed prooxidant action *in vitro* rather than under actual physiological conditions or *in vivo* [30].

It is important to note that prooxidant substances increase the production of reactive oxygen species (ROS) in cancer cells to a hazardous level but do not have the same effect on normal cells [30]. Therefore, the ability of antioxidant compounds to cause oxidation in biological systems is not naturally or often detrimental and can be employed as a therapeutic approach for treatment. The use of large doses of vitamin C for various cancers and the use of porphyrin compounds in photodynamic therapy have both served as evidence of this [32]. To effectively create chemical strategies against oxidative stress using natural antioxidants, it is strongly recommended to fully investigate their dual nature.

In this context, the current study evaluated the antioxidant activity of algae extracts and pure pigments and examined their prooxidant activity. The current study investigated the antioxidant activity of algae extracts (**Chapter 3**) as well as purified pigments (**Chapter 4**) on different types of free radicals, employing TEAC, DPPH, and FRAP assays. Because the crude extracts may contain a variety of bioactive compounds, like polyphenols and organic acids, it is not trivial to identify the real contribution of each photosynthetic pigment to the total antioxidant activity of extracts that are high in photosynthetic pigments.

In this study, a comparative analysis of the antioxidant activity of the pure compounds and their extracts using assays was carried out. The general cytotoxicity of the purified pigments and intracellular ROS production were also tested. The results showed that the antioxidant activity of the purified pigments increases in the following order: methyl pheophorbide *a* > pheophorbide *a* > pheophytin *a* > phycocyanin. This difference in radical scavenging activity is due to the chemical structure of the chromophores present in the dyes, as discussed in **Chapter 4**. Our comparative analysis of the antioxidant activity of the purified pigments also showed that the intracellular results are in line with the results observed in the DPPH, TEAC, and FRAP assays.

This study also investigated the prooxidant activity of purified pigments by measuring the ability of the pigments to produce singlet oxygen. The results showed that pheophorbide a, pheophytin a, and methyl pheophorbide a are effective in singlet oxygen generation, while phycocyanin shows only marginal activity when compared to chlorophyll derivatives. The results also indicate that the efficacy of methyl pheophorbide a to generate singlet oxygen is slightly higher than that of pheophorbide *a* and pheophytin *a*.

6.1.4 Conclusion: evaluation of sustainability of extraction methods

Sustainability refers to the capacity of a process or system to continue working while effectively addressing the requirements of future generations. Sustainability is a significant factor in the creation of production systems that attempt to save energy and material resources for the future. Microalgae has great potential as a raw material. However, there is ongoing discussion on the sustainability of industrial systems that depend on algae. The market size for products derived from algae in the commercial sector is currently limited. To produce commercially viable products, it is necessary to efficiently extract primary metabolites using cost-effective technologies [33].

Hence, the present work assessed the procedures for extracting photosynthetic pigments from dried *Spirulina* on a small scale in a laboratory setting (**Chapter 5**). This study compared three extraction methods: standard maceration (SLE), ultrasound-assisted (AUE), and freeze-thaw (FT) methods,

commonly regarded as sustainable techniques. A life cycle assessment (LCA) was performed to assess the environmental impact of these procedures following an evaluation of their efficiency. Based on the LCA analysis, the primary factor in most impact categories was the electricity usage by extraction equipment, with solvent production and emissions being the next significant contributor. Simultaneously, the findings of this study indicate that selecting environmentally friendly solvents for extraction may not consistently yield advantageous outcomes. Substituting acetone with ethanol enhances the sustainability of extraction procedures but results in a reduction in the pigment yield. Consequently, there is an urgent need for studies that aim to develop solvents that are both sustainable and effective.

6.2 General conclusions

This thesis establishes a comprehensive workflow for the isolation, purification, and characterization of photosynthetic pigments from several marine algae species. In addition, the antioxidant and prooxidant properties of the four isolated pigments were evaluated. The comparative extraction results of the four marine algae showed that *Spirulina* powder can provide a good yield of protein pigments such as phycocyanin and non-protein pigments such as chlorophyll and carotenoids. To determine the appropriate solvent for extracting protein pigments, water was used as a solvent, but in the case of non-protein pigments, acetone and ethanol were chosen. Acetone showed higher extraction efficiency than ethanol, although the latter has a lower environmental footprint. Therefore, in the current study, a new method was developed to extract both non-protein and protein pigments from spirulina powder using water as a solvent.

Water has shown suitability in terms of efficiency and sustainability for the extraction of both types of pigments using mechanical separation techniques such as sonication and centrifugation, and non-mechanical techniques such as freezing/thawing. These steps can efficiently enhance the overall separation process because they exploit the properties of the material, such as its size, shape, and density, as well as the average viscosity and solubility of the chosen medium. The effects of the extraction processes on the environment

were studied. The LCA analysis showed that the biggest cause in most impact categories was the electricity used by the extraction equipment, followed by the production of solvents and emissions.

Column chromatography can effectively purify non-protein pigments and convert chlorophyll pigments into more stable compounds, leading to a purification yield ranging between 0.9 and 2.6 g. Additionally, precipitation with salts can purify phycocyanin to a degree similar to the four-cycle freeze-thaw method. This lowers the amount of energy needed, which is an important part of making any process sustainable. The purity of non-protein pigments was more than 95%, while the purity of phycocyanin in other dyes was 100%, based on HPLC analysis.

Results evaluating the antioxidant activity of pigment extracts from the four algae indicated that the increase in antioxidant activity parallels the increase in pigment content in the extracts by different methods, highlighting the potential contribution of photosynthetic pigments and their metabolites and/or degradation products to antioxidant activity. After purifying the extracts, the chlorophyll derivatives appeared to be more effective as antioxidants and pro-oxidants than phycocyanin, where methyl pheophorbide showed the strongest antioxidant and pro-oxidant capacity compared to other pure compounds.

6.3 Future directions

The pigments derived from macroalgae and microalgae have significant potential for use in several industries. Particularly, β-carotene, astaxanthin, and phycocyanin are already being commercially marketed [34]. Nevertheless, the process of extracting and the technologies currently available are still connected to substantial expenses for both implementation and operation. Furthermore, energy demands play a crucial role in ensuring the ongoing sustainability of a process.

Thus, standardizing the process for extracting pigments from biomass continues to be a difficult task. Developments in technology have led to the establishment of increasingly efficient and quick methods for extracting and separating pigments. These methods, such as UAE, MAE, SFE, and PLE, can

meet the demands of high-throughput screening, selectivity, stability of target extracts, and process safety. Several of these environmentally friendly technologies have become standard procedures for preparing samples for the purpose of analysis [35]. Due to the limitations of these technologies, manufacturing most microalgal pigments is currently confined to the experimental stage, and their commercial production is not yet realistic. Therefore, additional investigations are required to enhance current methodologies and take advantage of untapped technological possibilities. When it comes to isolation, obtaining pure pigments from complex mixtures is still a challenge. Currently, we are not able to achieve this in a single step. However, by using more selective methods for extraction, fractionation, and purification, we can reduce the time it takes to isolate the biological material and obtain the final purified compound.

In respect to biological studies, numerous experiments have revealed the biological impacts of photosynthetic pigments obtained from algae [36]. Hence, it is imperative to conduct further investigations to define the distinct biological impacts of pigments at the cellular level and their application in preventive settings. In the future, it will be essential to focus research efforts on investigating the interactions between photosynthetic pigments and other compounds, such as polyphenols, to identify potential synergistic or antagonistic effects. This is particularly relevant because most nutritional supplements consist of a combination of different components. Therefore, it's crucial to look at how different pigments interact with one another to understand the specific role that each component plays in a complex combination. The bioavailability of pigments is an essential aspect that affects the biological action of chemicals. Currently, there is still incomplete knowledge on the absorption, metabolism, and secretion of photosynthetic pigments. Hence, comprehending the bioavailability of this set of pigments, together with its correlation with the food matrix, becomes essential to understanding its impact on human health.

Ultimately, there is a distinct and increasing interest in extracting and isolating natural substances and their advantageous uses. These applications additionally modify the extraction methods employed, as well as the stationary phases and mobile phases utilized in these procedures. Hence, the most

advantageous approach for maximizing the value of microalgae and cyanobacteria is to employ a biorefinery process. This method improves economic feasibility by enabling the utilization of several by-products that can be utilized separately. Nevertheless, the existing literature primarily consists of limited-scale experiments and theoretical investigations, which generally yield equivocal results when seeking to apply them on a larger scale.

6.4 Reference

1. Pradhan, B., et al., *Bioactive metabolites from marine algae as potent pharmacophores against oxidative stress-associated human diseases: A comprehensive review.* Molecules, 2020. **26**(1): p. 37.

2. Pereira, L. and J.M. Neto, *Marine algae: biodiversity, taxonomy, environmental assessment, and biotechnology*. 2014: CRC Press.

3. Sonani, R.R., et al., *Recent advances in production, purification and applications of phycobiliproteins.* World journal of biological chemistry, 2016. **7**(1): p. 100.

4. Pagels, F., et al., *Phycobiliproteins from cyanobacteria: Chemistry and biotechnological applications.* Biotechnology Advances, 2019. **37**(3): p. 422-443.

5. Pérez-Gálvez, A., I. Viera, and M. Roca, *Carotenoids and chlorophylls as antioxidants.* Antioxidants, 2020. **9**(6): p. 505.

6. Pan-utai, W. and S. Iamtham, *Extraction, purification and antioxidant activity of phycobiliprotein from Arthrospira platensis.* Process Biochemistry, 2019. **82**: p. 189-198.

7. Wu, Q., et al., *The antioxidant, immunomodulatory, and anti-inflammatory activities of Spirulina: an overview.* Archives of toxicology, 2016. **90**(8): p. 1817-1840.

8. Mishra, T., et al., *Spirulina: The Beneficial Algae.* International Journal of Applied Microbiology Science., 2013. **2**.

9. Moorhead, K., B. Capelli, and G. Cysewski, *SPIRULINA nature's superfood.* Hawaii: Cyanotech Corporation, 1993: p. 1-63.

10. Abugri, D.A. and W.H. McElhenney, *Extraction of total phenolic and flavonoids from edible wild and cultivated medicinal mushrooms as affected by different solvents.* J Nat Prod Plant Resour, 2013. **3**(3): p. 37-42.

11. Lotito, S.B. and B. Frei, *The increase in human plasma antioxidant capacity after apple consumption is due to the metabolic effect of fructose*

on urate, not apple-derived antioxidant flavonoids. Free Radic Biol Med, 2004. **37**(2): p. 251-8.

12. Minchev, I., N. Petkova, and I. Milkova-Tomova, *Ultrasound-assisted extraction of chlorophylls and phycocyanin from Spirulina platensis.* Biointerface Res Appl Chem, 2020. **11**: p. 9296-9304.

13. Macías-Sánchez, M., et al., *Comparison of supercritical fluid and ultrasound-assisted extraction of carotenoids and chlorophyll a from Dunaliella salina.* Talanta, 2009. **77**(3): p. 948-952.

14. Tavanandi, H.A. and K. Raghavarao, *Ultrasound-assisted enzymatic extraction of natural food colorant C-Phycocyanin from dry biomass of Arthrospira platensis.* Lwt, 2020. **118**: p. 108802.

15. Kong, W., et al., *Optimization of ultrasound-assisted extraction parameters of chlorophyll from Chlorella vulgaris residue after lipid separation using response surface methodology.* Journal of food science and technology, 2014. **51**: p. 2006-2013.

16. Scoglio, S., *Microcystins in water and in microalgae: Do microcystins as microalgae contaminants warrant the current public alarm?* Toxicology reports, 2018. **5**: p. 785-792.

17. Stramarkou, M., et al., *Comparative analysis of different drying techniques based on the qualitative characteristics of Spirulina platensis biomass.* Journal of Aquatic Food Product Technology, 2021. **30**(5): p. 498-516.

18. Xia, S., et al., *Production, characterization, and antioxidant activity of fucoxanthin from the marine diatom Odontella aurita.* Marine drugs, 2013. **11**(7): p. 2667-2681.

19. Wiberg, K., et al., *Peak purity determination with principal component analysis of high-performance liquid chromatography–diode array detection data.* Journal of Chromatography A, 2004. **1029**(1-2): p. 13-20.

20. Machmudah, S., et al., *Supercritical fluids extraction of valuable compounds from algae: Future perspectives and challenges.* Engineering Journal, 2018. **22**(5): p. 13-30.

21. Yan, S.-G., et al., *Single-step chromatography for simultaneous purification of C-phycocyanin and allophycocyanin with high purity and recovery from Spirulina (Arthrospira) platensis.* Journal of Applied Phycology, 2011. **23**: p. 1-6.

22. Song, W., C. Zhao, and S. Wang, *A large-scale preparation method of high purity C-phycocyanin.* International journal of bioscience, biochemistry and bioinformatics, 2013. **3**(4): p. 293-297.

23. Yin, L., et al., *Orthogonal test design for optimization of suitable conditions to separate C-phycocyanin from Spirulina platensis by high-speed counter-current chromatography using reverse micelle solvent system.* Journal of separation science, 2011. **34**(11): p. 1253-1260.

24. Purohit, A., et al., *Processing-independent extracellular production of high purity C-phycocyanin from Spirulina platensis.* ACS Biomaterials Science & Engineering, 2019. **5**(7): p. 3237-3245.

25. Chethana, S., et al., *Single step aqueous two-phase extraction for downstream processing of C-phycocyanin from Spirulina platensis.* Journal of Food Science and Technology, 2015. **52**: p. 2415-2421.

26. Faieta, M., et al., *High hydrostatic pressure treatment of Arthrospira (Spirulina) platensis extracts and the baroprotective effect of sugars on phycobiliproteins.* Innovative Food Science & Emerging Technologies, 2021. **70**: p. 102693.

27. Pham-Huy, L.A., H. He, and C. Pham-Huy, *Free radicals, antioxidants in disease and health.* International journal of biomedical science: IJBS, 2008. **4**(2): p. 89.

28. Lanfer-Marquez, U.M., R.M. Barros, and P. Sinnecker, *Antioxidant activity of chlorophylls and their derivatives.* Food research international, 2005. **38**(8-9): p. 885-891.

29. Roohbakhsh, A., et al., *Neuropharmacological properties and pharmacokinetics of the citrus flavonoids hesperidin and hesperetin—A mini-review.* Life sciences, 2014. **113**(1-2): p. 1-6.

30. Eghbaliferiz, S. and M. Iranshahi, *Prooxidant activity of polyphenols, flavonoids, anthocyanins and carotenoids: updated review of*

mechanisms and catalyzing metals. Phytotherapy Research, 2016. **30**(9): p. 1379-1391.

31. Usuki, R., Y. Endo, and T. Kaneda, *Prooxidant activities of chlorophylls and pheophytins on the photooxidation of edible oils.* Agricultural and Biological Chemistry, 1984. **48**(4): p. 991-994.

32. Chen, Q., et al., *Pharmacologic doses of ascorbate act as a prooxidant and decrease growth of aggressive tumor xenografts in mice.* Proceedings of the National Academy of Sciences, 2008. **105**(32): p. 11105-11109.

33. Vuppaladadiyam, A.K., et al., *Sustainability analysis of microalgae production systems: a review on resource with unexploited high-value reserves.* Environmental science & technology, 2018. **52**(24): p. 14031-14049.

34. Sun, H., et al., *Microalgae-derived pigments for the food industry.* Marine drugs, 2023. **21**(2): p. 82.

35. Zhang, Q.-W., L.-G. Lin, and W.-C. Ye, *Techniques for extraction and isolation of natural products: A comprehensive review.* Chinese medicine, 2018. **13**: p. 1-26.

36. Pangestuti, R. and S.-K. Kim, *Biological activities and health benefit effects of natural pigments derived from marine algae.* Journal of functional foods, 2011. **3**(4): p. 255-266.

Appendix A Supporting information of Chapter 3

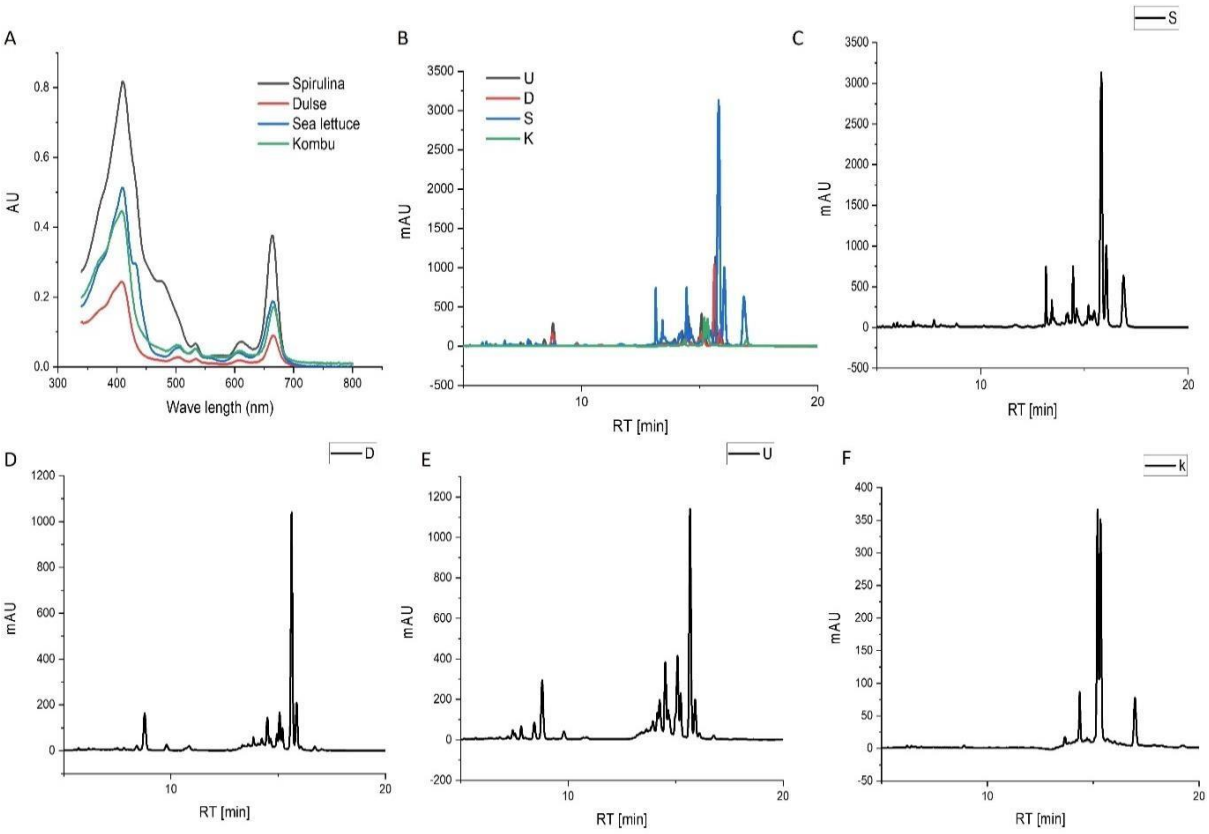


Figure A.1 Chromatograms of algae extracts in HPLC/DAD at 410 nm according to the acetone ultrasound-assisted extraction method. **A**) UV-vis spectra of all algae extracts, **B**) chromatogram for all algae species, **C**) chromatogram for *Spirulina*, **D**) chromatogram for *Dulse*, **E**) chromatogram for *Kombu* and F) chromatogram for *Sea lettuce*.

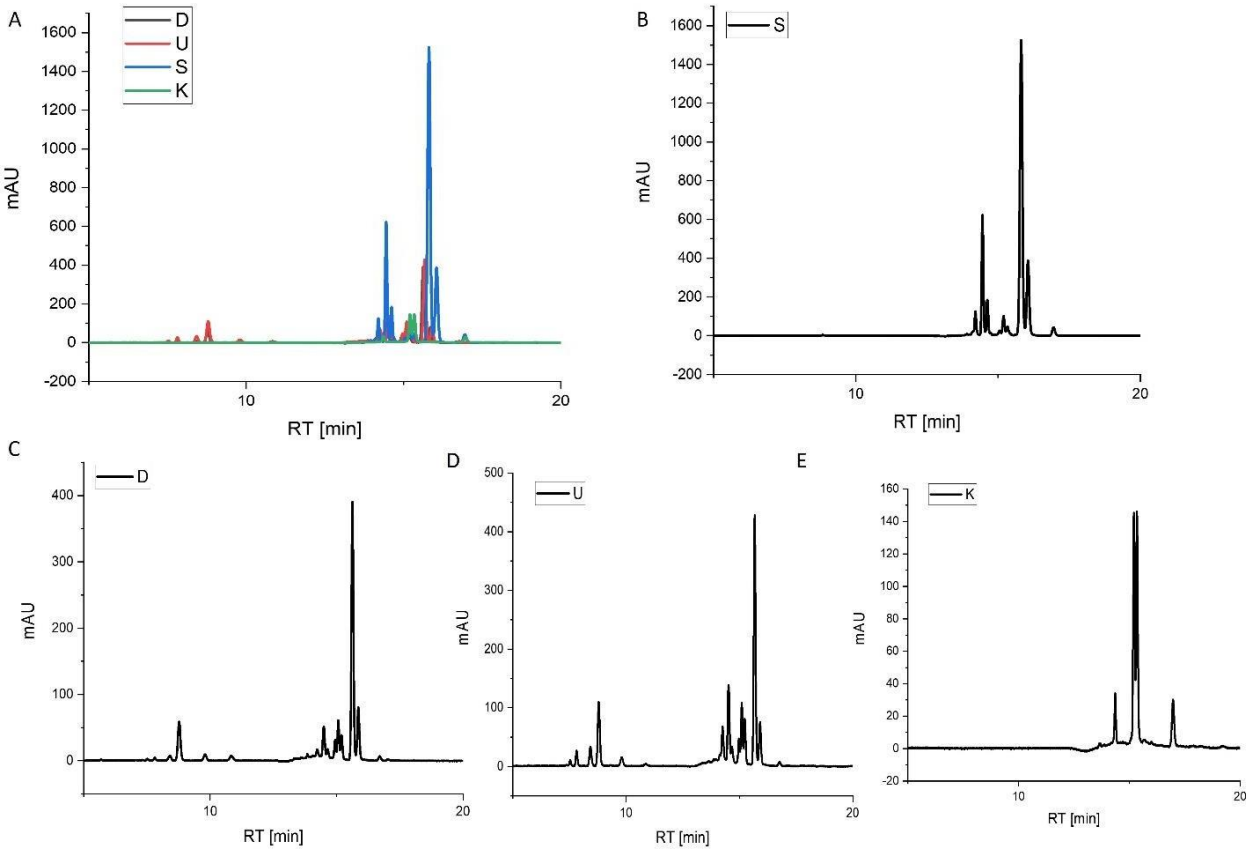


Figure A.2 Chromatograms of algae extracts in HPLC/DAD at 670 nm according to the acetone ultrasound-assisted extraction method. **A**) chromatogram for all algae species, B) chromatogram for *Spirulina*, C) chromatogram for *Dulse*, D) chromatogram for *Sea lettuce* and E) chromatogram for *Kombu*.

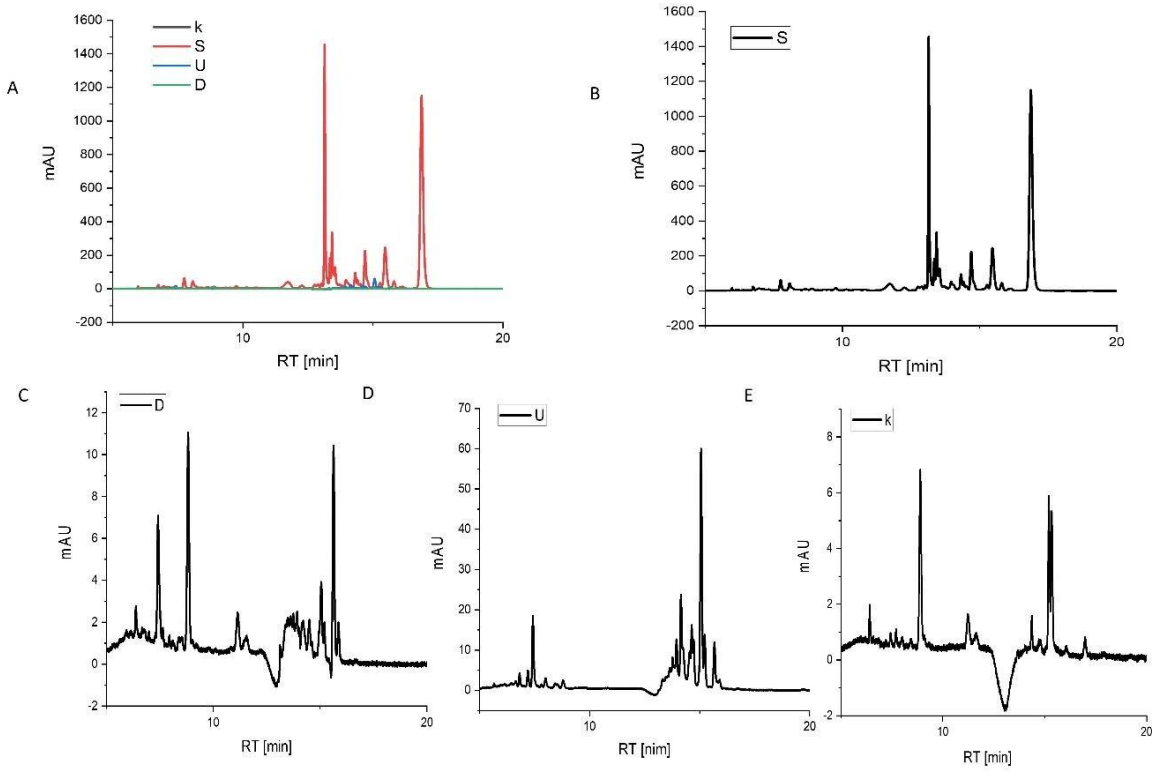


Figure A.3 Chromatograms of algae extracts in HPLC/DAD at 450 nm according to the acetone ultrasound-assisted extraction method. **A**) chromatogram for all algae species, B) chromatogram for *Spirulina*, C) chromatogram for *Dulse*, D) chromatogram for *Sea lettuce* and E) chromatogram for *Kombu*.

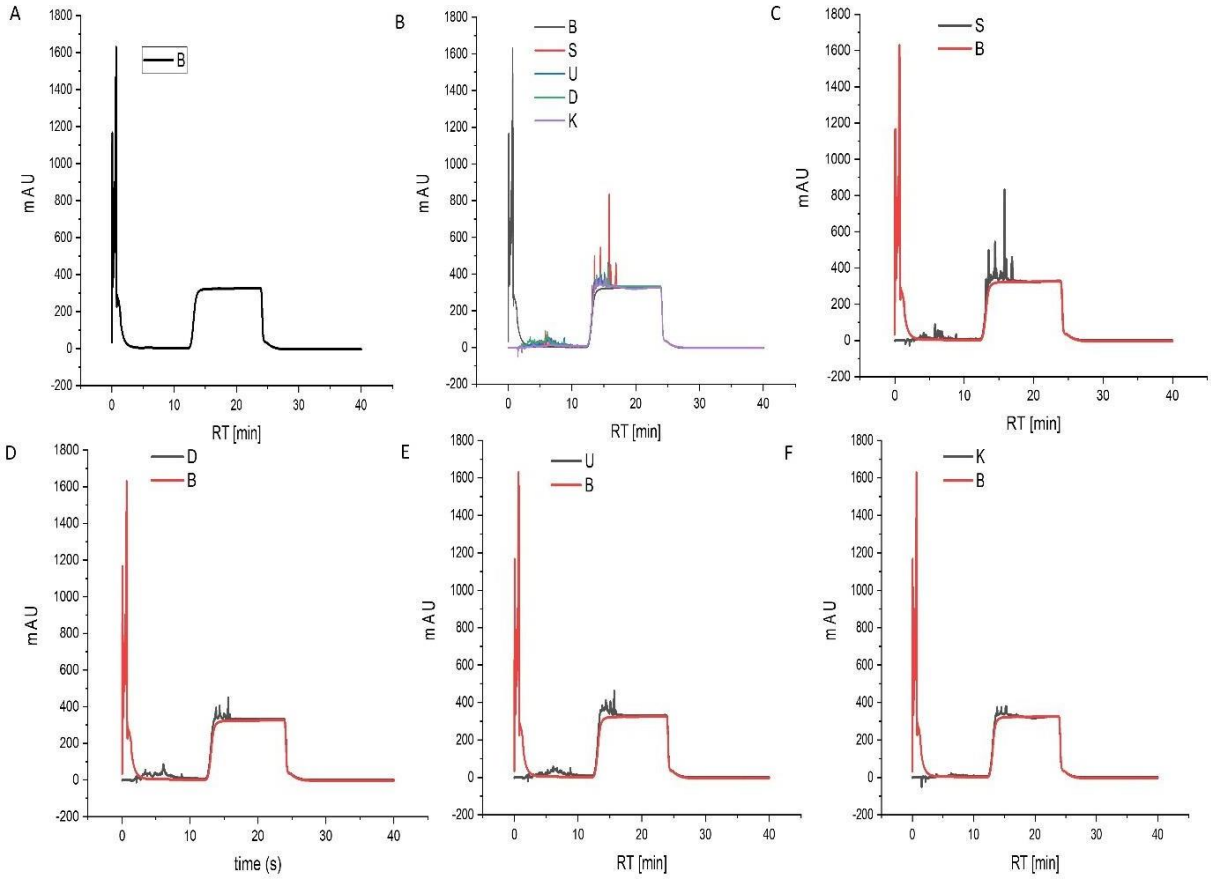
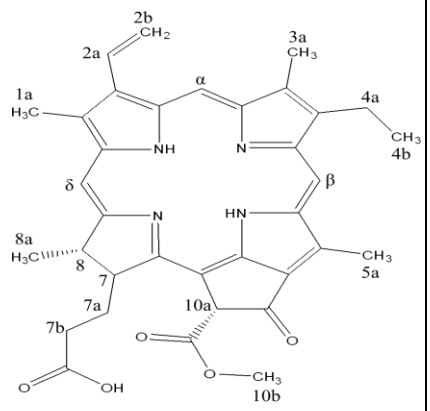
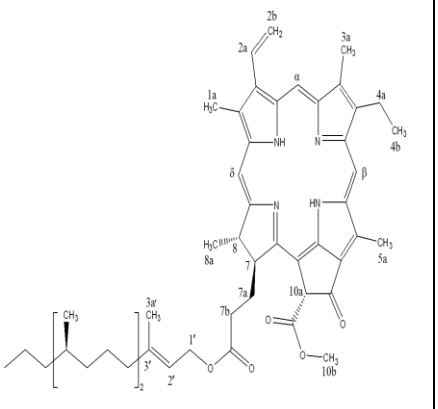


Figure A.4 Chromatograms of algae extracts in HPLC/DAD at 254 nm according to the acetone ultrasound-assisted extraction method. A) chromatogram for blank. B) chromatogram for all algae species A) chromatogram for *Spirulina*, B) chromatogram for *Dulse*, C) chromatogram for *Sea lettuce* and D) chromatogram for *Kombu*.

Appendix B Supporting information of Chapter4

Table B.1. Summary of characteristic data for pheophorbide a and pheophytin

Pigment	1H NMR (400 MHz, acetone-d6)		UV-Vis	MS	Weight
	δH (m, J-values in Hz)	H group			
Pheophorbide *a*	9.70 (s, 1H) 9.40 (s, 1H) 8.83 (s, 1H)	***Meso-*** β-H α-H δ-H	λ_{max} (acetone): 666 nm, 406nm	887.569	2.6 g
	8.10 (dd, 1H, *J*= 12 Hz, 18 Hz) 6.24 (dd, 1H, J = 2 Hz, 18 Hz) 6.11 (dd, 1H, *J* = 2 Hz, 12 Hz)	**Vinyl** 2a-Hx 2b-Hb 2b-Ha			
	3.77 (s, 3H) 3.57 (s, 3H) 3.37 (s, 3H) 3.14 (s, 3H,) 1.75 (d, 3H) 1.57 (t, 3H)	**Methyl** 10b-CH_3 5a-CH_3 la-CH_3 3a-CH3 8a-CH_3 4b-CH_3			
	6.31 (s, 1H,) 4.64 (m, 1H) 4.18 (m, 1H) 3.65 (q, 2H) 2.70 (m, 4H)	**Other H** 10a-H 7-H 8-H 4a-CH_2 7a, 7b-CH_2			
Pheophytin *a*	9.78 (s, 1H) 9.74 (s, 1H) 8.86 (s, 1H)	***Meso-*** β-H α-H δ-H	λ_{max} (acetone): 666 nm, 406nm	593.277	1.4 g
	8.05 (dd,1H, J = 12 Hz, 18 Hz) 6.29 (dd, 1H, J = 2 Hz, 18 Hz) 6.09 (dd, 1H, J = 2 Hz, 12 Hz)	**Vinyl** 2a-Hx 2b-Hb 2b-Ha			
	3.87 (s, 3H) 3.60 (s, 3H) 3.40 (s, 3H) 3.12 (s, 3H) 1.83 (d, 3H) 1.62 (t, 3H)	**Methyl** 10b-CH_3 5a-CH_3 la-CH_3 3a-CH_3 8a-CH_3 4b-CH_3			
	3.48 (q, 2H) 2.38 (m, 4H) 6.37 (s, 1H) 4.64 (m, 1H, *J* = 7 Hz,) 4.18 (m, 1H, *J* = 7 Hz,)	**Other H** 4a-CH2 7a, 7b-CH_2 10- OH 7-H 8-H			
	5.21 (t, 1H) 5.05 (m) 1.48 (s, 3H) 1.27 (s, 3H) 1.09 (m, 18H) 0.65-0.85 (m, 12H)	**Phytyl** 2'-H l'-H 3a'-CH_3 7'-15'-CH 4'-14'-CH_2 7a'-16'-CH_3			

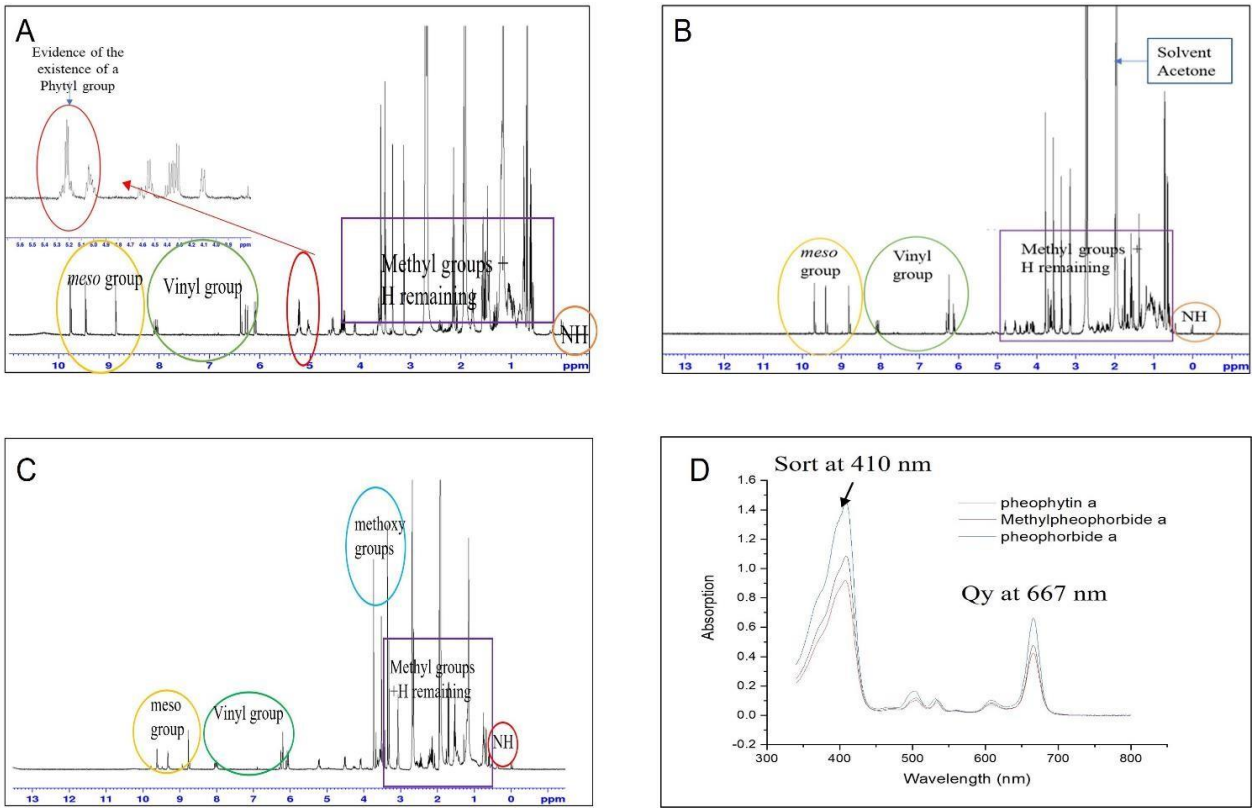


Figure B.1. The characteristic absorption regions in ^{1}HNMR and UV-Vis of pheophytin *a* (A), pheophorbide *a* (B), methyl pheophorbide *a* (C), UV-Vis for all pigments (D).

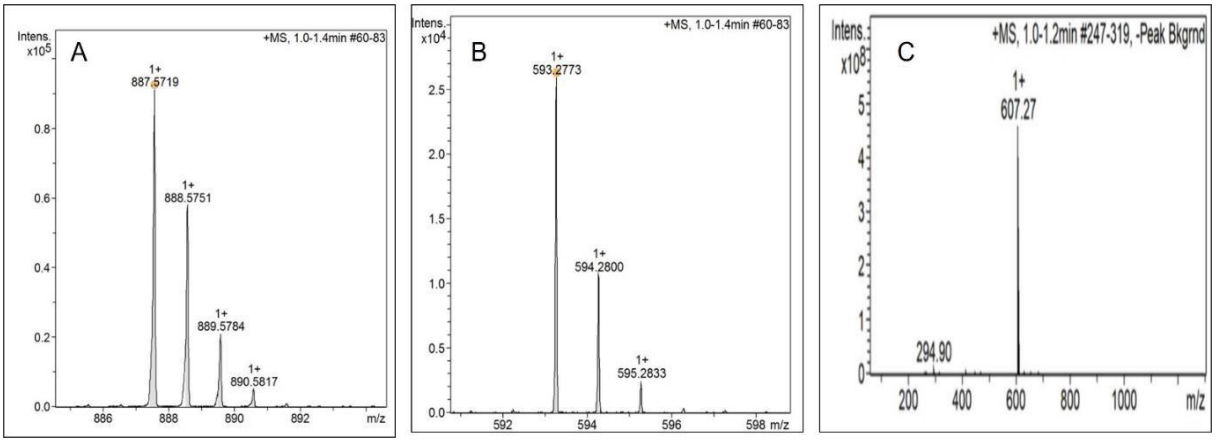


Figure B.2. MS spectra for **A)** pheophytin *a*, **B)** pheophorbide *a*, **C)** methyl pheophorbide *a*.

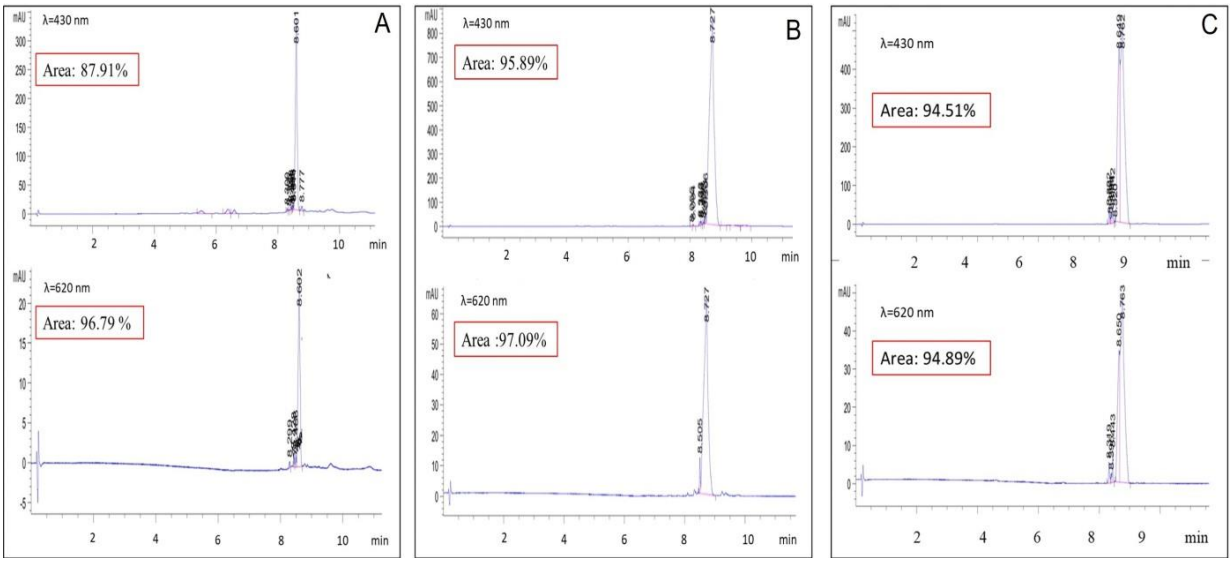


Figure B.3. HPLC-DAD chromatograms of purified pigments a at 430 nm and 620 nm. **A)** pheophytin a, **B)** pheophorbide a, **C)** methyl pheophorbide a.

3. Characterization of phycocyanin

Table B.2. The distinct regions of phycocyanin in UV-Vis and FT-IR, the percentage purity of the phycocyanin in HPLC, the grade and weight of the phycocyanin.

FT-IR	**UV - Vis**	**Purity% (HPLC)**	**Purity (A_{620}/A_{280})**	**Weight**
3466 cm^{-1}N-H stretching vibrations presence of amine (proteins) groups, O-H in alcohol or N-H in amide groups 1638 cm^{-1} C=C in alkene groups 1460 cm^{-1} C=C in pyrrole groups 1101 cm^{-1} C-O stretching in alcohol groups	λ_{max} (H_2O) at 620 nm	100%	Food grade (0.6)	15 g

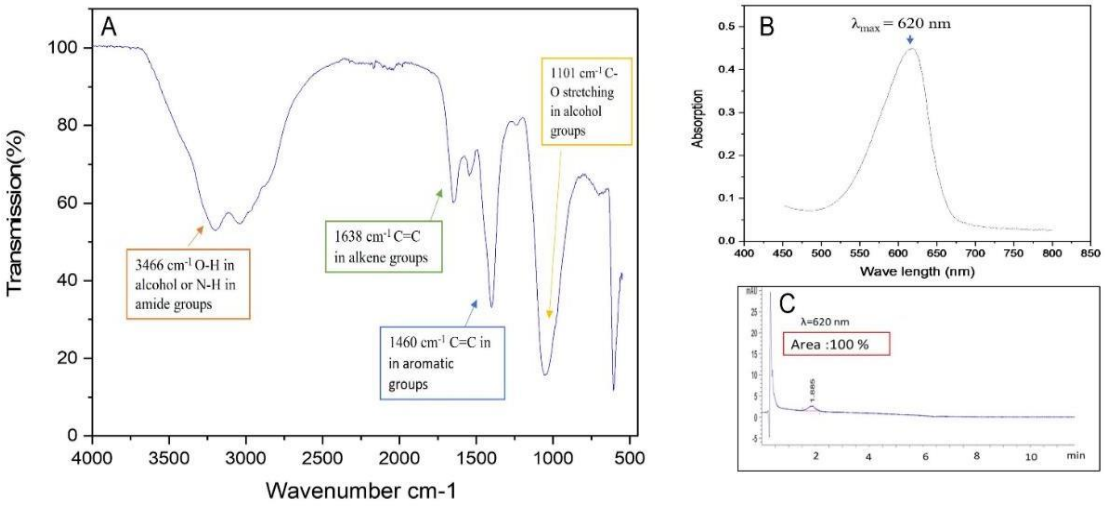


Figure B.4. The distinct absorption regions of phycocyanin **A**) in FT-IR, **B**) UV-Vis spectra, **C)** HPLC-DAD chromatograms of purified phycocyanin (detection: 620 nm).

4. Antioxidant activity of pigments

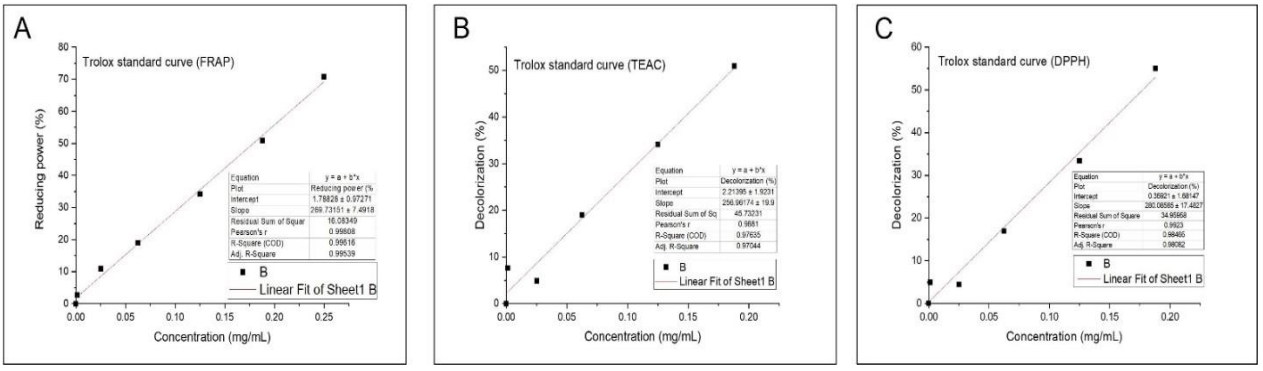


Figure B.5. Trolox standard curve representing decolorization (%) at different concentrations for Trolox in the range 0-1000 Mm to evaluation of the antioxidant efficacy of naturally occurring pigments isolated from spirulina by TEAC, DPPH and FRAP.

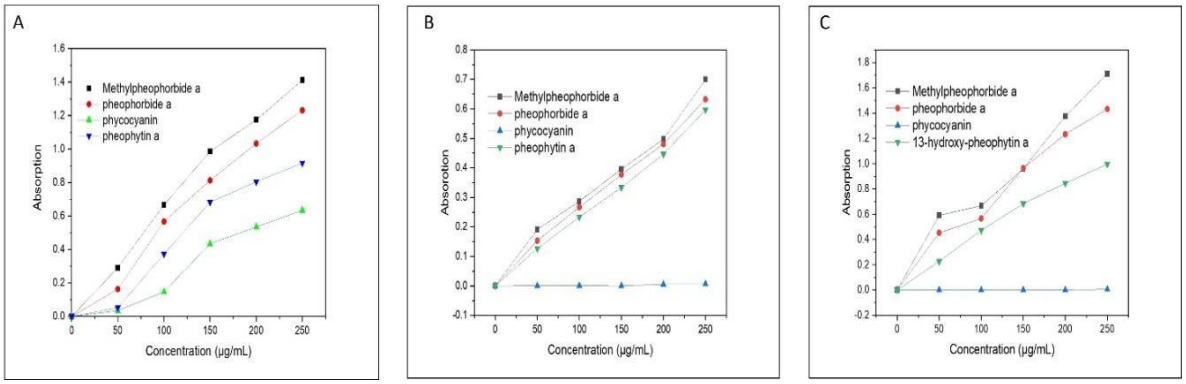


Figure B.6. Colour interference in pigments with the assays' wavelength. A) Pigments absorption measured in FRAP assay at 593 nm, B) Pigments absorption measured in TEAC assay at 743 nm. C) Pigments absorption measured in DPPH assay at 515nm.

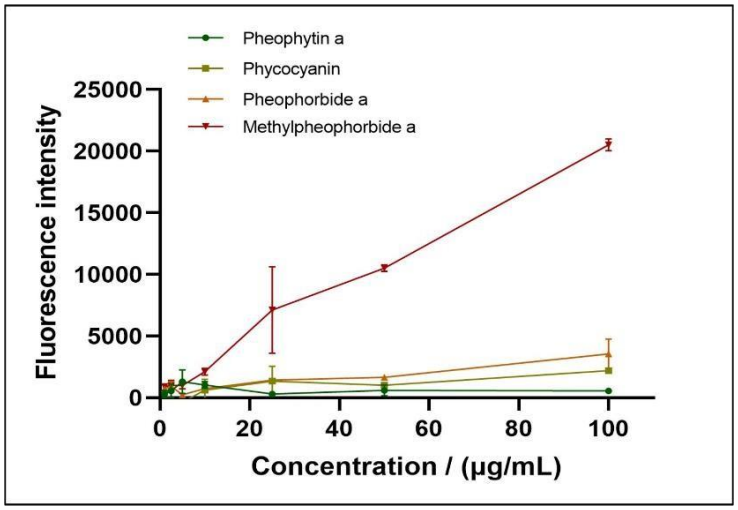


Figure B.7. Colour interference of samples at excitation of 485/20 and emission of 535/25 in ROS assay. The results were presented as mean value ± SD.

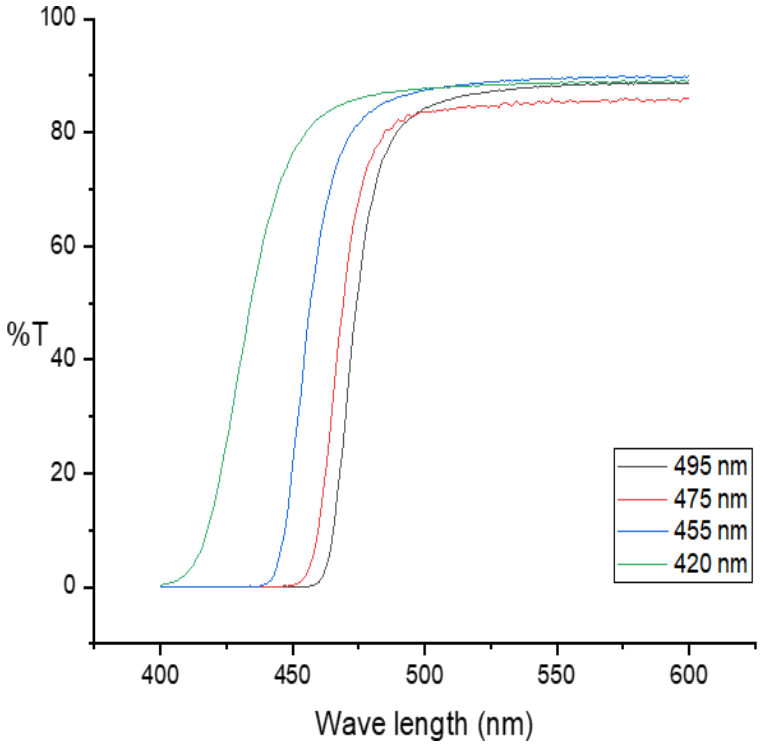


Figure B.8 Cut-off filters were checked before the experiment was conducted to ensure that they were wide-range cutter filters.

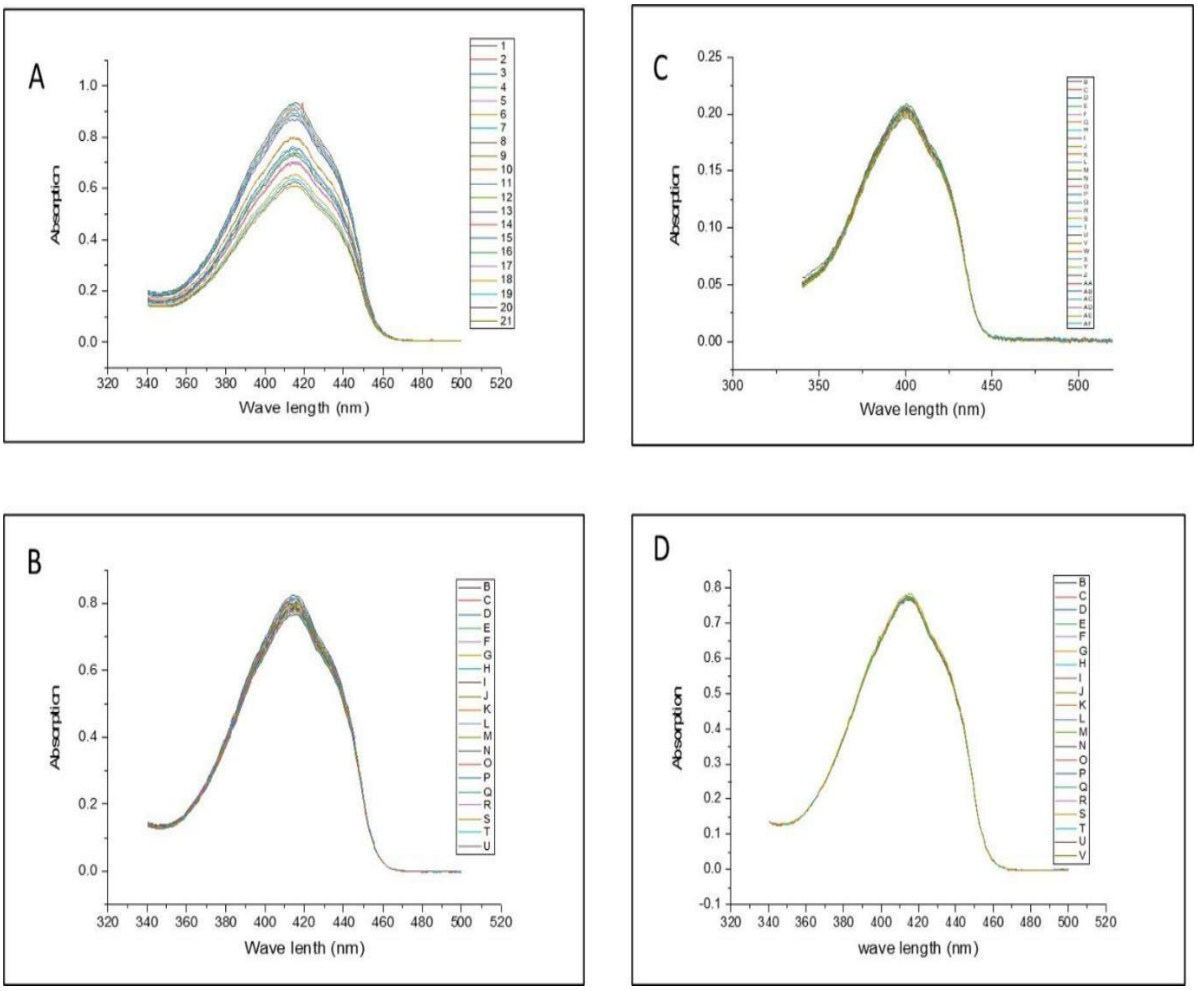


Figure B.9. The photostability of DPBF on its own at 410 nm when illuminated by visible light (Xenon lamp - 50 W) using four cut-off filters at every 6 s for 2 min. A) Filter 420 nm, B) Filter 455 nm, C) Filter 475 nm, D) Filter 495 nm.

Appendix C Supporting information of Chapter 5

Table C.1. Environmental impact potentials for the extraction of 1 mg/g of photosynthesis pigments by SLE and UAE using acetone or ethanol as solvent for extraction.

No	Impact category	Amount SLE acetone	Amount UAE acetone	Amount SLE ethanol	Amount UAE ethanol	Unit
1	Fine particulate matter formation	1.97E-06	1.30E-06	1.90E-05	1.30E-06	Kg PM2.5 eq
2	Fossil resource scarcity	0.00000	0.00000	0.00000	0.00000	kg oil eq
3	Freshwater ecotoxicity	5.93E-02	3.63E-02	4.73E-02	2.35E-02	kg 1,4-DCB
4	Freshwater eutrophication	1.82E-06	1.10E-06	1.44E-06	6.94E-07	kg P eq
5	Global warming	9.44E-03	6.17E-04	5.71E-04	4.36E-04	kg CO_2 eq
6	Human carcinogenic toxicity	3.30E-10	2.12E-10	2.68E-10	1.47E-10	kg 1,4-DCB
7	Human non-carcinogenic toxicity	9.93E-10	6.00E-10	7.88E-10	3.81E-10	kg 1,4-DCB
8	Ionizing radiation	1.00E-03	5.63E-04	7.75E-04	3.19E-04	kBq Co-60 eq
9	Land use	5.35E-03	3.06E-03	4.17E-03	1.78E-03	m2a crop eq
10	Marine ecotoxicity	6.33E-5	2.21E-06	2.86E-06	2.21E-06	kg 1,4-DCB
11	Marine eutrophication	3.24E-06	2.57E-06	4.20E-08	2.47E-08	kg N eq
12	Mineral resource scarcity	5.10E-08	4.12E-08	4.10E-08	3.12E-08	kg Cu eq
13	Ozone formation, Human health	0.0023	0.0011	0.0013	0.0003	kg NOx eq
14	Ozone formation, Terrestrial ecosystems	0.0037	0.0017	0.0027	0.0007	kg NOx eq
15	Stratospheric ozone depletion	8.50E-10	4.72E-10	6.55E-10	2.62E-10	kg CFC^{11} eq
16	Terrestrial acidification	2.14E-05	1.72E-05	1.90E-05	1.49E-05	kg SO2 eq
17	Terrestrial ecotoxicity	0.0032	0.00012	0.0021	0.0002	kg 1,4-DCB
18	Water consumption	1.71E-04	1.03E-04	1.36E-04	6.53E-05	m3 water eq

Table C.2. Environmental impact potentials for the extraction of 1 mg/g of photosynthesis pigments by FT and FT+UAE using water as solvent for extraction.

No	Impact category	Amount FT	Amount FT+UAE	Amount FT+UAE new approach	Unit
1	Fine particulate matter formation	1.57E-06	1.90E-06	1.90E-06	Kg PM2.5 eq
2	Fossil resource scarcity	0	0	0	kg oil eq
3	Freshwater ecotoxicity	2.53E-04	2.73E-04	2.73E-04	kg 1,4-DCB
4	Freshwater eutrophication	1.22E-06	1.14E-06	1.14E-06	kg P eq
5	Global warming	9.44E-05	9.71E-05	9.71E-05	kg CO_2 eq
6	Human carcinogenic toxicity	1.30E-10	1.68E-10	1.68E-10	kg 1,4-DCB
7	Human non-carcinogenic toxicity	3.93E-10	3.88E-10	3.88E-10	kg 1,4-DCB
8	Ionizing radiation	1.00E-04	1.75E-04	1.75E-04	kBq Co-60 eq
9	Land use	5.35E-04	6.17E-04	6.17E-04	m2a crop eq
10	Marine ecotoxicity	6.33E-06	6.86E-06	6.86E-06	kg 1,4-DCB
11	Marine eutrophication	3.24E-06	3.20E-06	3.20E-06	kg N eq
12	Mineral resource scarcity	5.23E-08	5.10E-08	5.10E-08	kg Cu eq
13	Ozone formation, Human health	5.50E-11	5.55E-11	5.55E-11	kg NOx eq
14	Ozone formation, Terrestrial ecosystems	7.33E-10	7.44E-10	7.44E-10	kg NOx eq
15	Stratospheric ozone depletion	8.45E-10	8.55E-10	8.55E-10	kg CFC^{11} eq
16	Terrestrial acidification	1.14E-05	1.06E-05	1.06E-05	kg SO2 eq
17	Terrestrial ecotoxicity	3.20E-04	3.10E-04	3.10E-04	kg 1,4-DCB
18	Water consumption	0.00307	0.00336	0.00336	m3 water eq

Table C.3. Main contributors to the environmental impact categories for extraction by SLE.

No	Impact category	Market for electricity	Market for acetone	Other
1	Fine particulate matter formation	90.83%	9.13%	0.04%
2	Fossil resource scarcity	0	0	0
3	Freshwater ecotoxicity	65.46%	34.46%	0.08%
4	Freshwater eutrophication	62.21%	37.76%	0.03%
5	Global warming	76.71%	23.27%	0.02%
6	Human carcinogenic toxicity	74.33%	25.54%	0.13%
7	Human non-carcinogenic toxicity	62.42%	37.47%	0.11%
8	Ionizing radiation	63.65%	36.32%	0.03%
9	Land use	55.48%	44.36%	0.16%
10	Marine ecotoxicity	91.61%	8.37%	0.02%
11	Marine eutrophication	79.28%	20.64%	0.08%
12	Mineral resource scarcity	91.61%	8.37%	0.02%
13	Ozone formation, Human health	3.86%	84.06%	12.08%
14	Ozone formation, Terrestrial ecosystems	3.86%	84.06%	12.08%
15	Stratospheric ozone depletion	70.91%	28.96%	0.13%
16	Terrestrial acidification	92.07%	7.91%	0.02%
17	Terrestrial ecotoxicity	86.86%	13.12%	0.02%
18	Water consumption	61.67%	38.04%	0.29%

Table C.4. Main contributors to the environmental impact categories for extraction by UAE.

No	Impact category	Market for electricity	Market for acetone	Other
1	Fine particulate matter formation	91.72%	8.23%	0.05%
2	Fossil resource scarcity	0	0	0
3	Freshwater ecotoxicity	64.35%	35.56%	0.09%
4	Freshwater eutrophication	60.20%	39.76%	0.04%
5	Global warming	75.71%	24.25%	0.04%
6	Human carcinogenic toxicity	72.32%	27.54%	0.14%
7	Human non-carcinogenic toxicity	63.42%	36.47%	0.11%
8	Ionizing radiation	63.65%	36.32%	0.03%
9	Land use	53.47%	46.35%	0.18%
10	Marine ecotoxicity	90.61%	9.35%	0.04%
11	Marine eutrophication	74.28%	20.64%	5.08%
12	Mineral resource scarcity	88.61%	8.37%	3.02%
13	Ozone formation, Human health	2.08%	84.06%	13.86%
14	Ozone formation, Terrestrial ecosystems	3.86%	84.06%	12.08%
15	Stratospheric ozone depletion	69.91%	28.94%	1.15%
16	Terrestrial acidification	91.07%	8.91%	0.02%
17	Terrestrial ecotoxicity	85.86%	14.12%	0.02%
18	Water consumption	60.67%	39.04%	0.29%

Table C.5. Main contributors to the environmental impact categories for extraction by FT.

No	Impact category	Market for electricity	Market for water	Other
1	Fine particulate matter formation	90.83%	0.13%	9.04%
2	Fossil resource scarcity	0	0	0
3	Freshwater ecotoxicity	65.46%	0.046%	34.49%
4	Freshwater eutrophication	62.21%	0.076%	37.71%
5	Global warming	76.71%	0 %	23.29%
6	Human carcinogenic toxicity	74.33%	0 %	25.67%
7	Human non-carcinogenic toxicity	62.42%	0 %	37.58%
8	Ionizing radiation	63.65%	0.032%	36.32%
9	Land use	55.48%	0.36%	44.16%
10	Marine ecotoxicity	91.61%	0.37%	8.02%
11	Marine eutrophication	79.28%	0.64%	20.08%
12	Mineral resource scarcity	91.61%	0.37%	8.02%
13	Ozone formation, Human health	6.86%	0 %	93.14%
14	Ozone formation, Terrestrial ecosystems	3.86%	0 %	96.14%
15	Stratospheric ozone depletion	70.91%	0 %	29.09%
16	Terrestrial acidification	92.07%	0.091%	7.84%
17	Terrestrial ecotoxicity	86.86%	0.12%	13.02%
18	Water consumption	59.67%	40.04%	0.29%