Rodrigo Borges

Valorization of Microalgal Biomass in a Biorefinery Perspective

Master's in Biotechnology

The present work was performed under the supervision of:

Dr. Raúl Barros
Dr. Sara Raposo

Universidade do Algarve

Faculdade de Ciências e Tecnologia

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Declaration of Authorship

I hereby declare being the sole author of the present work. It is original and unprecedented. The researched works and authors are properly cited both in the text and bibliographical references included.

Rodrigo Borges

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“I don’t want to believe, I want to know.”
– Carl Sagan

Resumo

A valorização da biomassa de microalgas verdes foi avaliada e melhorada, utilizando como modelo a microalga verde não-axénica *Chlorella sorokiniana* para a caracterização do perfil de pigmentos e ácidos gordos presentes. Foi efetuada a avaliação de diferentes solventes para a extração de pigmentos, incluindo solventes verdes, ou seja, menos prejudiciais para o ambiente, tal como a extração sequencial de ácidos gordos através de uma segunda etapa de extração. Um segundo ciclo de extração à mesma biomassa foi efetuado para avaliar a necessidade deste para a extração de pigmentos com solvente fresco. Este estudo foi efetuado para tanto biomassa fresca como liofilizada. Utilizando os resultados dos ensaios realizados foi feito um estudo preliminar da sustentabilidade económica de um processo industrial com base na extração sequencial de ambos metabolitos.

Ultimamente, a utilização de microalgas para a extração e criação de produtos de elevado valor tem sido estudada e atualmente já é implementada em alguns setores. Devido à versatilidade destes microrganismos, podem ser utilizados para a produção de uma variedade de bens, como biocombustíveis e bioplásticos. As principais vantagens das microalgas são o seu baixo custo de manutenção e não competirem por terreno com atividades agrícolas. As microalgas também possuem o potencial de ser cultivadas em águas residuais como forma de tratamento destas, utilizando os nutrientes presentes nestas para crescer.

A eficiência de extração dos solventes utilizados foi comparada pela adição de solvente à biomassa e homogeneização por vortex. A extração foi auxiliada por uma hora de banho de ultrassons. As fases sólidas e líquidas foram separadas por centrifugação e a presença de pigmentos foi analisada por espectrofotometria e cromatografia líquida. A mesma biomassa foi utilizada para avaliar a eficiência de um segundo ciclo de extração.

A luteína, clorofila *a*, clorofila *b*, juntamente com algumas clorofilas não identificadas foram considerados os pigmentos principais da *C. sorokiniana*. Outros pigmentos como neoxantina, betacaroteno, feoforbide *a* e feofitina *a* foram encontrados em menores quantidades. Relativamente à extração de pigmentos, a extração de pigmentos de biomassa fresca obteve rendimentos de extração mais elevados que a biomassa liofilizada para todos os solventes. O solvente com a taxa

de extração mais elevada para os pigmentos quantificados foi o metanol para ambas biomassa fresca e liofilizada, atingindo para a biomassa fresca rendimentos de extração de 13.83 ± 0.70 mg/g biomassa para luteína, 18.61 ± 2.91 mg/g para clorofila *a*, 29.93 ± 1.87 mg/g para clorofila *b*, e 3.15 ± 0.40 mg/g para betacaroteno. A mistura de clorofórmio-metanol, tal como o acetato de metilo, demonstraram ser bastante eficientes na extração de pigmentos. O isopropanol e o éter etílico apresentaram os rendimentos de extração de pigmentos mais baixos. O segundo ciclo de extração de pigmentos utilizando solvente novo para a mesma biomassa provou não ser muito eficiente, especialmente para os solventes com rendimentos de extração mais elevados. Os resultados obtidos na quantificação de pigmentos por espectrofotometria e *ultra high-performance liquid chromatography (UHPLC)* demonstraram bastante variação, sendo que a clorofila *a* demonstrou valores superiores na quantificação por espectrofotometria enquanto a clorofila *b* demonstrou valores mais elevados na quantificação por UHPLC. Os carotenoides totais demonstraram valores bastante aproximados no entanto foram ligeiramente superiores na quantificação por UHPLC.

A extração sequencial de ácidos gordos e pigmentos a partir da mesma biomassa foi avaliada por uma extração inicial de pigmentos da biomassa com os solventes que apresentaram melhor extração, seguida de uma extração de ácidos gordos pelo método de Bligh & Dyer (1959). Os resultados obtidos foram comparados com o valor de ácidos gordos totais obtidos para biomassa sem extração de pigmentos prévia. A quantificação dos ácidos gordos foi feita através da transesterificação destes e análise por cromatografia de gás.

A caracterização de ácidos gordos revelou o ácido palmítico como o presente em maior quantidade na biomassa de *C. sorokiniana*, correspondendo a 40.5% do total de ácidos gordos. O ácido palmitoleico corresponde a 8.1% do total, o ácido oleico a 10.7% do total de ácidos gordos e o ácido linolénico a 15.5% do total de ácidos gordos. Os restantes ácidos gordos apresentaram frações menores. De forma semelhante ao que se sucedeu na extração de pigmentos, a extração de ácidos gordos demonstrou ser mais eficiente para biomassa fresca, extraindo 118.1 mg/g biomassa, em comparação à liofilizada onde foi extraído apenas 57.9 mg/g biomassa.

Apesar de o metanol apresentar a melhor taxa de extração de pigmentos demonstrou remover a totalidade de ácidos gordos da biomassa fresca durante a extração de pigmentos. Os solventes utilizados que apresentaram menor extração de

ácidos gordos durante a extração de pigmentos foram o acetato isopropílico e o acetato etílico, no entanto estes solventes apresentaram apenas valores medíocres na extração de pigmentos. Devido a esta fraca extração de ácidos gordos por parte do acetato etílico durante a extração de pigmentos foi possível extrair aproximadamente 77% do total de ácidos gordos presentes na biomassa durante a fase de extração de ácidos gordos.

A avaliação preliminar do processo de extração sequencial de ambos pigmentos e ácidos gordos da biomassa de *C. sorokiniana* a uma escala industrial foi efetuada através de uma simulação utilizando o software SuperPro Designer. O processo industrial foi projetado com base nos resultados obtidos no trabalho prático mantendo as proporções de matérias usadas e produtos obtidos. O acetato etílico foi escolhido como solvente para a simulação devido a possuir uma extração sequencial mais equilibrada entre pigmentos e ácidos gordos. Os resultados obtidos indicam a sustentabilidade económica do processo se os produtos forem comercializados a valores elevados. Os preços dos produtos seriam, 10 000 euros/kg para os pigmentos e 15 euros/kg para os ácidos gordos. Apesar de os valores serem elevados, é possível que se enquadrem no mercado real devido à especificidade e pureza do produto.

Futuramente, deveria ser feita uma avaliação mais profunda à extração sequencial de pigmentos e ácidos gordos, possivelmente incluindo a co-extração de mais metabolitos de elevado valor da mesma biomassa. A valorização de microalgas seria uma contribuição no avanço de para uma maior sustentabilidade ambiental.

Palavras-chave: microalgas, biorefinaria, biomassa, pigmentos, ácidos gordos, sustentabilidade.

Abstract

The valorisation of microalgae biomass was assessed and enhanced by the characterization of pigment and fatty acid profiles using the microalga *Chlorella sorokiniana* as a model, increasing pigment extractability by comparison of multiple extraction solvents including green solvents and sequential extractability of fatty acids from the same biomass after pigment extraction, including both fresh and freeze-dried biomass. A preliminary assessment of the sequential extractability of both pigments and fatty acids process at industrial scale was simulated to assess its viability.

Lutein, chlorophyll *a*, chlorophyll *b* and unidentified chlorophylls were the most abundant pigments present in *C. sorokiniana*. For both fresh and freeze-dried biomass, the solvent with the highest extraction yields for the quantified pigments was methanol. For fresh biomass, methanol achieved extraction yields of 13.83 ± 0.70 mg/g biomass for lutein, 18.61 ± 2.91 mg/g for chlorophyll *a*, 29.93 ± 1.87 mg/g for chlorophyll *b*, and 3.15 ± 0.40 mg/g for beta-carotene (β-carotene). A second pigment extraction cycle from the same biomass was shown to be inefficient, especially for the most efficient solvents.

The fatty acids (FA) present in the highest concentrations in *C. sorokiniana* were palmitic acid (40.5% of total fatty acids), palmitoleic acid (8.1% of total FA), oleic acid (10.7% of total FA) and linoleic acid (15.5% of total FA). The sequential extraction of fatty acids in different stages from the same biomass was not efficient using methanol, however isopropyl acetate and ethyl acetate were able to extract pigments from the biomass without removing a large portion of fatty acids.

For both metabolites and all solvents, fresh biomass was shown to achieve higher extraction yields. This benefits the processing operations for this biomass by removing freeze-drying costs.

An industrial scale process for the sequential extraction of both metabolites, using ethyl acetate for its average pigments extractability and poor fatty acid removal, from the same biomass was found to be profitable by selling the pigments at 10,000 euros/kg and the fatty acids at 15 euros/kg, which might be suitable for the purity of the obtained products.

Keywords: microalgae, biorefinery, biomass, pigments, fatty acids, sustainability.

Table of Contents

Declaration of Authorship .... ***i***

Copyright .... ***ii***

Acknowledgements .... ***iii***

Resumo .... ***vii***

Abstract .... ***xi***

Figure Index .... ***xv***

Table Index .... ***xvii***

Equation Index .... ***xviii***

List of Abbreviations .... ***xix***

1. Introduction ***1***

- **1.1. Sustainability** **1**
- **1.2. Microalgae** **2**
  - *1.2.1. Chlorella sorokiniana* 3
- **1.3. Photosynthetic Pigments** **4**
  - 1.3.1. Chlorophylls 5
  - 1.3.2. Carotenoids 8
  - 1.3.3. Pigment Extraction 10
  - *1.3.4.* Pigments found in *C. sorokiniana* 12
- **1.4. Fatty Acids** **13**
  - 1.4.1. Saturated, Monounsaturated and Polyunsaturated Fatty Acids 15
  - 1.4.2. Fatty Acid Extraction 18
  - *1.4.3.* Fatty Acid Profile of *C. sorokiniana* 20
- **1.5. Industry and Biorefinery** **20**
- **1.6. Objectives** **22**

2. Material and Methods ***24***

- **2.1. Cell Culture and Maintenance** **24**
- **2.2. Pigment Extraction** **24**
  - 2.2.1. Extraction Conditions 24
  - 2.2.2. Analytical Conditions 25
- **2.3. Fatty Acid Extraction** **27**
  - 2.3.1. Pigment Pre-Extraction 27
  - 2.3.2. Total Lipid Extraction Conditions 28
  - 2.3.3. Acid-Catalyzed Transesterification 29
  - 2.3.4. Analytical Conditions 29
- **2.4. Economic and Sustainability Assessment** **30**
- **2.5. Data Analysis** **31**

3. Results and Discussion ***32***

- **3.1. Pigment Extraction** **32**
  - 3.1.1. Pigment Characterization 32
  - 3.1.2. Solvent Extraction Efficiency 36
  - 3.1.2.1. Lutein Extraction 36
  - 3.1.2.2. Chlorophyll *a* Extraction 38

3.1.2.3. Chlorophyll *b* Extraction 40
3.1.2.4. Beta-carotene Extraction 42
3.1.3. Second Extraction 43
3.1.4. Spectrophotometry Vs. Chromatography 46
3.2. Fatty Acid Extraction 47
3.2.1. Fatty Acid Profile of *C. sorokiniana* 47
3.2.2. Sequential extraction of Pigments and Fatty Acids 50
3.3. Industrial Sustainability 52
4. Conclusions 55
5. Cited Literature 57

Figure Index

Figure 1 - Chemical structure of 1) chlorophyll a (chlorin) and 2) chlorophyll c1 (porphyrin) adapted from (Fernandes et al., 2020). *6*

Figure 2 – Absorbance spectrum profile of 1) Chl a, 2) Chl b, 3) Chl c2 and 4) phytin a, adapted (Clementson & Wojtasiewicz, 2019). *7*

Figure 3 - Chemical structure of 1) beta-carotene; 2) lutein; 3) fucoxanthin; 4) violaxanthin; 5) Neoxanthin; and 6) Zeaxanthin, adapted from (Scheer, 2003). *8*

Figure 4 - Absorbance spectrum profile of 1) β-carotene, 2) lutein, 3) fucoxanthin, 4) violaxanthin, 5) neoxanthin, and 6) zeaxanthin as adapted from (Clementson & Wojtasiewicz, 2019). *9*

Figure 5 - General derivation mechanism of stearic acid into gamma-linolenic acid in microalgae (Muñoz et al., 2021; Rustan & Drevon, 2005). *18*

Figure 6 - Representation of the industrial scale module schematic for the sequential extraction of both pigments (stream S-110) and fatty acids (stream S-123) from the microalga Chlorella sorokiniana, recovering both biomass and used solvent (streams S-117 and S-107, respectively) for commercialization to reduce production cost. *30*

Figure 7 - Chromatogram from the pigment analysis of a fresh biomass sample extracted with chloroform-methanol (2:1, v/v) including pigment name, spectrum match factor relative to standard and retention time. The chromatogram is represented in signal intensity (mAU) over time (min). Pigments identified include: neoxanthin (peak 3), pheophorbide a (peak 7) and derivates (peak 5 and 6), lutein (peak 8) and derivates (peak 9 and 10), chlorophyll a derivates (peak 15, 17 and 18), chlorophyll b (peak 16) and derivates (peak 11, 12, 13, and 14), pheophytin a (peak 23) and derivates (peak 17 and 18). *33*

Figure 8 – Chromatogram obtained from the pigment analysis of a fresh biomass sample extracted with ethyl acetate including pigment name, spectrum match factor relative to standard and retention time. The chromatogram is represented in signal intensity (mAU) over time (min). Pigments identified include: neoxanthin (peak 3), pheophorbide a (peak 5) and derivates (peak 9 and 14), lutein (peak 10) and derivates (peaks 11 and 12), chlorophyll a (peak 21) and derivates (peak 17 and 19), chlorophyll b (peak 18) and derivates (peak 13, 15, 16, and 20), β-carotene (peak 23), pheophytin a (peak 30) and derivates (peak 26, 27 and 31). *34*

*Figure 9 - Extraction yield (mg/g of biomass dry weight) of lutein from (a) freeze-dried biomass and (b) fresh biomass by Chloroform-Methanol (2:1, v/v), Methanol, Ethanol, Acetone, Isopropanol, Isopropyl Acetate, Methyl Acetate, Ethyl Acetate, Ethyl Ether (n=4; mean ± SD). Equal letters indicate the absence of statistical significance of mean variation and are independent for each graph. * - n=3.* ... *37*

*Figure 10 - Extraction yield (mg/g of biomass dry weight) of chlorophyll a from freeze-dried biomass (a) and fresh biomass (b) by Chloroform-Methanol (2:1, v/v), Methanol, Ethanol, Acetone, Isopropanol, Isopropyl Acetate, Methyl Acetate, Ethyl Acetate, Ethyl Ether (n=4; mean ± SD). Equal letters indicate the absence of statistical significance of mean variation and are independent for each graph. * - n=3.* *39*

*Figure 11 - Extraction yield (mg/g of biomass dry weight) of chlorophyll b from freeze-dried biomass (a) and fresh biomass (b) by Chloroform-Methanol (2:1, v/v), Methanol, Ethanol, Acetone, Isopropanol, Isopropyl Acetate, Methyl Acetate, Ethyl Acetate, Ethyl Ether (n=4; mean ± SD). Equal letters indicate the absence of statistical significance of mean variation and are independent for each graph. * - n=3. .... 41*

Figure 12 - Extraction yield (mg/g of biomass dry weight) of β*-carotene from freeze-dried biomass (a) and fresh biomass (b) by Chloroform-Methanol (2:1, v/v), Methanol, Ethanol, Acetone, Isopropanol, Isopropyl Acetate, Methyl Acetate, Ethyl Acetate, Ethyl Ether (n=4; mean ± SD). Equal letters indicate the absence of statistical significance of mean variation and are independent for each graph. * - n=3. .... 42*

*Figure 13 - Extraction yields (mg/g of biomass dry weight) of lutein from the first and second extraction cycles for both freeze-dried biomass (a) and fresh biomass (b) by Chloroform-Methanol (2:1, v/v), Methanol, Ethanol, Acetone, Isopropanol, Isopropyl Acetate, Methyl Acetate, Ethyl Acetate, Ethyl Ether (n=4; mean ± SD). Equal letters indicate the absence of statistical significance of mean variation and are independent for each solvent and graph. * - n=3. .... 44*

*Figure 14 - Extraction yields (mg/g of biomass dry weight) of chlorophyll b from the first and second extraction cycles for both freeze-dried biomass and fresh biomass by Chloroform-Methanol (2:1, v/v), Methanol, Ethanol, Acetone, Isopropanol, Isopropyl Acetate, Methyl Acetate, Ethyl Acetate, Ethyl Ether (n=4; mean ± SD). Equal letters indicate the absence of statistical significance of mean variation and are independent for each solvent and graph. * - n=3; ** - n=2. .... 45*

Figure 15 – Representation of the calculated extraction yields for chlorophyll a, chlorophyll b and total carotenoids from the pigment extraction of fresh biomass by methanol, using the Lichtenthaler (1987) equations with the values obtained from Ultra High-Performance Liquid Chromatography (UHPLC). Different letters represent the correlation of means and are independent for each pigment (paired t-test, α*=0.05). 46*

Figure 16 – Chromatogram obtained from the analysis of fatty acid methyl esters from freeze-dried C. sorokiniana biomass including methyl ester name and retention time. The chromatogram is represented in signal intensity (mV) over time (min). FAMES identified include: methyl tridecanoate (peak 1), methyl myristate (peak 2), methyl myristoleate (peak 5), methyl palmitate (peak 9), methyl palmitoleate (peak 10), methyl heptadecanoate (peak 13), methyl stereate (peak 14), methyl oleate/elaidate (peak 15), methyl linoleate/linoelaidate (peak 17), and methyl linolenate (peak 18). 48

Table Index

Table 1 - Visual description of Chlorophyll colours, adapted from (Fernandes et al., 2020). 7

Table 2 - Literature review of main pigments present in C. sorokiniana, solvent used and extraction method, as well as respective extraction yield represented in $\mu g\ g^{-1}$ of biomass. 13

Table 3 - Common name, systematic name and abbreviation for some of the most common saturated FAs (Haynes, 2016). 15

Table 4 - Common name, systematic name, double bond (DB) position and abbreviation of a few monounsaturated FAs and their isomers (Kazaz et al., 2022). 16

Table 5 - Common name, systematic name, omega (ω) classification, double bond (DB) position and abbreviation of some polyunsaturated FAs and their isomers. 17

Table 6 - Percentage values for total lipids (w/w) and main fatty acids (% of total lipids) of C. sorokiniana with different extraction methods in the reviewed literature. 20

Table 7 - Solvent gradient ratios for UPLC method. 26

Table 8 - Pigments found in this assay for the different solvent and biomass conditions. 35

Table 9 - Identified fatty acid methyl esters (FAMEs) corresponding to the peaks of the chromatogram represented in figure 16, respective common fatty acid names, abbreviation, average relative area (%; n=4) and standard deviation (SD). 49

Table 10 - Fatty acid extraction yield (mg/g of biomass) for both fresh and freeze-dried biomass, including mean, standard deviation (SD), number of replicates (n), and yield extraction of the means relative to the means of direct extraction for samples pre-extracted with the different solvents. 51

Equation Index

Equation 1 – Lichtenthaler's chlorophyll a *equation for methanol 100%*.......................................25
Equation 2 – Lichtenthaler's chlorophyll b *equation for methanol 100%*.......................................25
Equation 3 – Lichtenthaler's total carotenoids equation for methanol 100%.................................25
Equation 4 – Obtained equation for lutein UHPLC standard curve………………………………..…26
Equation 5 – Obtained equation for chlorophyll a *UHPLC standard curve*…………………………..27
Equation 6 – Obtained equation for chlorophyll b *UHPLC standard curve*…………………………27
Equation 7 – Obtained equation for beta-carotene UHPLC standard curve………………………...27

List of Abbreviations

ANOVA – Analysis of Variance

β-carotene – Beta-carotene;

C_2H4O_2 – Acetic acid;

CH_2O_2 – Formic acid;

Chl – Chlorophyll;

DAD – Diode Array Detector;

DHA – Docosahexaenoic acid;

DPA – Docosapentaenoic acid;

EPA – Eicosapentaenoic acid;

ETA – Eicosatetraenoic acid;

FA – Fatty acids;

FAME – Fatty acid methyl esters;

FID – Flame ionization detector;

GC – Gas chromatography;

GC-MS – Gas chromatography mass spectrometry;

IBVF – Institute of Plant Biochemistry and Photosynthesis;

M – Millions;

MUFA – Monounsaturated fatty acid;

PUFA – Polyunsaturated fatty acid;

Phide – Pheophorbide;

Phytin – Pheophytin;

PTFE – Polytetrafluoroethylene;

R&D – Research and development;

SD – Standard Deviation;

SFE – Supercritical fluid extraction;

SMV- Significance of mean variation;

TAP – Tris-acetate-phosphate

UHPLC – Ultra high-performance liquid chromatography;

ω-3 – Omega-3

ω-6 – Omega-6

1. Introduction

Microalgae are an alternative sustainable source of high-value compounds used for several different product industries, such as cosmeceutical, pharmaceutical, nutraceutical, as well as biofuel and bioplastic production (Chisti, 2020). This thesis will be focused on the valorization of microalgae from an industrial biorefinery perspective, using the non-axenic microalga *Chlorella sorokiniana* as a model. In this section a brief introduction will be given on the many topics covered by the thesis goals.

1.1. Sustainability

The global dependence on traditional natural resources used to produce domestic everyday consumables, has risen 65% from the year 2000 to the year 2019. This increase in consumption is most likely related to the approximate 9 million hectares of forests cut down for agricultural expansion and the continuous increase in carbon dioxide emissions over the last century. The increase in carbon dioxide emissions and greenhouse effect gases are related to rising global warming and the acidification of the oceans. All these occurrences affect the biodiversity and ecosystems around the world (United Nations, 2022). Recycling the waste produced in any given industry (circular economy) is one of the most important steps in assuring a more sustainable usage of natural resources. The main drawbacks of this approach are high needs for research and development and structural investment, as most industries are not prepared for implementation. However, a circular economy alone is not enough, especially considering non-convertible waste such as the waste produced from fossil fuels, thus it is imperative to explore and take advantage of alternative natural resources which are renewable, recyclable, reusable and have less impact on the environment (Sharma *et al.*, 2021).

A large part of the carbon dioxide released and the natural resources consumed are due to the production of energy as many countries still depend on fossil fuels for the production of electrical energy (Razmjoo *et al.*, 2021). Alternative energy sources such as solar, wind, hydroelectric or geothermal are used regularly and proven to be efficient but with the high energy demand, which is continuously growing, these sustainable energies will not substitute fossil fuel in the foreseeable future (Jabeen *et al.*, 2020). Thus, finding various sources of raw materials with the capacity to produce energy and making the process economically viable would be a major contribution, especially if the process is based on a circular economy, for example using carob waste for the production of bioethanol (Raposo *et al.*, 2017).

Another reason for the high carbon dioxide emissions and natural resources consumed in mass is the unbalanced large-scale industrial production which tends to use a few select unsustainable raw materials or materials which compete in other markets (Yang *et al.*, 2022). The traditional plastics now indispensable in todays society are produced from crude, the majority of ethanol produced is derived from sugarcane or corn, and fatty acids used in nutraceutical products are derived mainly from fish oil. These are a few among many other products derived from unsustainable sources (EIA, 2022; Nielsen *et al.*, 2020; Otero *et al.*, 2021). However, alternatives for these raw materials are being studied, for example, the production of the same products from different types of wastes, microbial biomass or algal biomass (Battista *et al.*, 2022; Mathiot *et al.*, 2019).

1.2. Microalgae

For some time, microalgae have been studied for their many valuable characteristics, in particular, because of their low-cost maintenance, large potential in biorefinery and not competing for agricultural land. Microalgae possess a variety of high-value metabolites that can have many sustainable applications, including increasing the nutritional value of human food, being used for animal feed, used as compost for fertilizing agricultural land, as well as the production of biofuels and bioplastic (Chisti, 2020). In addition, microalgae can accumulate carbon from the atmosphere through photosynthesis. For example under certain conditions, *Chlorella vulgaris* was able to capture approximately its weight worth of carbon, which it uses to convert into organic compounds such as sugars (Aghaalipour *et al.*, 2020).

The nutritional value of microalgae can be attested by the presence of fatty acids essential for human nutrition, as well as protein, minerals, pigments and its energy value (Tokuşoglu & Ünal, 2003; Wang *et al.*, 2022). Despite the challenges still present in the large-scale production of microalgae and the downstream process of the biomass into the final products, the use of microalgae for the enrichment of less nutritious foods is a viable and sustainable option to aid in the mitigation of world hunger issues without the need for genetic engineering techniques (Gohara-Beirigo *et al.*, 2022).

With the recent findings, concerns have risen dramatically about the amounts and variety of micro and nanoplastic particles present in the environment, most notably in marine environments. These can sorb chemical pollutants and cause disruptive damage when ingested by living organisms (Guo & Wang, 2019). The most common plastics in everyday objects are synthetic polymers derived from fossil resources and are, for the most part undegradable. However, bioplastics can be produced from polymers and monomers obtained from microalgae which are for the most part biodegradable (Mohan *et al.*, 2022).

Among the many other applications of microalgae, they can also be grown in wastewater and used as a treatment for contaminants such as phosphorus, chemical oxygen demand, ammonium, nitrate, and other inorganic nitrogen forms. Some microalgae can degrade these contaminants or use them as a nutrient source to grow (Wang *et al.*, 2010). Due to their capacity to produce fermentable sugars and different lipids especially when in consortia, microalgal biomass grown in wastewater can be harvested and used to produce third-generation biofuels (Moreno-García *et al.*, 2021).

1.2.1. Chlorella sorokiniana

The *Chlorella* genus was first described in the late nineteenth century as a coccoid green alga by Beijerinck (1890). After mass culturing, this microalga was introduced as a model for plant biology research and became one of the most studied green algae. The *Chlorella* genus is integrated in the Chlorellaceae family, Trebouxiophyceae class and Chlorophyta phylum. There are currently three species of the *Chlorella* genus, which are *Chlorella vulgaris, Chlorella lobophora and Chlorella sorokiniana* (Huss *et al.*, 1999; Luo *et al.*, 2010).

The *C. sorokiniana* species was first isolated by Sorokin & Myers (1953), from a sample obtained from a creek in Texas, USA. It was of high interest and very well studied by Dr. Sorokin due to its fast growth rate and ability to grow without organic nutrients and at high temperatures. The *C. sorokiniana* species can also grow in mixotrophic cultures, growing both with light and in darkness (Shihira & Kraus, 1965). Since it was first identified several strains of this species were discovered all over the world (Guiry & Guiry, 2022).

1.3. Photosynthetic Pigments

As previously stated, microalgae accumulate nutrients through photosynthesis. Very briefly, photosynthesis is a well-known process through which some organisms such as plants, algae and some bacteria species can transform light energy and water into chemical energy through electron and proton transfers. The di-hydrogen atoms in the H_2O molecule are used in the Calvin cycle which converts captured carbon dioxide into carbohydrates, such as sugars, releasing oxygen into the atmosphere. These reactions are initiated by the change in excitation states of light-absorbing pigment molecules, mainly chlorophylls, due to interactions with photons (Johnson, 2016).

Aside from their use as food dyes, pigments have many benefits in different areas. Both chlorophylls and carotenoids have been recognized for their antioxidant and antimicrobial properties. The antioxidant activity of carotenoids has been associated with a decrease in cancer risk (Farkas *et al.*, 2020; Fernandes *et al.*, 2020). According to Moeller *et al.* (2000), lutein has a role in preventing age-related eye disorders such as cataracts. Beta-carotene is a precursor to Vitamin A (all-trans-retinol) and can be absorbed by the human body, being converted into Vitamin A which has immune regulatory functions (O'Byrne & Blaner, 2013). The antioxidant effect of pigments, in particular, astaxanthin has been shown to increase immune response, growth and nutritional value in aquatic animals, thus bringing increased benefits to aquaculture feed (Chen *et al.*, 2021). Furthermore, pigments can be used in cosmeceutical products such as sunscreen, tanning and anti-ageing skin products due to their antioxidant properties and in the case of yellow/red pigments, such as canthaxanthin which can lodge themselves in the epidermis and subcutaneous fat, darkening the skin (Pangestuti *et al.*, 2020).

In the cells of photosynthetic eukaryotes, pigments can be found bound to each other by specific proteins in light-harvesting complexes located in the thylakoid membranes of the chloroplasts (Nelson & Cox, 2000). Pigments can be divided into three main types, chlorophylls, carotenoids and phycobiliproteins. Chlorophylls absorb blue and red light, reflecting mostly green light, carotenoids absorb blue and green light, reflecting mostly yellow and red, and phycobiliproteins absorb mostly green and yellow light, reflecting either blue or red light. However, phycobiliproteins are pigmented proteins mostly present in red macroalgae and cyanobacteria and since the present study was based on the green microalgae *C. sorokiniana* this class of pigments will not be further discussed (Johnson, 2016; Mesquita *et al.*, 2021; Pagels *et al.*, 2020). In the following subsections, chlorophylls, carotenoids, and pigment extraction will be reviewed in greater detail.

1.3.1. Chlorophylls

Chlorophylls (Chls) are one of the most important pigment classes in the photosynthetic process, present in most organisms capable of light-harvesting. They are responsible mainly for the capture of light photons, except for those organisms where the chlorophyll function is performed by phycobiliproteins (Mesquita *et al.*, 2021). There are two major types of Chls, distinguished by their structure: chlorins (Chl *a*, Chl *b*, Chl *d*) and porphyrins (Chl *c1*-*c3*). While chlorins possess a magnesium-coordinated tetrapyrrole connected to a phytyl chain through an ester group, porphyrins have a carboxylic functional group instead of the ester-phytyl group. Small variations in the terminal groups of the other outer tetrapyrrole carbons originate from the different chlorins and porphyrins (Fernandes *et al.*, 2020; Merritt & Loening, 1980; Scheer, 2003). An example of a chlorin and a porphyrin chemical structure is represented in Figure 1.

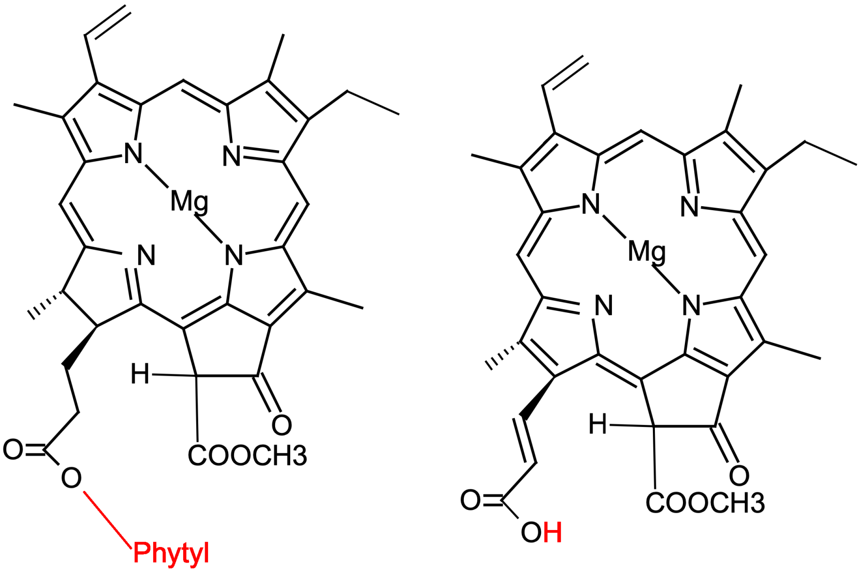

Figure 1 - Chemical structure of 1) chlorophyll a *(chlorin) and 2) chlorophyll* c1 *(porphyrin) adapted from (Fernandes et al., 2020).*

Even though chlorophylls and their structures and functions have been studied since they were first described and named in 1817, discoveries are still made on this subject to this day (Pelletier & Caventou, 1817). Chlorophyll *f* was only first described in 2010 after being discovered in filamentous cyanobacteria, it is thought to assume an auxiliary role in photosynthesis, enhancing energy transfer (Chen *et al.*, 2010; Kato *et al.*, 2020). There are various possible changes to the tetrapyrrole structure that can affect the nature of the chlorophyll, making possible the existence of a very large number of chlorophylls and derivates. For example, bacteriochlorophylls have the same structure as Chls, however, the core metal is not Mg, but rather zinc. Other examples are pheophytins (phytin) and pheophorbides (phide), phytin a is a derivate of Chl *a* where the tetrapyrrole is not metal coordinated, phide *a* is a derivate of phytin *a* where the phytyl group is replaced by the carboxylic group, very much like Chls *c* (Merritt & Loening, 1980; Scheer, 2003).

As previously stated, chlorophylls absorb blue and red light reflecting in the green spectra, yet small shifts in the precise wavelength of the main peaks cause changes in the emission color (Chen *et al.*, 2010). Table 1 presents a description of the color variation for the main chlorophylls:

Table 1 - Description of chlorophyll colors adapted from (Fernandes et al., 2020).

Chlorophyll	**Color Description**
Chl *a*	Blue-Green
Chl *b*	Brilliant Green
Chl *c* (1-3)	Yellow-Green
Chl *d*	Brilliant Green
Chl *f*	Emerald Green

In literature, the exact wavelength of the absorption maxima for pigments can slightly vary. The two main responsible factors for this variation are the solvent in which the pigments are dissolved and the equipment used (Lichtenthaler, 1987; van Heukelem *et al.*, 1994). Despite these small variations, the absorption spectra of pigments are very useful for identification, considering patterns, peak shape and absorption maxima. Small variations such as protrusions, humps or indentures in the spectrum shape can be the factor that distinguishes two pigments (Clementson & Wojtasiewicz, 2019). In Figure 2 are the spectra for some of the main chlorophylls obtained from standards.

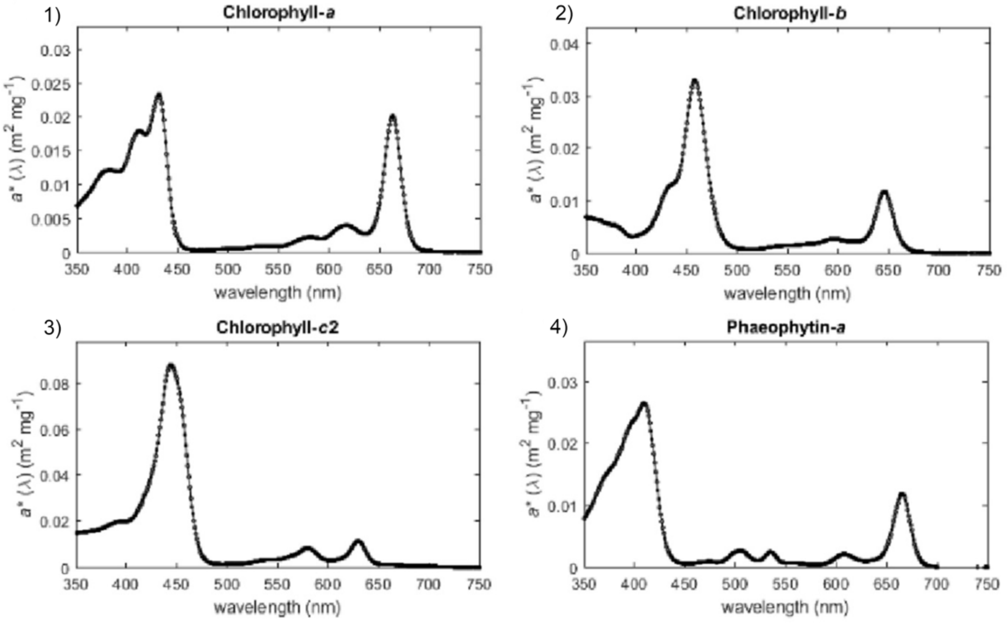

Figure 2 – Absorbance spectrum profile of 1) Chl a, 2) Chl b, 3) Chl c2 and 4) phytin a, adapted from Clementson & Wojtasiewicz (2019).

1.3.2. Carotenoids

The function of carotenoids in photosynthesis, besides contributing to the capture of light, is to protect the photosynthesis complexes from degradation by excessive light. Carotenoids can be divided into two groups: carotenes, for example beta-carotene (β-carotene), and xanthophylls, which are very similar to carotenes but have hydroxy groups (e.g., lutein). Each carotenoid can have many derivations by changing from *cis* to *trans* isomers in the different chiral points (Scheer, 2003). There are currently over 700 carotenoids reported, some of which perhaps have only been reported from a specific species and have derived from one of the main carotenoids. These derivations can occur easily since carotenoids are long hydrocarbon chains, and thus the molecules are more susceptible to reaction (Britton *et al.*, 2004). In some cases, carotenoids may vary from each other by different and unique functional groups (Dembitsky & Maoka, 2007). For most oxygenic phototrophs (with few exceptions) all carotenoids are dicyclic, meaning they have two carbon rings, one at each extremity of the molecule. Despite this, the common precursor to all carotenoids is the acyclic carotenoid lycopene, which in turn is derived from phytoene. Lycopene is converted by a lycopene cyclase enzyme into either alpha-carotene or β-carotene, further modified into all other carotenoids through the carotenogenesis pathway (Córdova *et al.*, 2018; Krubasik & Sandmann, 2000; Takaichi, 2020). The molecular structures of a few common carotenoids are depicted in Figure 3.

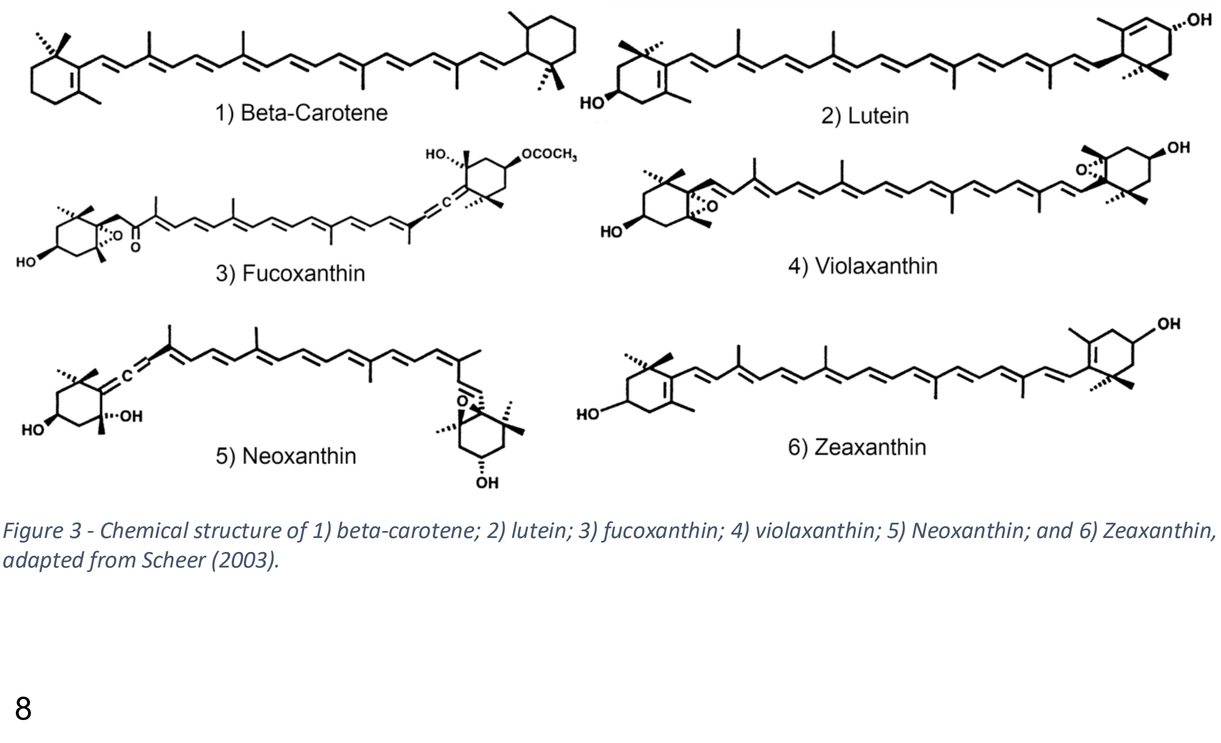

Figure 3 - Chemical structure of 1) beta-carotene; 2) lutein; 3) fucoxanthin; 4) violaxanthin; 5) Neoxanthin; and 6) Zeaxanthin, adapted from Scheer (2003).

During the late seasons, autumn and winter, leaves fester and turn to dark shades of orange and light brown, this is the result of a faster deterioration of chlorophylls relative to carotenoids (Johnson, 2016). The longevity of carotenoids could be due their high antioxidant activity, facilitated by the exposed double-bonds along the chain which allow interactions with dioxidene ($^1O_2$ or singlet oxygen) and free radicals (Young & Lowe, 2018). When carotenoids react with the dioxidene molecule, they become excited while converting the singlet oxygen to triplet oxygen, a less reactive state and release harmless heat to stabilize. When reacting with a free radical, the carotenoid structure facilitates electron transfers which result in a stable carotenoid radical that may later become a secondary derivative (Fiedor & Burda, 2014).

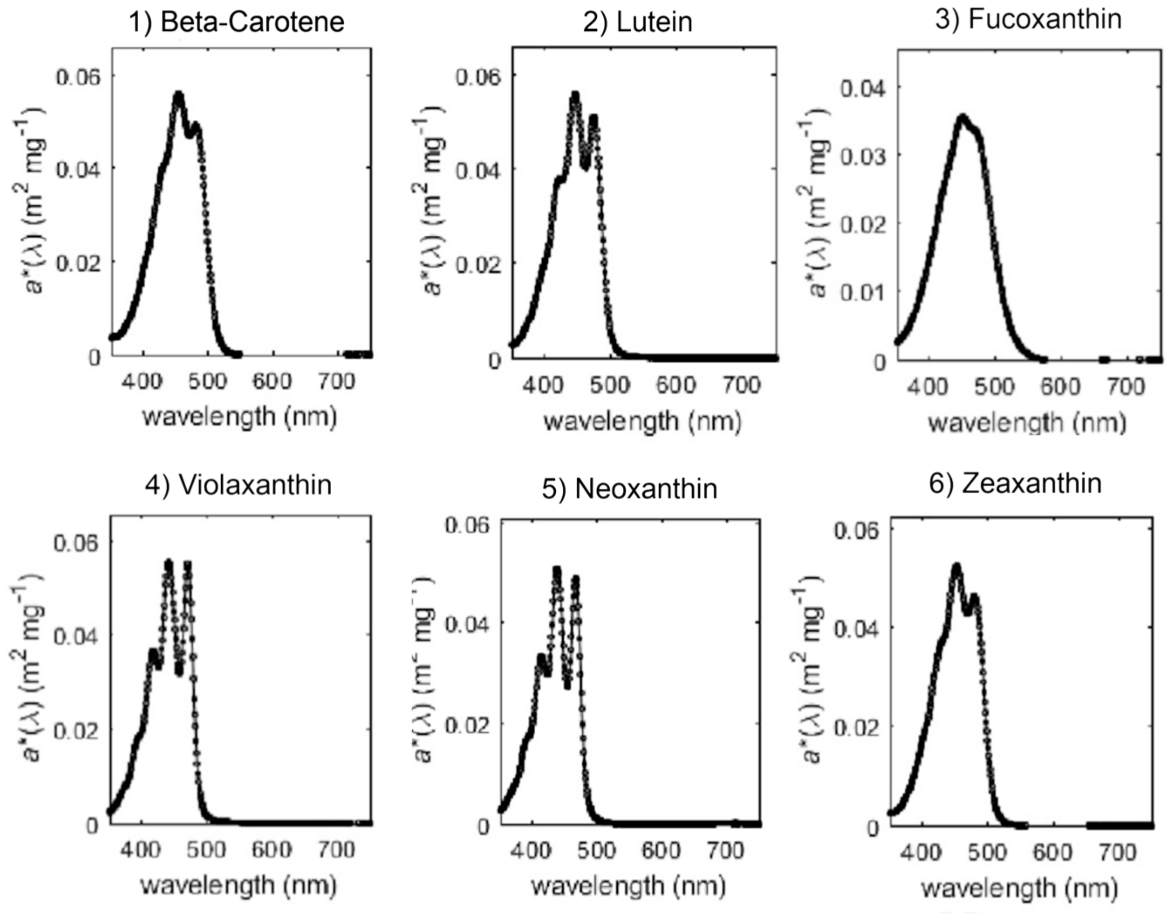

Figure 4 - Absorbance spectrum profile of 1) β-carotene, 2) lutein, 3) fucoxanthin, 4) violaxanthin, 5) neoxanthin, and 6) zeaxanthin as adapted from Clementson & Wojtasiewicz (2019).

As was mentioned carotenoids absorb in the blue and green spectrum. Similar to chlorophylls, carotenoids have their own spectrum shape and absorption maxima that aid in their identification. Carotenoid spectra have usually three shapes: one big round peak, a forked peak with a slight shoulder on the left side, or a three-forked peak

(Britton *et al.*, 2004; Clementson & Wojtasiewicz, 2019). Despite this, the similarity of some pigment spectra, including those of chlorophylls, can difficult the identification of certain pigments. However, the separation of pigments in a chromatographic column, for example through Ultra High-Performance Liquid Chromatography (UHPLC), with the help of the absorption maxima and spectrum shape it is possible to identify and quantify pigments with a great level of accuracy (Sanz *et al.*, 2015). Figure 4 presents a few examples of carotenoid spectra.

1.3.3. Pigment Extraction

To identify the pigments, present in biomass and further isolate them for any use it is first necessary to extract them from the cellular membranes. Different methods allow the extraction of pigments, some rely on increasing the membrane permeability, some create disruptions or pores in the membranes and others involve the breakage of the cell wall. There are three main extraction techniques and a few assistance methods that increase the extraction yield (Ngamwonglumlert *et al.*, 2017). The extraction can be performed by classical solvent extraction, supercritical fluid extraction (SFE) or enzymatic extraction. The enzymatic and SFE techniques involve high-cost infrastructures or materials, and the processes can be hard to perform as well as demand too much precision. Still, these methods are more efficient than solvent extraction (Pagels *et al.*, 2021). Nonetheless, the efficiency of solvents in the extraction of pigments can be improved as it relies heavily on the chemical and physical properties of the extraction. The characteristics of the biomass used, for example, pigment and solvent polarity, as well as the solvent ability to penetrate the cell wall are important factors to consider in solvent extraction (Hagerthey *et al.*, 2006). Solvent extraction is based on the higher chemical affinity of the pigments and the penetration of the cell wall by the solvent, inflating and rupturing the membranes and carrying the pigments out (Grima *et al.*, 2013).

The different extraction assistance methods that can be applied to solvent extraction can highly increase its efficiency. However, due to differences in biomass characteristics no single solvent extraction protocol is ideal for all the different organisms (Cartaxana & Brotas, 2003). There are several physical disruption methods such as freeze-thaw cycles and maceration as biomass pre-treatment, however the most popular methods for assisting classical solvent extraction are microwave-assisted, ultrasound-assisted, pressure-assisted, and electric pulse-assisted techniques (Pagels *et al.*, 2021; Salinas-Salazar *et al.*, 2019).

The heat and electromagnetic radiation of the microwave appear to increase the cell wall disruption by enlarging the pore size. However, it should be considered that some pigments are susceptible to thermal degradation and microwave-assisted treatment could prove not to be the best choice (Aparicio-Ruiz *et al.*, 2011; Cheng *et al.*, 2013; Latorre *et al.*, 2013). Ultrasonic-assisted extraction is based on the principles of the cavitation phenomenon caused by the pressure oscillation of waves in water by being exposed to certain frequencies, this results in the creation and destruction of several small bubbles producing instantaneous high temperatures and pressures that result in the disruption of the cell walls (Capelo *et al.*, 2005). Pressure-assisted techniques include high-pressure homogenization, which refers to passing the solvent-biomass suspension through small holes at high pressures creating high-shear forces that force the cell wall to break, as well as pressurized liquid extraction, where the solvent-biomass suspension is submitted to high temperatures and pressures destabilizing the cell wall and increasing the permeability (Lee *et al.*, 2017; Mandal *et al.*, 2015). The electric pulse-assisted method consists in passing high-intensity electric surges in the solvent suspension containing the biomass, causing small pores to open in the membranes facilitating the extraction of internal compounds such as pigments (Günerken *et al.*, 2015).

In the reviewed literature, methanol, acetone and ethanol appear to be the most used solvents in pigment extraction (Henriques *et al.*, 2007; Safafar *et al.*, 2015). There are a few parameters that should influence the selection of a solvent such as the solubility of the desired extractable in the solvent, its inefficiency in extracting unwanted compounds, and ideally the solvent creates a two-phase system with water to remove contaminants, as well as being volatile to facilitate its removal (Grima *et al.*, 2013). The solubility of pigments in solvents is especially relevant in terms of polarity. Some pigments can be very hydrophobic while others can be the opposite, usually chlorophylls are for the most part, either as hydrophilic as, or more hydrophilic than carotenoids, which have a more fluctuating polarity. Thus, Chls in general have higher extraction yields in methanol and the most hydrophobic carotenoids have higher extraction yields in acetone (Esquivel-Hernández *et al.*, 2017; Martins *et al.*, 2021;

Ruivo *et al.*, 2014). However, many times the theoretical expectations do not match the practical results, for example as reported by Safafar *et al.* (2015) methanol had better extraction yields for β-carotene even though their solubility is incompatible than acetone as a possible result of the extracted matrix.

Even though the mixture of chloroform and methanol shows to be the most effective solvent it is also highly toxic, pollutant and very dangerous to the environment (Halim *et al.*, 2012; Solovchenko *et al.*, 2001). Thus, the search for efficient greener alternatives is a desirable goal for more sustainable industry practices. The meaning of "green" solvent can be quite wide and difficult to categorize, spanning from readily biodegradable to recyclable, solvents of biological production (such as bio-alcohols), if it is environmentally safe or if it has low toxicity. Common solvents such as ethanol, methanol, isopropanol, isopropyl acetate and ethyl acetate are considered safe and their valorization in extraction techniques as a substitute for fossil-derived solvents would be very beneficial (Capello *et al.*, 2007; Doble & Kruthiventi, 2007).

By using greener solvents which have been found thus far to be less efficient extractants than the classical harmful solvents it is conceivable that they will do less harm to the biomass and other metabolites, allowing the extraction of other metabolites, such as fatty acids, which when extracted with chloroform-methanol, remain in the chloroform phase (Solovchenko *et al.*, 2001). Taking advantage of the same biomass for the sequential extraction of two separate metabolites would improve the economic viability of the processes and the value of the biomass (Pagels *et al.*, 2021).

1.3.4. **Pigments found in *C. sorokiniana***

A brief literature review of the main pigments found in *C. sorokiniana* along with extraction methods and solvents used as well as extraction yields were summarized in Table 2.

Table 2 - Literature review of main pigments present in C. sorokiniana, solvent used and extraction method, as well as respective extraction yield represented in $\mu g\ g^{-1}$ of biomass.

Method	**Solvent**	**Chlorophyll *a* ($\mu g\ g^{-1}$)**	**Chlorophyll *b* ($\mu g\ g^{-1}$)**	**Lutein ($\mu g\ g^{-1}$)**	**Beta-carotene ($\mu g\ g^{-1}$)**	**Author**
Solvent extraction cycles and saponification.	Methanol:Ethyl Acetate	___	___	2830.3	458.5	Diprat *et al.* (2020)
Sonication-assisted solvent extraction	Ethanol 96%	14780	7730	___	___	(Kuznetsova *et al.*, 2020)
Homogenization of solvent and biomass.	Methanol	___	___	3366.9	469.8	(Matsukawa *et al.*, 2000)
Sonication-assisted solvent extraction	Methanol	615.3	725	3220	1039	(Safafar *et al.*, 2015)[1]
Sonication-assisted solvent extraction	Methanol	1455	389.3	2069	614.4	(Safafar *et al.*, 2015)[2]
Solvent extraction cycles and saponification.	Methanol:Ethyl Acetate	___	___	908.48	198.48	(Fernandes *et al.*, 2020)
Solvent extraction cycles with maceration.	Methanol and Ethyl Acetate	12660	4810	___	___	(Morcelli *et al.*, 2021)

[1] – Grown under higher light intensity; [2] – Grown under lower light intensity;

Other carotenoids seem to be consistently present in trace quantities, such as alpha-carotene, zeaxanthin, violaxanthin, and neoxanthin however the main carotenoids consistently in high quantities are lutein and β-carotene (Diprat *et al.*, 2020; Fernandes *et al.*, 2020). The analytical methods used for the quantification of pigments in the references mentioned in Table 2 are always either HPLC or spectrophotometric absorbance at specific wavelengths, for example as described by Lichtenthaler (1987). The variation of values observable in Table 2 is likely to be due to the differences in *C. sorokiniana* strains and growth conditions, which can affect the amount of pigments produced (Safafar *et al.*, 2015; Ziganshina *et al.*, 2022).

1.4. Fatty Acids

Fatty acids (FA) can make up to 20% of microalgae dry weight, furthermore, depending on the species the FA composition may be very diversified. Microalgae are usually rich in FA with 16 and 18-long carbon chains (Yun *et al.*, 2020a). Having a source of diversified FAs would be ideal for any industry, as FA have various applications such as nutrition, both for humans and for animal feed, in particular for aquaculture feed. Other uses for FAs include the production of third-generation biofuels, not competing with other foods for agricultural land, cosmetics and pharmaceuticals (Maltsev & Maltseva, 2021).

The transesterification of FAs into fatty acid methyl esters (FAMEs) is essential in the production of biofuels. The FAs profile of microalgae is usually similar within each other as well as with other land plants (Graham *et al.*, 2012; Griffiths & Harrison, 2009). All of these are appropriate for the production of biofuels as they promote an acceptable balance to the viscosity, combustion speed, oxidative stability and the cold flow of fuels (Moser, 2014).

Furthermore, clinical trials have shown that a FA-rich diet can decrease the risk of atherosclerotic cardiovascular disease and in general improve cardiovascular health (Wu *et al.*, 2020). Other trials have also found a connection between Omega-3 rich diets, more specifically rich in docosahexaenoic acid (DHA), a natural ligand of the peroxisome proliferator-activated receptor gamma complex. This complex is activated by DHA and plays an anti-inflammatory role in the cardiovascular system also having been reported to inhibit cancer cell proliferation by apoptosis (Naeini *et al.*, 2020). The anti-inflammatory activity of fatty acids has also been reported to reduce the intensity of rheumatoid arthritis symptoms (Vadell *et al.*, 2020).

Fatty acids are usually amphipathic organic acids containing a non-polar long hydrocarbon chain and a polar head composed of a carboxylic group. In nature, FAs are produced usually with a stable even number of carbons in their chain for which the number of double bonds can affect its function. Usually, these molecules are derived from triglycerides and phospholipids (Chen & Liu, 2020; Wiktorowska-Owczarek *et al.*, 2015). In microalgae, FAs can be synthesized by the standard aerobic pathway, where the acetyl coenzyme A and malonyl coenzyme A, originated from the tricarboxylic acid cycle, are usually converted into basic saturated 16 and 18-carbon chain FAs, known as palmitic acid and stearic acid respectively, later in the metabolic pathway these can be derived into unsaturated FAs. On the other hand, anaerobic microalgae can also produce FAs in the absence of oxygen through the polyketide synthase pathway in a very similar fashion to the aerobic pathway (Sun *et al.*, 2018).

According to the number of double bonds in their carbon chain, FAs can be divided into three categories: saturated, monounsaturated and polyunsaturated. An alternative way to identify FAs other than the IUPAC or common name is their number of carbons and the number of double bonds. For example, linoleic acid, which has 18 carbons in its hydrocarbon chain and two double bonds can be identified as C18:2

(Wiktorowska-Owczarek *et al.*, 2015). The fatty acids referred to in this section are generally free fatty acids, that are not bound to any other molecule such as alcohols or proteins (Brondz, 2016). The characteristics and details of these types of FAs are further discussed in the next section.

1.4.1. Saturated, Monounsaturated and Polyunsaturated Fatty Acids

Saturated fatty acids are the only type of fatty acids that may not have a hydrocarbon chain, as in the case of formic (CH_2O_2) and acetic acid ($C_2H_4O_2$) and they do not have any double bonds in their hydrocarbon chain (Brondz, 2016). Even though necessary for a balanced diet, diets high in saturated fatty acids can lead to several cardiovascular, inflammation and weight health problems (Zhou *et al.*, 2020). In Table 3 is a short list of a few stable saturated FAs found in nature from four carbons long (C4) to twenty-four carbons long (C24).

Table 3 - Common name, systematic name and abbreviation for some of the most common saturated FAs (Haynes, 2016).

Common Name:	**Systematic name:**	**Abreviation:**
Butyric Acid	Butanoic Acid	C4:0
Caproic Acid	Hexanoic Acid	C6:0
Caprylic Acid	Octanoic Acid	C8:0
Capric Acid	Decanoic Acid	C10:0
Lauric Acid	Dodecanoic Acid	C12:0
Myristic Acid	Tetradecanoic Acid	C14:0
Palmitic Acid	Hexadecanoic Acid	C16:0
Stearic Acid	Octadecanoic Acid	C18:0
Arachidic Acid	Icosanoic Acid	C20:0
Behenic Acid	Docosanoic Acid	C22:0
Lignoceric Acid	Tetracosanoic Acid	C24:0

Monounsaturated fatty acids (MUFA) keep the same number of carbons and base structure of saturated FAs however they must have only one double bond in their hydrocarbon chain. There are many configurations that MUFAs with longer chains can acquire as the placement of the double bond in the hydrocarbon chain can affect the function and behavior of the molecule. For example, the petroselinic, oleic and vaccenic acids all have a similar structure composed of 18 carbons and one double bond, yet for each the double bond is found in a different carbon (carbons 6, 9, and

11, respectively) (Andersson & Holman, 1974; Kazaz *et al.*, 2022). Despite the established relationship between *trans* configurations of MUFAs and cardiovascular health disorders, a balanced diet rich in MUFAs, present in high amounts from plant-derived oils such as olive oil, has been associated with cardiovascular health improvement, reduced cholesterol levels, better regulated glycemic control in patients with diabetes, overall better weight control and improved immune systems (Gillingham *et al.*, 2011; Kris-Etherton, 1999). A few examples of the many existing MUFAs from 16 to 24 carbons long and some of their isomers can be found in Table 4, along with the double bond position.

Table 4 - Common name, systematic name, double bond (DB) position and abbreviation of a few monounsaturated FAs and their isomers (Kazaz et al., 2022).

Common Name:	Systematic name:	DB position:	Abreviation:
Sapienic Acid	(6Z)-Hexadecenoic Acid	10	C16:1
Palmitoleic Acid	(9Z)-Hexadecenoic Acid	7	C16:1
Petroselinic Acid	(6Z)-Octadecenoic Acid	12	C18:1
Oleic Acid	(9Z)-Octadecenoic Acid	9	C18:1
Vaccenic Acid	(11Z)-Octadecenoic Acid	7	C18:1
-	(5Z)-Eicosaenoic Acid	15	C20:1
Gondoic Acid	(11Z)-Eicosaenoic Acid	9	C20:1
Paullinic Acid	(13Z)-Eicosaenoic Acid	7	C20:1
-	(5Z)-Docosenoic Acid	17	C22:1
Euric Acid	(13Z)-Docosenoic Acid	9	C22:1
Nervoic Acid	(5Z)-Tetracosenoic Acid	9	C24:1

The last type of FAs are the polyunsaturated fatty acids (PUFAs), which similarly to MUFAs, maintain the base structures of saturated FAs with the addition of having more than one double bond in their hydrocarbon chains. Very much like MUFAs, they can have many isomers as the location of the double bonds in their structure may vary. These are regarded as the most beneficial and coveted fatty acids, being also the most valuable and since they are not produced by the human body they must be acquired by ingestion of foods containing them (Ferreira *et al.*, 2020). The omega-3 (ω-3) and omega-6 (ω-6) fatty acids, which are two classes of PUFAs, are famously recognized for their healthy properties mainly for the cardiovascular system but also for the nervous system and for having anti-inflammatory properties (Wiktorowska-Owczarek *et al.*, 2015). The ω-3 and ω-6 classes are identified by the position of the first double bond in relation to the outer end of the hydrocarbon tail, for example linoleic acid (C18:2) which has a double bond in carbon 9 and another in 12 is an ω-6 PUFA as the double bond in carbon 12 is 6 carbons away from carbon 18 (Rustan & Drevon, 2005). Table 5 lists some high interest PUFAs commonly reported, along with the double bond positions in their hydrocarbon chain.

Table 5 - Common name, systematic name, omega (ω) classification, double bond (DB) position and abbreviation of some polyunsaturated FAs and their isomers.

Common Name:	**Systematic name:**	**ω**	**DB position:**	**Abrev.:**	**Reference:**
Linoleic Acid	(97,12Z)-octadeca-9,12-dienoic acid	6	9, 12	C18:2	(Rustan & Drevon, 2005)
a-Linolenic Acid	(9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid	3	9, 12, 15	C18:3	(Cholewski *et al.*, 2018)
y-Linolenic Acid	(6Z,9Z,12Z)-octadeca-6,9,12-trienoic acid	6	5, 9, 12	C18:3	(Gocen *et al.*, 2018)
Eicosatetraenoic Acid (ETA)	(8Z,11Z,14Z,17Z)-icosa-8,11,14,17-tetraenoic acid	3	8, 11, 14, 17	C20:4	(Cholewski *et al.*, 2018)
Arachidonic Acid	(5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraenoic acid	6	5, 8, 11, 14	C20:4	(Gocen *et al.*, 2018)
Eicosapentaenoic Acid (EPA)	(5Z,8Z,11Z,14Z,17Z)-icosa-5,8,11,14,17-pentaeonoic acid	3	5, 8, 11, 14, 17	C20:5	(Cholewski *et al.*, 2018)
Adrenic Acid	(7Z,10Z,13Z,16Z)-docosa-7,10,13,16-tetraenoic acid	6	7, 10, 13, 16	C22:4	(Gocen *et al.*, 2018)
Docosapentaenoic Acid n-3 (DPA)	(7Z,10Z,13Z,16Z,19Z)-docosa-7,10,13,16,19-pentaenoic acid	3	7, 10, 13, 16, 19	C22:5	(Cholewski *et al.*, 2018)
Docosapentaenoic Acid n-6 (DPA)	(4Z,7Z,10Z,13Z,16Z)-docosa-4,7,10,13,16-pentaenoic acid	6	4, 7, 10, 13, 16	C22:5	(Kumon *et al.*, 2003)
Docosahexaenoic Acid (DHA)	(4Z,7Z,10Z,13Z,16Z,19Z)-docosa-4,7,10,13,16,19-hexaenoic acid	3	4, 7, 10, 13, 16, 19	C22:6	(Cholewski *et al.*, 2018)

As is succinctly represented in Figure 5, mono and polyunsaturated fatty acids are derived from their saturated main structure by *desaturase* enzymes in very complex metabolic mechanisms which vary in different organisms (Muñoz *et al.*, 2021). Different types of FAs can be successively derived from each other by enzymes such as *desaturases* and *elongases*, however a class of PUFAs such as ω-3 cannot be derived into different classes, for example into ω-6 PUFAs (Rustan & Drevon, 2005).

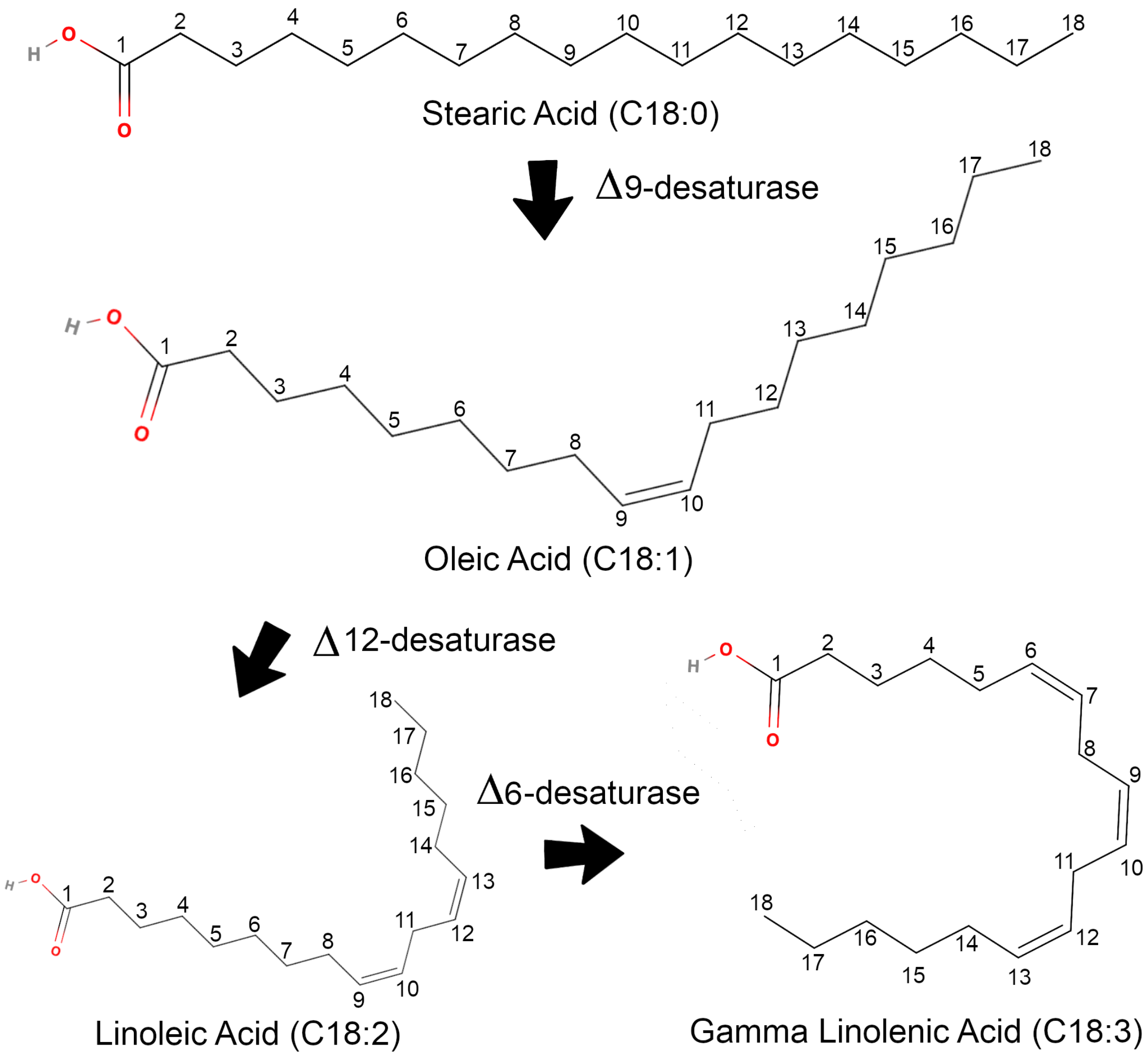
Stearic Acid (C18:0)
Δ9-desaturase
Oleic Acid (C18:1)
Δ12-desaturase
Linoleic Acid (C18:2)
Δ6-desaturase
Gamma Linolenic Acid (C18:3)

Figure 5 - General derivation mechanism of stearic acid into gamma-linolenic acid in microalgae (Muñoz et al., 2021; Rustan & Drevon, 2005).

1.4.2. Fatty Acid Extraction

Similar to pigment extraction, FA extraction is based on techniques that allow the penetration of cell walls and permeation through complex matrices. One of the first techniques used for the extraction of lipids was the Soxhlet method, developed in the second half of the 19th century. It is based on solvent extraction, boiling the sample in solvent and recovering the extractant by condensation. However, the apolar nature of FAs is important when choosing the extraction solvent or solvents (Zygler *et al.*, 2012). Later came two very similar methods considered to be the *classics*: Bligh & Dyer

(1959) and Folch *et al.* (1957). Both are very efficient and significantly faster than the Soxhlet extraction, based on the homogenization of a sample in a mixture of chloroform-methanol.

Over time, the most common extraction methods were modifications of these two methods, varying solvents or mixtures, solvent ratios and extraction conditions to better suit the type of sample extracted, in particular, the use of assisting techniques such as microwave and sonication or including the use of cell disrupting pretreatment techniques (Akondi *et al.*, 2017; Billakanti *et al.*, 2013; Gorgich *et al.*, 2020). However, in the last decades supercritical fluid extraction has been introduced as a more environmentally friendly and efficient extraction method for FAs, although its drawbacks are the same as for pigment extraction, such as high-cost infrastructures (Lee *et al.*, 2021; Li *et al.*, 2014). Under the right conditions and with the proper assisting method, solvent extraction can achieve higher extraction results in less time than supercritical fluid, or Soxhlet extractions (Reboleira *et al.*, 2022). The concepts of wall breakage or perforation in FA extraction assisting methods, as well as the methods themselves, are the same as the ones for pigment extraction, described in section 1.3.3. (Gorgich *et al.*, 2020).

It is important to note that one of the most common techniques for the identification and quantification of FAs is by their transesterification into FAMEs and analysis through gas-chromatography (GC), either with a flame ionization detector (FID) or more recently using a mass spectrometry detector (GC-MS), the latter being considered more reliable (Nkwonta *et al.*, 2016). As previously mentioned, FAMEs are also essential for the production of biofuels and as such the derivatization of FAs is an important step that can be performed by reaction with diazomethane, a reaction catalyzed by a base or, the most common option, an acid-catalyzed reaction which is usually performed after extraction (Christie, 1984). However, there are also methods for direct transesterification of FAs in the biomass, such as the method developed by Lepage & Roy (1986), which itself allows direct derivatization and extraction of the methyl esters, which is still used as described or with minor modifications to increase efficiency (Arif *et al.*, 2021).

1.4.3. Fatty Acid Profile of *C. sorokiniana*

This section focuses on reviewing the main fatty acids found in *C. sorokiniana*. Table 6 shows the concentrations of the main FAs found and the extraction methods used in recent literature.

Table 6 - Percentage values for total lipids (w/w) and main fatty acids (% of total lipids) of C. sorokiniana with different extraction methods in the reviewed literature.

Method	**Total Lipids (%)**	**C16:0 (%)**	**C16:1 (%)**	**C18:0 (%)**	**C18:1 (%)**	**C18:2 (%)**	**C18:3 (%)**	**Author**
Bligh & Dyer extraction	29.3	37.2	3.0	5.1	3.1	21.5	25.9	(Diprat *et al.*, 2020)
Suspended in Methanol-Chloroform (1:1)	23.28	43.25	3.48	2.64	5.13	17.87	13.42	(Yun *et al.*, 2020b)^1
Suspended in Methanol-Chloroform (1:1)	23.12	40.98	3.75	2.55	5.09	18.02	14.3	(Yun *et al.*, 2020b)^2
Suspended in n-Hexane:Isopropanol (2:1)	23.1	14.54	13.58	3.99	31.96	15.99	5.01	(Papapolymerou *et al.*, 2022)
Bligh & Dyer extraction	19.78	20.93	2.73	4.87	18.05	32.41	8.84	(Eladel *et al.*, 2019)
Sohxlet with Ethanol-n-Hexane (1:9)	13.5-20	17.80	0.8	1.72	18.35	18.12	26.06	(Bazarnova *et al.*, 2019)

1 - *C. sorokiniana* KNUA114; 2 - *C. sorokiniana* KNUA122

Values can fluctuate along with different culture and treatment conditions however 16 and 18 carbon FAs appear to be consistently the main fatty acids produced by *C. sorokiniana*, especially palmitic (C16:0) and linoleic (C18:2) acid (Papapolymerou *et al.*, 2022; Qiu *et al.*, 2017). Similarly, to the literature review of pigments for *C. sorokiniana*, smaller amounts of other fatty acids are found varying both quantities and FAs reported. The fatty acid profile of microalgae can be influenced by several factors including culture temperature (Yun *et al.*, 2020b).

1.5. Industry and Biorefinery

For any industry, one of the main steps and also the most expensive is the research and development (R&D) of techniques or products. The money invested in this step can help increase the quality of a product, the production speed, the amount of materials spent, and minimize the cost of materials used, having long-term return benefits. Innovation of current industrial processes can help increase the sustainability of a process by including cheaper and more ecological materials or increasing the efficiency of the process to reduce waste (Straathof *et al.*, 2019). Most of the R&D and innovation of biotechnological and pharmaceutical industries is achieved by collaboration with universities. Although there is debate that these collaboration techniques have limits and could withstand improvement, it has been proven to benefit the industrial process (Lange & Wagner, 2021; Melnychuk *et al.*, 2021).

Biotechnology is a field with a broad span of sectors from agriculture and food to pharmaceuticals. Over the years biotechnology has been at the center of the creation and improvement of industrial processes, for example, the fermentation of beverages and the use of enzymes in textile and paper industries. Genetic engineering also played an important role in the food and pharmaceutical industries, producing more nutritive fruits and vegetables from more resistant plants or allowing the production of pharmaceutical compounds using bacteria or yeasts instead of living animals (Lokko *et al.*, 2018).

Theoretical discoveries and laboratorial experiments are vital as the base of almost all industries, although transitioning a recently discovered laboratory scale technique to a production facility is not easy and involves a few R&D steps before being feasible. The first step is to perfect the laboratory protocol, however, small-scale tools, equipment and material quality are either impossible to use at larger scales or are too expensive to be sustainable. Thus, the laboratory protocol must be adapted to a larger scale, maintaining the conditions as close as possible to the original protocol, and defining the design of the whole process. After the theoretical scale-up has been designed and calculated each operation of the process must be tested at larger scale conditions, usually through a pilot scale production facility. A pilot scale operation is one of the best options to predict process factors such as production costs, sustainability, input and output data, waste generated and environmental impact (Niazi & Brown, 2016; Piccinno *et al.*, 2016).

The assessment of all these factors before the establishment of any production units is very important for factories to be profitable. For example, as previously stated, microalgae contain different high-value compounds and a worldwide rising interest in alternative and more sustainable resources fueling programs and strategies for their integration (Hasport *et al.*, 2022). Nonetheless reports of the investment and operation costs of functioning productions with microalgae as the main resource can achieve extremely elevated values which are sometimes profitable and others not at all. As an example, a case study estimated by Özçimen *et al.* (2018) shows the possibility of revenue capable of covering the initial investment and the operation costs of the first year in only half a year for the production of β-carotene from microalgae. On the other hand the yearly revenue of the production of biodiesel would not cover 10% of its own operational cost. This is the reason why the discovery of new methods, their improvement or the reuse of resources in a circular economy is so important, as they can help reduce the cost of production and increase revenue. Microalgae used for the treatment of wastewaters could then be used for the extraction of valuable compounds and the resulting waste paste could be reapplied for other purposes (Bhatt *et al.*, 2022; Özçimen *et al.*, 2018).

1.6. Objectives

Recent world events such as the global pandemic and the situation presently taking place in the Ukraine, as well as the repercussions caused by these events, unfortunately, caused a rupture in the stock of materials necessary for the work proposed at the beginning of the thesis. Deliveries took more time than estimated causing delays in the practical assignments of this Master thesis plan, therefore a different approach had to be taken with a few alterations to the original plan.

The goal of the present study is to increase the valorization of microalgal biomass in a biorefinery perspective, using the microalga *Chlorella sorokiniana* as a model for green coccoid microalgae for the production of high value pigments and fatty acids. For this, the environmental sustainability of the process is also a significant factor, therefore, the extractability of pigments by different *green* or less pollutant solvents were tested, as well as a two-step sequential extractability of both pigments and fatty acids. The data collected from the extraction and sequential extraction of the high value products was used for a preliminary assessment of the economic and environmental sustainability of an industrial scaled process.

In summary, the steps taken to achieve these goals were:

- Characterization of pigments present in *C. sorokiniana*;
- To compare the extractability of different solvents, including green solvents;
- Characterization of the fatty acid composition of *C. sorokiniana*;
- Analysis of the sequential extractability of fatty acids from biomass previously used for the extraction of pigments;
- Preliminary economic sustainability assessment of the process using the software SuperPro Designer;

2. Material and Methods

2.1. Cell Culture and Maintenance

The non-axenic microalga *Chlorella sorokiniana* SAG 211-32 was supplied from the Institute of Plant Biochemistry and Photosynthesis (IBVF) (Seville, Spain). Culture conditions were based on those described by (Rodrigues *et al.*, 2020). The microalgae were grown in Tris-acetate-phosphate (TAP) medium in photomixotrophic conditions. The culture temperature was maintained at 20 ºC under continuous white light irradiation and aeration. Final culture volumes of 3 or 5 L were sequentially scaled-up from an initial inoculum of 50 mL. Once the culture reached its highest production phase (stationary growth), the culture was centrifuged and the biomass collected at 2164 g for 10 minutes (Hettich Zentrifugen Universal 320, Germany) and stored at 4 ºC for immediate use or stored at -20 ºC for longer periods. For fresh and freeze-dried biomass comparison in assays a portion of the biomass was lyophilized (Labconco FreeZone Plus 6, USA).

2.2. Pigment Extraction

The following subsections detail the extraction methods used for pigment extraction, identification and quantification from the non-axenic microalga *C. sorokiniana*.

2.2.1. Extraction Conditions

Pigment extraction was performed under dim lighting to avoid the oxidation of pigments (Schüler *et al.*, 2020). Both fresh and freeze-dried biomass were used for the extraction of pigments, other than this extraction conditions were maintained for all samples to evaluate the efficiency of different solvents. Conditions were based on the methods used by Schüler *et al.* (2020) and Wiltshire *et al.* (2000) and perfected upon trial. Extraction was carried out in quadruplicates for each solvent and biomass conditions, by suspending 2 mg of freeze-dried biomass in 3 mL of solvent. The dry weight of fresh biomass allowed to determine the water content to maintain the same biomass-solvent ratio in the extraction. The mixtures were vortexed for 30 s to maximize de-agglomeration of the biomass and placed in a sonic bath at 50/60 Hz for a period of 60 minutes (J.P. Selecta Ultrasons-H, Spain). The solvent was recovered by centrifugal force at 2795 g for 5 minutes (Hettich Zentrifugen Universal 320, Germany). The remaining biomass pellet was resuspended in fresh solvent and subjected to a second cycle of extraction. All extractant solvents except for methanol were evaporated by vacuum in a desiccator covered from light to avoid oxidation of pigments and resuspended in methanol.

The different solvents used for pigment extraction were: methanol, acetone, 96% ethanol, ethyl acetate, chloroform-methanol (2:1), isopropanol, isopropyl acetate, methyl acetate, ethyl ether.

2.2.2. Analytical Conditions

The resulting extracts were analyzed by spectrophotometry (Cintra 202, GBC Scientific Equipment Ltd., Australia) using methanol as blank. The absorbance was read at 470, 652 and 665 nm and the resulting absorbances were applied to the following formulas to calculate total chlorophyll *a* (Chla), chlorophyll *b* (Chlb) and total carotenoids (Carot) in milligrams per liter (mg/L) as described by (Lichtenthaler, 1987):

$$C_{Chla} = 16.72 \cdot A_{665} - 9.16 \cdot A_{652} \quad (1)$$

$$C_{Chlb} = 36.92 \cdot A_{652} - 15.28 \cdot A_{665} \quad (2)$$

$$C_{Carot} = \frac{1.44 \cdot A_{470} - 1.63 \cdot C_{Chla} - 104.96 \cdot C_{Chlb}}{221} \quad (3)$$

For the chromatographic analysis, the samples were filtered through a 0.2 μm PTFE syringe filter into amber glass vials and analyzed using a Thermo Fisher Dionex Ultimate 3000 UHPLC coupled with a diode array detector (DAD) (190-800 nm) (Thermo Fisher Scientific, USA), with a reverse-phase ACE Excel 3 C18-PFP (4.6 mm ID x 150 mm) column (Avantor, USA) and a Phenomenex SecurityGuard C18 pre-column (Phenomenex Inc., USA).

The mobile phase was composed of a two-solution gradient as described by Sanz *et al.* (2015). Solution A was composed of 225 mM ammonium acetate in Milli-Q and 100% HPLC grade methanol in a ratio of 18:82 and Solution B was composed of >99.7% HPLC Grade Ethanol pumped at 1 mL/min. Gradient ratios are described in Table 7.

Table 7 - Solvent gradient ratios for UHPLC method.

Time (min)	**Solution A %**	**Solution B %**
0	100	0
20	61.8	38.2
22	25	75
33	20	80
36	10	90
37	0	100
42	0	100

Pigment characterization was performed by retention time and order, cross-referenced with peak absorbance shape and maxims from a DHI standard mix (DHI, Denmark) and those described by Britton *et al.* (2004) and Clementson & Wojtasiewicz (2019). Quantification was performed using prepared standard curves with a sequence of known concentrations ranging from 0.1 to 5 mg/L of chlorophyll *a* (Sigma-Aldrich, USA), chlorophyll *b* (Sigma-Aldrich, USA), β-carotene (TCI, Japan) and lutein (Fisher Scientific, USA). Peaks with calculated areas inferior to 1% of the area of the highest peak were discarded from analysis as well as peaks that only appeared in less than 75% of quadruplicates.

These pigments were quantified by the respective areas in the chromatograms of the quadruplicates for each solvent used as well as for both fresh and freeze-dried biomass. The mean areas from the quadruplicates obtained were used to calculate the concentration of the pigments through the equations resulting from the calibration curves. These equations are represented below as the calibration equation for lutein (4), chlorophyll *a* (5), chlorophyll *b* (6), and β-carotene (7) as well as their respective determination coefficients (R^2).

$$Area\ (mAU * \min) = 3.6027\ C_{lutein}(mg * L^{-1}) - 0{,}615 \quad (4)$$
$$R^2 = 0.9912$$

$$Area\ (mAU * \min) = 2.3219\ C_{chl\ a}(mg * L^{-1}) - 0{,}0799 \quad (5)$$
$$R^2 = 0.9988$$

$$Area\ (mAU * \min) = 0.7484\ C_{chl\ b}(mg * L^{-1}) - 0{,}016 \quad (6)$$
$$R^2 = 1$$

$$Area\ (mAU * \min) = 1.965\ C_{beta-carotene}(mg * L^{-1}) - 0.453 \quad (7)$$
$$R^2 = 0.9961$$

Applying the corresponding peak areas to the equations above resulted in the determination of pigment concentration in milligrams per liter (mg/L). Multiplying the value obtained by the sample volume and dividing by the biomass extracted produced the yield of pigments extracted in milligrams per grams of biomass.

2.3. Fatty Acid Extraction

The next subsections describe the protocols used for the extraction of fatty acids from the biomass of *C. sorokiniana*, derivatization into fatty acid methyl esters (FAMEs), for characterization of the fatty acid profile and evaluation of the sequential extractability of fatty acids after one extraction cycle of pigments. During the practical procedures of this assay direct light exposure of the samples was avoided in order to reduce the risk of degradation of both pigments and fatty acids by oxidation (Cascant *et al.*, 2017; Schüler *et al.*, 2020).

2.3.1. Pigment Pre-Extraction

A pre-extraction of pigments was performed before with the same method used as in the previous assay with minor alterations. Both fresh and freeze-dried biomass were used for the extraction of pigments in the same conditions using the five solvents with the best extraction yields in the previous assay. For this extraction 0.1 g of freeze-dried biomass and its equivalent of fresh biomass, were suspended in 5 mL of solvent and vortexed for 30 s. Quadruplicates were made for each solvent and type of biomass. The samples were sonicated in a sonic bath at 50/60 Hz for a period of 60 minutes (J.P. Selecta Ultrasons-H, Spain). The tubes were centrifuged at 2795 g for 5 minutes (Hettich Zentrifugen Universal 320, Germany) and the solvents were removed, evaporated under vacuum in a desiccator and resuspended in 1 mL of methanol, except for those already in methanol. Samples were filtered and analysis of pigments was performed as originally described in the pigment analytical conditions subsection.

The biomass pellet resulting from the extraction was also dried under vacuum in a desiccator after which it was further dried in an oven for 30 minutes at 50 ºC to remove any solvent residue.

2.3.2. Total Lipid Extraction Conditions

The extraction of total lipids was performed according to Bligh & Dyer (1959) with minor alterations. The freeze-dried and fresh biomass samples after pigment extraction, as well as quadrupled fresh and freeze-dried biomass samples with no pigment pre-extraction were suspended in 3 mL of chloroform-methanol (1:2 v/v) and vortexed for 1 minute. After, 1 mL of chloroform was added and vortexed once more for 1 minute, adding 1 mL of a 0.88% potassium chloride aqueous solution afterwards to wash impurities (Folch *et al.*, 1957). The tubes containing the solutions were shaken and centrifuged at 2795 g for 10 minutes (Hettich Zentrifugen Universal 320, Germany). Using a glass Pasteur pipette, the chloroform layer was carefully removed and transferred to Eppendorf tubes, if necessary leaving small fractions of chloroform to avoid transfer of the other layers. The Eppendorf tubes were then centrifuged at 7826 g for 5 mins (Eppendorf Centrifuge 5415D, Germany) to further separate any possible unwanted solvent present, collecting 1 mL of chloroform phase and transferring it through a 0.2 µm polytetrafluoroethylene (PTFE) filter into weighted glass tubes. The chloroform was evaporated overnight in a dry bath at 50 ºC, after which the tube containing the extracted lipids was placed in an oven at 100ºC for 10 minutes to further evaporate residual chloroform.

The tubes were cooled at room temperature under vacuum in a desiccator, weighted to calculate the amount of total lipids and subjected to acid-catalyzed transesterification for Gas Chromatography analysis.

2.3.3. Acid-Catalyzed Transesterification

The derivatization of lipids into fatty acid methyl esters was performed according to Christie (1984) with a few alterations. After weighting the total lipids, the samples were resuspended in 1 mL of toluene, then added 2 mL of a 1% H_2SO_4 methanol solution in stoppered tubes. The tubes were heated at 70 ºC for 2 h in a dry bath, after which the tubes were cooled at room temperature in a desiccator and 5 mL of a 5% NaCl aqueous solution was added to wash impurities. The FAMEs were extracted with 5 mL of hexane, separating the layers with a glass Pasteur pipette without sacrificing any of the hexane layer. The hexane layer was evaporated under vacuum in a desiccator and resuspended in dichloromethane.

2.3.4. Analytical Conditions

Analysis of FAME composition was performed based on the methods described by Ray & Gangopadhyay (2021) with a few adaptations for better peak separation and enhanced resolution. The samples were analyzed using a Thermo Scientific 1300 Gas Chromatograph (Thermo Fisher Scientific, USA) equipped with a flame ionization detector (FID) set to 230 ºC and a TraceGOLD TG-WaxMS (30m, 0.53mm I.D., 1µm film) column (Thermo Fisher Scientific, USA). The injector temperature was set at 230 ºC. The samples were injected in splitless mode and carried by helium gas with a flow of 3 mL/min. Column temperature began at 100 ºC, held for 1 minute and increased at a rate of 5 ºC/min until 230 ºC. The temperature was held for another 20 minutes for a total run time of 47 minutes.

The fatty acids were quantified and identified using a standard curve of Supelco 37 FAME mix (Sigma-Aldrich, USA), prepared in a series of dilutions in triplicate, retention times were used as the only means of identification for the fatty acids.

2.4. Economic and Sustainability Assessment

An industrial-scale production from the sequential extraction of pigments and fatty acids from the biomass of *Chlorella sorokiniana* was simulated using the software SuperPro Designer (Intelligen, Inc., version 12.0), taking into account the results obtained from the practical assays described in this work. This simulation was made with the intention of performing a preliminary assessment of a large-scale process using ethyl acetate, considered a green solvent, which the results of the practical work demonstrated to have a balanced extraction of both metabolites. The ratios between biomass and the solvents were based on the same ratios used in the practical work performed. Initial infrastructure and additional costs were estimated using the built-in economic model of the software SuperPro Designer, as was the cost of solvents. Biomass cost was estimated as raw vegetable biomass material. Value of final products were studied and discussed.

Industrial scale operating equipment was chosen considering the fidelity to the practical conditions performed. The flow sheet for the operating module is represented in Figure 6.

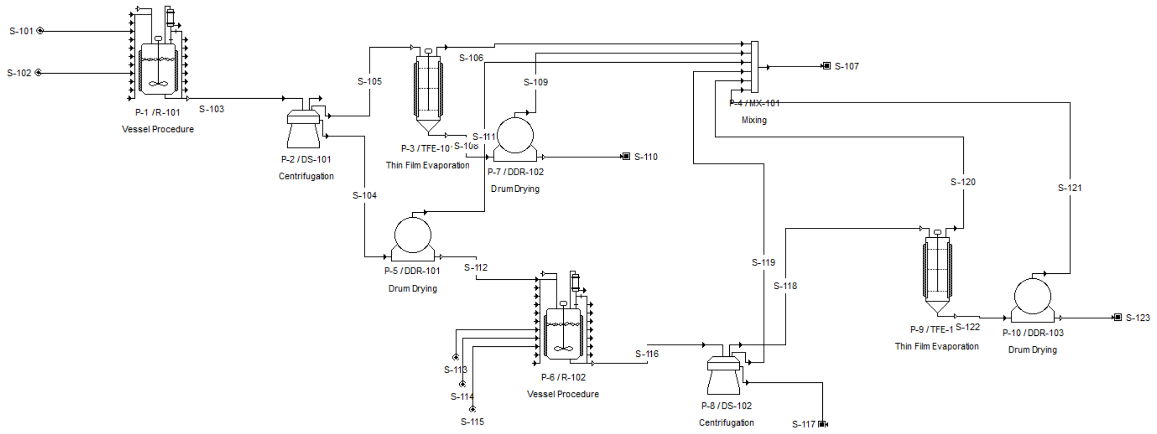
S-101
S-102
P-1 /R-101
Vessel Procedure
S-103
P-2 / DS-101
Centrifugation
S-105
S-104
P-3 / TFE-10
S-108
Thin Film Evaporation
S-106
S-109
S-111
P-7 / DDR-102
Drum Drying
S-110
P-4 / MX-101
Mixing
S-107
P-5 / DDR-101
Drum Drying
S-112
S-113
S-114
S-115
P-6 / R-102
Vessel Procedure
S-116
P-8 / DS-102
Centrifugation
S-117
S-119
S-118
S-120
S-121
P-9 / TFE-1
S-122
Thin Film Evaporation
P-10 / DDR-103
Drum Drying
S-123

Figure 6 - Representation of the industrial scale module schematic for the sequential extraction of both pigments (stream S-110) and fatty acids (stream S-123) from the microalga Chlorella sorokiniana, recovering both biomass and used solvent (streams S-117 and S-107, respectively) for commercialization to reduce production cost.

The extraction of pigments occurs in reactor R-101 fitted with an ultrasonic probe which is loaded with biomass and solvent, mixing and sonicating. The solvent and biomass are separated by centrifuge. The solvent is evaporated and recovered for reutilization. The biomass is further dried to remove residual solvent which is also recovered. The biomass is then loaded to reactor R-102 which gradually adds the chloroform, methanol and water in the proportions indicated in the fatty acid extraction protocol while mixing the solvent and biomass to improve the extraction of FAs. The resulting output is centrifuged and separated into three phases. Biomass, the solid phase, is recovered for commercialization, similarly to the alcoholic phase, methanol. The organic phase, containing the FAs, is evaporated and the resulting FAs are further dried to remove residual solvent. Fatty acids and pigments are collected at the end of streams S-123 and S-110, respectively and marketed for a profit.

2.5. Data Analysis

For each condition the procedure was performed in quadruplicate calculating the mean and standard deviation (SD) for each. The results were analyzed with GraphPad Prism (version 9.4.1, GraphPad Software, USA). Shapiro-Wilk test was used for data normality test and Two-Way Analysis of Variance (ANOVA) with a Tukey Honest Significant Differences or Holm-Šídák *post-hoc* tests and a 95% confidence interval (α=0.05) was used for the calculation of significance of mean variation (SMV).

3. Results and Discussion

In this section, the results obtained from the experimental procedures described in the materials and methods section will be presented, interpreted and discussed. Reviewed literature will be used as an aid for understanding the results and making inferences on their nature.

3.1. Pigment Extraction

The methods used for the extraction of pigments were designed to create direct comparisons of the extractability of the solvents used and the conditions of biomass used, that is fresh and freeze-dried biomass. The former can be directly used without the cost of freeze-drying, while the latter can be stored for longer periods (Papalia *et al.*, 2019). Another condition tested was the extractability of pigments in a second extraction cycle, which can help assess the viability of reusing the same biomass for a second extraction. The use of two quantification methods for pigments, the use of spectrophotometer with the wavelengths and concentration determination formulas perfected by Lichtenthaler (1987) as well as the identification and quantification of pigments using UHPLC allowed the comparison of both quantification methods.

3.1.1. Pigment Characterization

As it was previously stated in the introduction to pigment extraction, in the reviewed literature, it was found more than once that a mixture of chloroform and methanol is one of the most efficient solvents for the extraction of pigments (Molino *et al.*, 2018; Solovchenko *et al.*, 2001; Wood, 1985). Initially, the extraction results obtained from the chloroform-methanol (2:1, v/v) solvent were going to be used for the characterization of the biomass. However, as the polarity of pigments varies, observed extractability also varied according to the polarity of the used solvent, these results further discussed in the next section, verifying what was stated by Hagerthey *et al.*

(2006). Thus, for a better characterization of the non-axenic *Chlorella sorokiniana* pigments the results from more than one extraction had to be used.

In Figure 7 a chromatogram from one of the quadruplicates from the chloroform-methanol (2:1, v/v) extraction from fresh biomass is represented along with the names of identified chlorophylls and carotenoids.

As can be seen in Figure 7, chloroform-methanol (2:1, v/v) was able to extract from *C. sorokiniana* pigments such as lutein, chlorophyll *b* and chlorophyll *b* derivates, chlorophyll *a* derivates, neoxanthin, pheophorbide *a*, pheophytin *a*, and their derivates, along with a few other unknown pigments. The spectra of the unknown pigments detected in all the extractions from *C. sorokiniana*, suggest these are derivates of chlorophylls showing some similarity, although not resembling any particular chlorophyll spectra found in the standards or literature. Lutein, peak nr. 8, is the highest pigment present with an area representing 47.8% of the total.

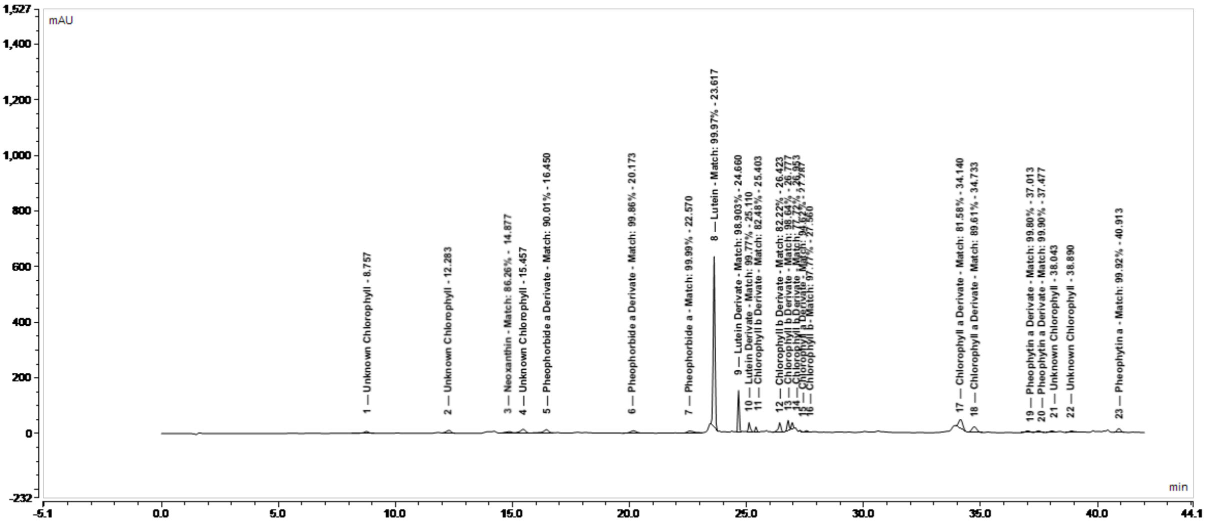

Figure 7 - Chromatogram from the pigment analysis of a fresh biomass sample extracted with chloroform-methanol (2:1, v/v) including pigment name, spectrum match factor relative to standard and retention time. The chromatogram is represented in signal intensity (mAU) over time (min). Pigments identified include: neoxanthin (peak 3), pheophorbide a (peak 7) and derivates (peak 5 and 6), lutein (peak 8) and derivates (peak 9 and 10), chlorophyll a *derivates (peak 15, 17 and 18), chlorophyll* b *(peak 16) and derivates (peak 11, 12, 13, and 14), pheophytin a (peak 23) and derivates (peak 17 and 18).*

In the extraction analyzed in Figure 7, lutein has two derivates (peaks 8 and 9), however, in other extractions, in particular with other solvents, there can be more derivates. The peaks identified as derivates of Chl *b* (peaks 11-14 and 16) show greater areas than Chl *b* and despite the absence of Chl *a*, there are three peaks identified as derivates of this pigment (peaks 15, 17, and 18). These peaks identified as derivates were either identified from the pigment standards or by a very high match factor with the main pigment spectra and proximity to the main pigment retention time. The pigment derivates could be divided in two types. As the derivates of carotenoids, have been catalogued and are found in nature as individual molecules characteristic of unique species (Britton *et al.*, 2004). Chlorophylls, on the other hand, can be more challenging to recognize. There are variations occurring in the structure of the tetrapyrrole caused by the natural pathways of different organisms, as mentioned in the introduction to chlorophylls, and there can also be allomerization of chlorophylls.

Carotenoids have been found to protect chlorophylls against allomerization, however once extracted they are more exposed to the conditions that enable it, for example, methanol has been found to increase the rate of allomerization (Hynninen, 1981).

These allomerizations have been reported by Hynninen (1981) to cause small deformations and deviations in the main pigment spectra. The derivatives of lutein, Chl *a*, and Chl *b* were quantified using the same standard curve as for the main pigment and when referring to the quantification of one of these pigments their derivates are included.

Another example is the extraction with ethyl acetate, for which the chromatogram is in Figure 8. Although not producing such high peaks did present peaks for β-carotene (peak 23) and the main peak of Chl *a* (peak 21).

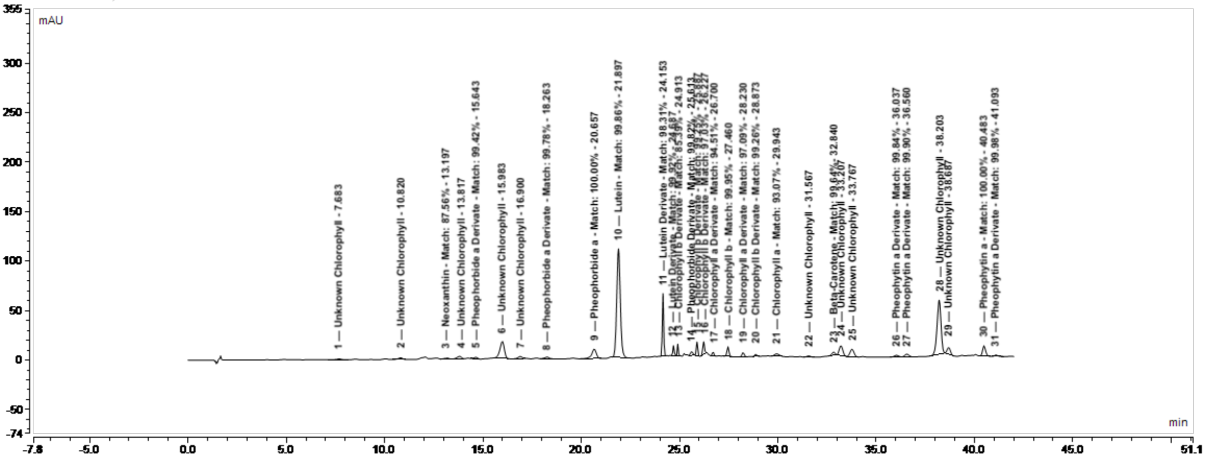

Figure 8 – Chromatogram obtained from the pigment analysis of a fresh biomass sample extracted with ethyl acetate including pigment name, spectrum match factor relative to standard and retention time. The chromatogram is represented in signal intensity (mAU) over time (min). Pigments identified include: neoxanthin (peak 3), pheophorbide a (peak 5) and derivates (peak 9 and 14), lutein (peak 10) and derivates (peaks 11 and 12), chlorophyll a *(peak 21) and derivates (peak 17 and 19), chlorophyll* b *(peak 18) and derivates (peak 13, 15, 16, and 20), β-carotene (peak 23), pheophytin a (peak 30) and derivates (peak 26, 27 and 31).*

The pigments extracted with different solvents, chromatograms in figures 7 and 8, did not vary significantly. Both present the peaks of lutein (peak 10), a few derivates and unknown chlorophylls. The same pigments were consistently observed in all other conditions with varying degrees of extractability, listed in Table 8, which appear to be the most common and abundant in *C. sorokiniana*. Lutein and β-carotene appear to be consistent with what is reported by Diprat *et al.* (2020) and Matsukawa *et al.* (2000), although no violaxanthin, alpha-carotene or zeaxanthin was found in these samples under these conditions. Safafar *et al.* (2015) described a more detailed report on different pigments found in two strains of *C. sorokiniana* which support the presence of chlorophylls *a* and *b*, neoxanthin, lutein, and β-carotene. Despite Safafar *et al.* (2015) reporting other pigments that apparently were not present in these samples under these conditions, such as fucoxanthin and zeaxanthin, they also report not finding violaxanthin or alpha-carotene in *C. sorokiniana* reported by the other two previous authors. The presence of astaxanthin was reported for one strain and not for the other, suggesting that the pigment profile could be specific to each strain. It could also be determined by culture conditions.

Table 8 - Pigments found in this assay for the different solvent and biomass conditions.

Pigments
Neoxanthin
Pheophorbide *a*
Pheophorbide a Derivates
Lutein
Lutein Derivates
Chlorophyll *b*
Chlorophyll *b* Derivates
Chlorophyll *a*
Chlorophyll *a* Derivates
Beta-Carotene
Pheophytin *a*
Pheophytin *a* Derivates

It should be noted that after the pigment analysis carried out on the samples extracted for fatty acid analysis, which involved using a larger amount of biomass, small concentrations of other pigments, such as violaxanthin and zeaxanthin were found. This information indicates the need for more research including higher volumes of biomass to concentrate and detect the presence of some pigments that could be present in lesser quantities as reported in the reviewed literature. Since this study was not possible due to the elevated quantity of biomass required, in the following sections the pigments in Table 8 will be considered the main pigments in the *C. sorokiniana* used in the present study, with an emphasis on the four quantified pigments.

3.1.2. Solvent Extraction Efficiency

In this section the extraction efficiency of the tested solvents with respect to the biomass used will be reported and discussed. For this, four of the main pigments present in the biomass were quantified, these were lutein, chlorophyll *a*, chlorophyll *b* and β-carotene. It is important to recall that the represented values for quantified pigments will also include their derivates as well unless explicitly stated otherwise.

As it has been previously stated, pigment extractability varies with its own and solvent polarity. The following section will describe and discuss in detail the extractability of each pigment.

3.1.2.1. Lutein Extraction

The resulting yields from the extraction of lutein from the non-axenic microalga *C. sorokiniana* for the different tested solvents are represented in Figure 9. At a first glance it is possible to observe an increased extractability in the fresh biomass compared to the freeze-dried biomass, this can be consistently observed throughout most pigment extractions and solvents. For lutein, this difference between fresh and freeze-dried biomass was found to be statistically significant for chloroform-methanol, methanol, methyl acetate, and ethyl acetate (two-way ANOVA, $\alpha = 0.05$). Although the sparse literature on this subject report similar or higher extractability from freeze-dried biomass, in this study the same was not observed (Damergi *et al.*, 2017; Hagerthey *et al.*, 2006). The reason for the lack of extractability might be explained by the results published by Pasquet *et al.* (2011), where freeze-dried biomass of the microalga *Dunaliella tertiolecta* was observed by scanning electron microscopy and described as agglomerated and flattened or compacted yet maintaining the cell shape. This could hamper the penetration of more polar solvents through the apolar phospholipidic membranes. In contrast, according to the argument laid out by the same authors, freeze-drying treatment was enough to turn the cell wall more susceptible to solvent penetration. The fact that this microalga was non-axenic could play a role in the extraction by affecting the extraction matrix and solvent-solute interactions.

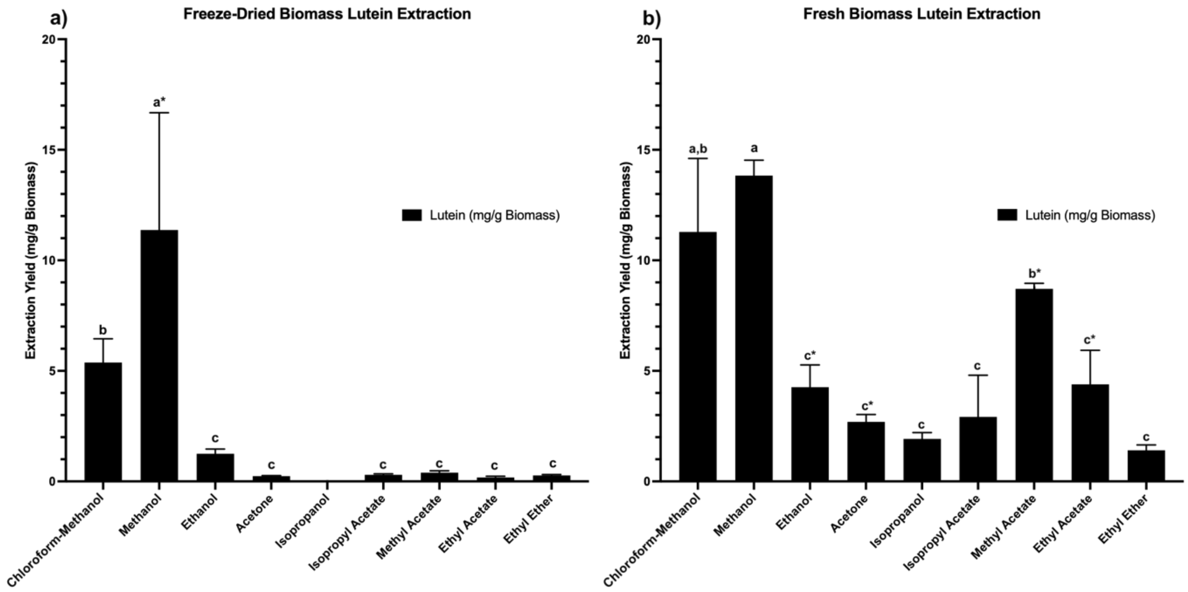

*Figure 9 - Extraction yield (mg/g of biomass dry weight) of lutein from (a) freeze-dried biomass and (b) fresh biomass by Chloroform-Methanol (2:1, v/v), Methanol, Ethanol, Acetone, Isopropanol, Isopropyl Acetate, Methyl Acetate, Ethyl Acetate, Ethyl Ether (n=4; mean ± SD). Equal letters indicate the absence of statistical significance of mean variation and are independent for each graph. * - n=3.*

Comparing the extraction of lutein by different solvents from freeze-dried biomass, represented in Figure 9 a, it is possible to observe a higher extraction yield from both chloroform-methanol and methanol alike. Ethanol appears to be the solvent with the third highest extractability. Isopropanol was not able to extract lutein from freeze-dried biomass. The remaining solvents show very little difference amongst each other as well as exhibiting very low extraction yields.

Fresh biomass, however, yielded different results (Figure 9 b). Methanol, and chloroform-methanol and methyl acetate, appear to display higher results in comparison to the other solvents. Methanol extraction of fresh biomass achieved the highest mean obtained for lutein extraction with a 13.83 ± 0.70 mg/g of biomass extraction yield. Methyl acetate appears to be the solvent with the third highest extractability yield. Ethyl ether appears to be the solvent with the least extractability capacity for lutein.

Chan *et al.* (2013) have reported very poor extractability of lutein by methanol from the microalga *Scenedesmus obliquus* arguing that such low extraction is due to its high polar nature, reporting as well, as high extractability of lutein by chloroform and ethyl ether, conflicting with the results obtained here. Although chloroform-methanol (2:1, v/v) had one of the highest extractability yields, ethyl ether did not demonstrate such results.

However, Safafar *et al.* (2015) report similar results to this work, where they obtained approximately 10 mg lutein/ g freeze-dried *C. sorokiniana* biomass extracted with methanol with high extraction rates.

On the other hand, the same authors also report very similar extraction results for acetone and ethanol compared to methanol which were not observed in this case. In fact, methanol has been reported more than once to achieve higher lutein extractability. Lee *et al.* (2021) report two-fold the extractability using methanol relative to acetone or ethanol from the microalga *Tetraselmis suecica*.

3.1.2.2. Chlorophyll *a* Extraction

The chlorophyll *a* extraction yields for different solvents using *C. sorokiniana* are represented in Figure 10. Apparently, a higher extractability can be seen for the fresh biomass yet this increase in extractability is only statistically relevant for methanol (two-way ANOVA, $\alpha = 0.05$). It is debated that since chlorophylls are more polar than carotenoids, the absence of water in the cell should not have high impact on their extractability, but rather assist their transition into more polar solvents (Ruivo *et al.*, 2014). Damergi *et al.* (2017) counter-argues this theory, suggesting that water in wet biomass is responsible for aiding in the extraction of polar solutes with non-polar solvents. Much like what was proposed for lutein, the problem with extraction from freeze-dried biomass might be related to solvent permeation through the cell walls due to the biomass conditions.

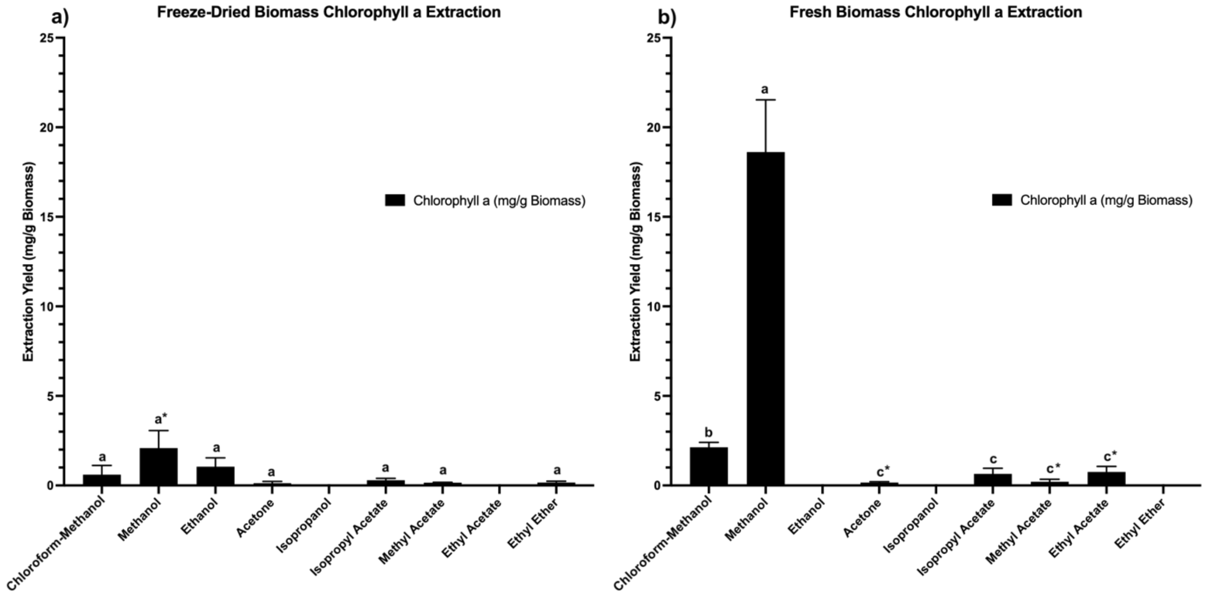

Figure 10 - Extraction yield (mg/g of biomass dry weight) of chlorophyll a *from freeze-dried biomass (a) and fresh biomass (b) by Chloroform-Methanol (2:1, v/v), Methanol, Ethanol, Acetone, Isopropanol, Isopropyl Acetate, Methyl Acetate, Ethyl Acetate, Ethyl Ether (n=4; mean ± SD). Equal letters indicate the absence of statistical significance of mean variation and are independent for each graph. * - n=3.*

From the graphical representation of Chl *a* extracted by different solvents from freeze-dried biomass in Figure 10 a, although there is no significance of mean variation (SMV), it is possible to observe that chloroform-methanol, methanol and ethanol had better extraction yields than the other solvents. Isopropanol and ethyl acetate appear to not have extracted any Chl *a*. The remaining solvents appear to have extracted a small amount of Chl *a* from freeze-dried biomass in relation to the highest extracting solvents.

Methanol had better results extracting the same pigment from fresh biomass (Figure 10 b) with an extraction yield mean of 18.61 ± 2.91 mg/g of biomass, being by far the best extracting solvent in this case. Ethanol, in contrast with its performance in freeze-dried biomass, did not extract any Chl *a* from fresh biomass. Chloroform-methanol was the second-best solvent with significantly lower results relative to methanol, yet still higher than the remaining solvents.

Lee *et al.* (2021) report extensive research on Chl *a* extraction from *Nannochloropsis sp.* with various solvents using dry biomass. Their results show an increased extractability of Chl *a* by isopropanol (70%) followed by ethanol (49%), and with lower extraction yields by methanol (36%) and acetone (32%) which contrasts with what is presently reported. Hagerthey *et al.* (2006) also shows results opposite to those found here. For unknown microalgae and cyanobacteria consortia freeze-dried biomass, methanol and acetone had high extraction yields and similar values, depending on biomass pre-treatments. The same authors reported very low extraction rates with fresh biomass independent of pre-treatment.

Solvent behavior for chlorophyll *a* extraction seem to be inconsistent with the literature reviewed, however Ogbonna *et al.* (2021) achieved chlorophyll *a* extraction yields of approximately 20 mg/g of biomass with 90% methanol for fresh *C. sorokiniana* which appears consistent with the values reported in the work. Safafar *et al.* (2015) report lower values of approximately 10 mg/g of freeze-dried *C. sorokiniana* biomass by methanol with similar values for acetone and ethanol. They also report a lower efficiency of ethyl acetate however it is still a substantial amount compared to the results obtained. Varaprasad *et al.* (2019) also achieved higher Chl *a* extraction with methanol (16.6 µg/mL) compared to ethanol (13.7 µg/mL), acetone (14.9 µg/mL) and ethyl ether (10.2 µg/mL) for *Chlorella vulgaris*.

The lower extraction yields obtained by solvents other than methanol could be explained by chlorophyllase enzyme activity which has been reported to remain active in some solvents and degrades chlorophylls (Wiltshire *et al.*, 2000). Even though there is a possibility of chlorophyll *a* degradation by chlorophyllase, it is more likely that degradation of chlorophylls might have occurred by oxidation during solvent vacuum evaporation in the desiccator despite all attempts to avoid direct light and air exposure during the procedure.

3.1.2.3. Chlorophyll *b* Extraction

The obtained yields from the different extractions of chlorophyll *b* from *C. sorokiniana* are represented in Figure 11. Once more, a higher extraction yield can be observed in fresh biomass in relation to freeze-dried biomass. This increase was statistically significant for chloroform-methanol, methanol, ethanol, and methyl acetate (two-way ANOVA, $\alpha = 0.05$). This phenomenon might have the same explanation as for chlorophyll *a*.

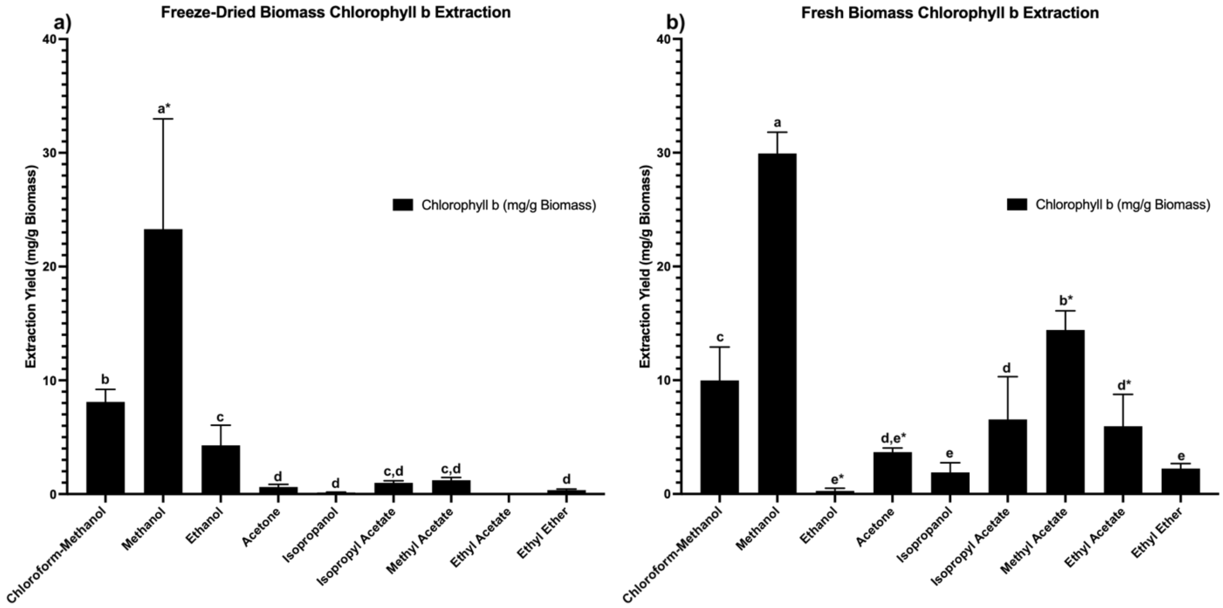

Figure 11 - Extraction yield (mg/g of biomass dry weight) of chlorophyll b *from freeze-dried biomass (a) and fresh biomass (b) by Chloroform-Methanol (2:1, v/v), Methanol, Ethanol, Acetone, Isopropanol, Isopropyl Acetate, Methyl Acetate, Ethyl Acetate, Ethyl Ether (n=4; mean ± SD). Equal letters indicate the absence of statistical significance of mean variation and are independent for each graph. * - n=3.*

The results for freeze-dried biomass represented in Figure 11 a show, much like for lutein and chlorophyll a, that the three extractant solvents with the highest results are chloroform-methanol, methanol and ethanol. The remaining solvents show a lower extractability of Chl *b* compared to the first three solvents. Ethyl acetate appears not to be able to extract Chl *b* in the same way it was not able to extract Chl *a*.

The results shown for fresh biomass (Figure 11 b) demonstrate the highest extraction yield of Chl *b* by methanol at 29.93 ± 1.87 mg/g biomass. Methyl acetate achieved the second highest extraction yield, followed by chloroform-methanol. Isopropyl acetate and ethyl acetate are tied in fourth place. From all the solvents used, ethanol extracted the lowest amount of Chl *b* from fresh biomass.

A publication by Varaprasad *et al.* (2019) comparing the extractability of chlorophylls by four different solvents from *Chlorella vulgaris* biomass show a higher extractability of Chl *b* by ethanol closely followed by acetone. Methanol only achieved approximately half the yield of the first two solvents, exhibiting lower yields than ethyl ether. Likewise, Morcelli *et al.* (2021) report higher extractability yields with ethanol compared to acetone and ethyl acetate for *C. sorokiniana* freeze-dried biomass. Piasecka & Baier (2022) report on extractions of up to 20 mg Chl *b* / g *C. sorokiniana* biomass using dimethyl sulfoxide as extraction solvent.

The higher extractability of Chl *b* by methanol compared to the other solvents, is not equivalent to what was found in the literature. Similarly to Chl *a*, chlorophyllase activity or oxidation during evaporation could be responsible for the lower extraction yields for other solvents.

3.1.2.4. Beta-carotene Extraction

The yields resulting from β-carotene extraction with different solvents from the *Chlorella* biomass are represented in Figure 12. It can be observed that fresh biomass had a much higher extraction yield for most solvents than freeze-dried, even though no statistical significance can be applied to this difference (two-way ANOVA, $\alpha = 0.05$). The difference between fresh and freeze-dried biomass can be seen for all the quantified pigments, there is the possibility that the freeze-drying process could cause degradation of the pigments. Besides the same factor that might affect the inefficiency of other pigments extraction from freeze-dried biomass, the non-polar nature of β-carotene is another factor that might increase extractability from fresh biomass as its higher affinity would assist in its transfer to the extracting solvent (Lee *et al.*, 2021).

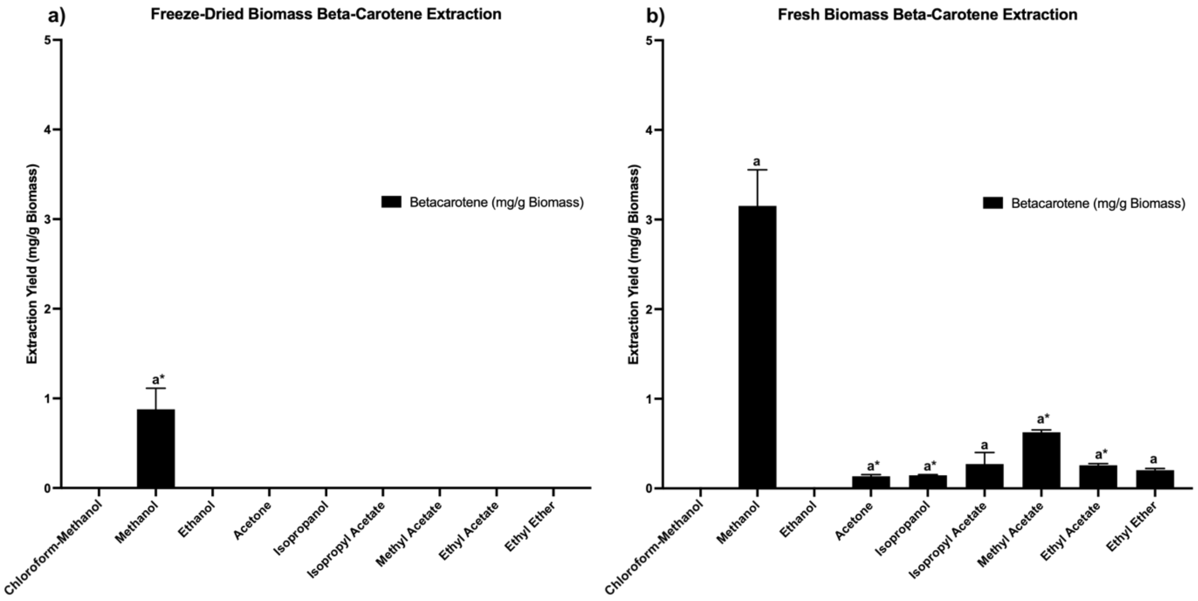

*Figure 12 - Extraction yield (mg/g of biomass dry weight) of β-carotene from freeze-dried biomass (a) and fresh biomass (b) by Chloroform-Methanol (2:1, v/v), Methanol, Ethanol, Acetone, Isopropanol, Isopropyl Acetate, Methyl Acetate, Ethyl Acetate, Ethyl Ether (n=4; mean ± SD). Equal letters indicate the absence of statistical significance of mean variation and are independent for each graph. * - n=3.*

Methanol appears to have been the only solvent able to extract β-carotene from freeze-dried biomass (Figure 12 a). However, more solvents were able to extract β-carotene from fresh biomass (Figure 12 b). Methanol differs from other solvents by achieving the highest extraction yield of β-carotene at 3.15 ± 0.40 mg/g biomass, although not statistically significantly. The second solvent with the highest extraction yield for β-carotene appears to be methyl acetate. Chloroform-methanol did not appear to extract any β-carotene in either fresh or freeze-dried biomass which is somewhat odd and does not match the reviewed literature since β-carotene should have higher affinity for organic solvents rather than more polar alcohols such as methanol and ethanol (Morcelli *et al.*, 2021).

Safafar *et al.* (2015) report extraction yields approximately 2 and 3 mg β-carotene / g *C. sorokiniana* biomass for methanol and acetone, respectively, which seems to be consistent with the values reported here. On the other hand, Diprat *et al.* (2020) report lower values of approximately 0.5 mg/g of biomass extracted with ethyl acetate and methanol.

As it has been stated previously, carotenes are more resistant to oxidation than chlorophylls, thus oxidation is less likely to explain the results obtained for β-carotene extraction (Hynninen, 1981). One explanation for the results obtained could be the matrix effect of the extract, possibly affected by the non-axenic nature of the microalga.

3.1.3. Second Extraction

For each biomass condition and solvent used a second extraction cycle was performed using the same biomass and fresh solvent under the same conditions as the first cycle, as described in the methods section, with the objective of obtaining a complete extraction. In this section the extraction efficiency of a second cycle is reported and discussed to assess the necessity of a second extraction cycle.

The results for the first extraction cycle for the different pigments lutein and chlorophyll *b* have a higher extractability than other pigments for most solvents tested. For the sake of briefness, these two pigments were chosen for the assessment of the second extraction cycle efficiency.

The relation between the first and the second lutein extraction cycles for all solvents used with both fresh and freeze-dried biomass can be found in Figure 13. Lutein extraction revealed a significant reduction in extraction efficiency from the first to the second cycle for all solvents with higher extractions in the first cycle, for both freeze-dried and fresh biomass. This decrease in extractability was does not appear observable for solvents presenting low extraction efficiency.

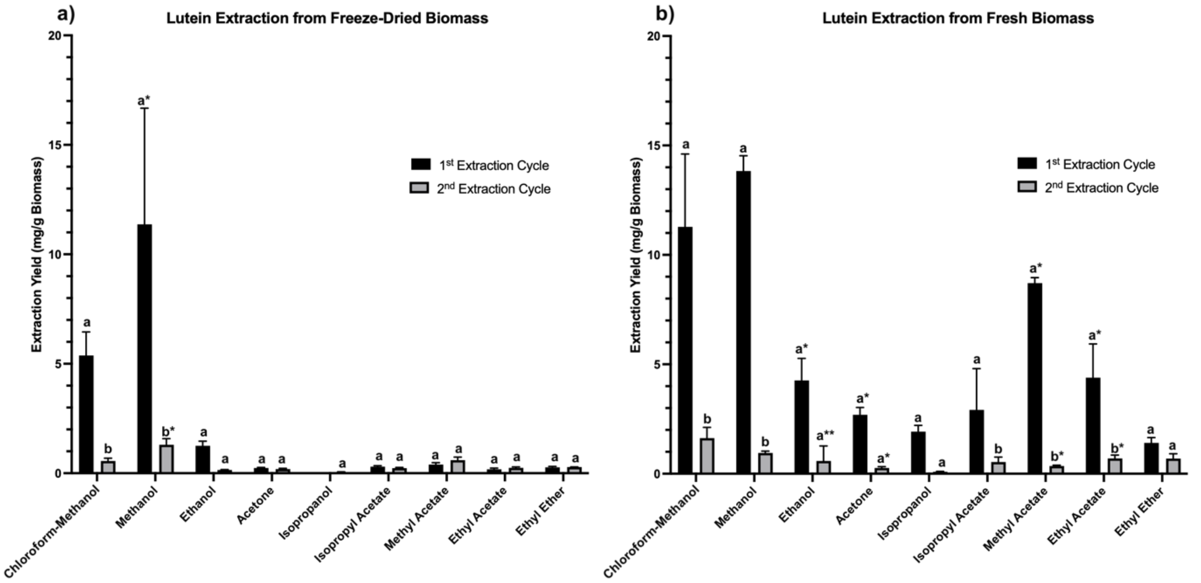

*Figure 13 - Extraction yields (mg/g of biomass dry weight) of lutein from the first and second extraction cycles for both (a) freeze-dried biomass and (b) fresh biomass by Chloroform-Methanol (2:1, v/v), Methanol, Ethanol, Acetone, Isopropanol, Isopropyl Acetate, Methyl Acetate, Ethyl Acetate, Ethyl Ether (n=4; mean ± SD). Equal letters indicate the absence of statistical significance of mean variation and are independent for each solvent and graph. * - n=3; ** - n=2.*

For freeze-dried biomass (Figure 13 a) the extraction mean of chloroform-methanol for the second extraction cycle was only 10.5% of the first and for methanol only 11.4%. For fresh biomass (Figure 13 b) the highest variation between cycles was observed with methyl acetate, extracting in the second cycle only 4.0% of what it extracted in the first cycle, followed by isopropanol and methanol with 4.9% and 6.9%, respectively. In contrast, the second cycle of extraction for ethyl ether extracted as much as 49.4% of the first cycle.

From Figure 14, it is possible to observe that chlorophyll *b* had a similar outcome to lutein. Both fresh and freeze-dried biomass show a significant extraction yield decrease for all solvents that had higher extraction yields in the first cycle. The second cycle of methanol extraction of freeze-dried biomass (Figure 14 a) extracted only 13.3% of the first cycle. For fresh biomass (Figure 14 b), in the second cycle methanol was able to extract only 2.7% of what it extracted in the first.

Solvents displaying a low second extraction yield relative to the first extraction cycle could be a good indicator of the solvent efficiency in the first extraction. Chan *et al.* (2013) report a second extraction cycle for lutein using ethyl ether with 47.7% of the yield from the first cycle, a high value that seems consistent with what was reported here but attests to the solvent's low efficiency in the first extraction. However, a relation between the solvent and biomass ratio has also been observed (Mäki-Arvela *et al.*, 2014). In this assay a large solvent/biomass ratio was used to compare solvent extractability without solvent saturation limitation.

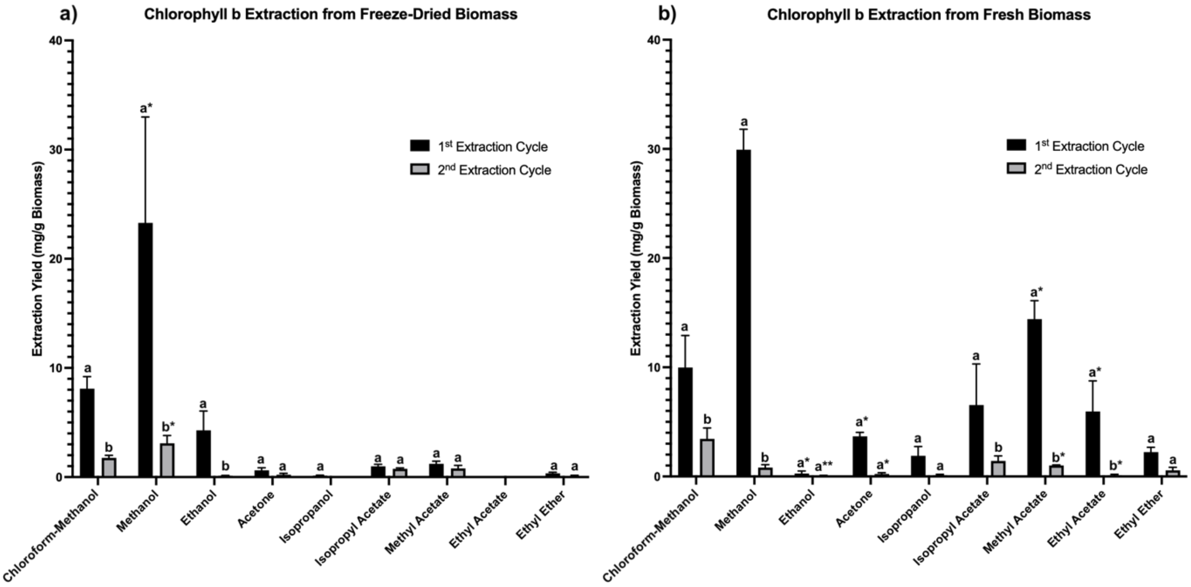

Figure 14 - Extraction yields (mg/g of biomass dry weight) of chlorophyll b *from the first and second extraction cycles for both (a) freeze-dried biomass and (b) fresh biomass by Chloroform-Methanol (2:1, v/v), Methanol, Ethanol, Acetone, Isopropanol, Isopropyl Acetate, Methyl Acetate, Ethyl Acetate, Ethyl Ether (n=4; mean ± SD). Equal letters indicate the absence of statistical significance of mean variation and are independent for each solvent and graph. * - n=3; ** - n=2.*

Assuming as a hypothetical argument the use of methanol for extraction of pigments such as lutein and chlorophyll *b* from fresh *C. sorokiniana* biomass, a second extraction would require twice the volume of methanol only to extract 6.9% of lutein or 2.7% of Chl *b* of the first cycle. It seems very unlikely that an economic assessment of the second extraction would support its feasibility. Moreover, an environmental assessment would also appear not to be a sensible choice, even including solvent recovery and reuse which would raise the cost of production.

3.1.4. Spectrophotometry Vs. Chromatography

Before identification and quantification of pigments present in the extracts by UHPLC, a less precise spectrophotometric method described in the Materials and Methods section was performed for a preliminary assessment of pigment extractability. As previously mentioned, this method is based on the equations derived by Lichtenthaler (1987) from specific absorption coefficients determined with a UV-Vis spectrometer at the indicated wavelengths.

In this section, the spectrophotometer and UHPLC results for the methanol extraction of pigments from the microalga *C. sorokiniana* fresh biomass will be compared for the assessment of the accuracy of the Lichtenthaler equations. Since these equations only account for chlorophyll *a*, chlorophyll *b* and total carotenoids, lutein being shown in figures 7 and 8 to represent most carotenoids extracted, will be considered as the equivalent to the total carotenoids. Figure 15 illustrates the differences in concentration calculated for spectrophotometric and liquid chromatography methods.

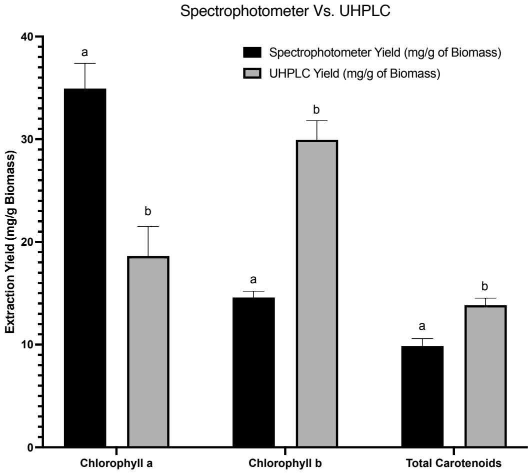

Figure 15 – Representation of the calculated extraction yields for chlorophyll a*, chlorophyll* b *and total carotenoids from the pigment extraction of fresh biomass by methanol, using the Lichtenthaler (1987) equations with the values obtained from Ultra High-Performance Liquid Chromatography (UHPLC). Different letters represent the correlation of means and are independent for each pigment (paired t-test,* α*=0.05).*

As seen in Figure 15, there was a considerable overestimation by the spectrophotometer quantification method for Chl *a*, almost doubling the results of what was obtained by UHPLC quantification. A possible explanation for this is the quantification of pheophorbide a and pheophytin a as well as the remaining unknown chlorophylls along with chlorophyll *a*. On the other hand, it appears the chlorophyll *b* quantified by the spectrophotometer method is close to half that quantified by UHPLC.

The results for the quantification of total carotenoids appear to be the closest for both methods. However, despite lutein representing the majority of carotenoids in *C. sorokiniana* it is not the only one yet the concentration of lutein from quantified using UHPLC is superior to that of the total carotenoid calculated by the spectrophotometer method. A study performed by Biehler *et al.*, (2010) shows that the nature of the extracted biomass can affect the results obtained in each method. Spinach (*Spinaceia oleracea*) for example, presented twice the concentration when analyzed by the Lichtenthaler (1987) method compared to UHPLC and leek (*Allium ampeloprasum*) had the opposite response, demonstrating higher results for UHPLC.

The spectrophotometry method is much cheaper and faster than liquid chromatography. Despite the results obtained for both methods not being the same they were on the same scale and as stated their accuracy can vary with the source of the pigments. The spectrophotometric method has shown to be helpful for a fast estimation of pigment concentration.

3.2. Fatty Acid Extraction

The fatty acid (FA) profile of the microalga *Chlorella sorokiniana* was analyzed by gas chromatography through acid transesterification of the FAs. Total fatty acids were also quantified for both fresh and freeze-dried biomass which allowed to compare the extractability of FAs for both types of biomasses. Another assay performed was the evaluation of the potential of fatty acid sequential extraction from biomass previously used for the extraction of pigments with different solvents for the valorization of the biomass. The following sections report and discuss the results of these experiments.

3.2.1. **Fatty Acid Profile of *C. sorokiniana***

The characterization of the fatty acid (FA) profile of *C. sorokiniana* was conducted by extraction of the FAs directly from freeze-dried biomass. The resulting FAs were transesterified and analyzed by gas chromatography as detailed in the

Materials and Methods section. The extraction of total lipids from freeze-dried biomass yielded 57.94 ± 27.56 mg/g of biomass while total lipids from fresh biomass yielded 118.08 ± 9.49 mg/g of biomass. Figure 16 represents one of the chromatograms obtained from the fatty acid methyl esters analysis by GC.

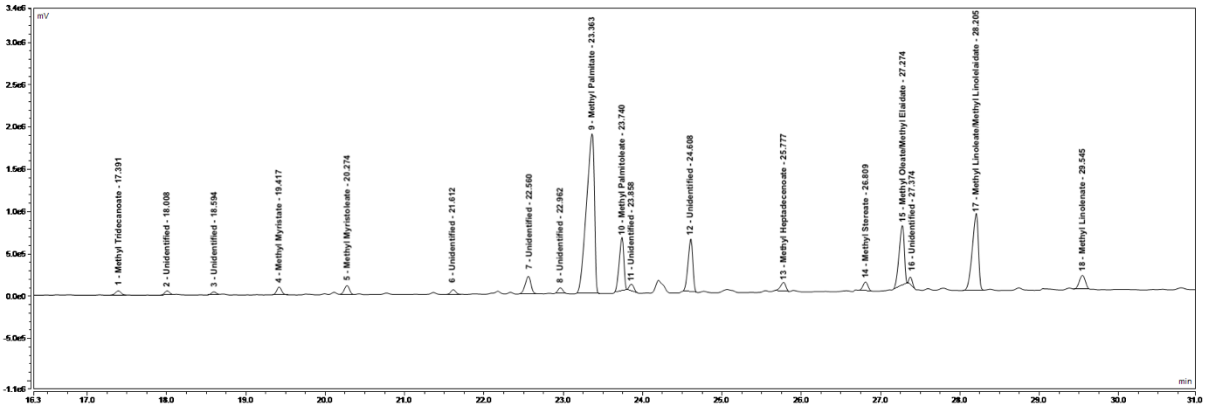

Figure 16 – Chromatogram obtained from the analysis of fatty acid methyl esters from freeze-dried C. sorokiniana biomass including methyl ester name and retention time. The chromatogram is represented in signal intensity (mV) over time (min). FAMES identified include: methyl tridecanoate (peak 1), methyl myristate (peak 2), methyl myristoleate (peak 5), methyl palmitate (peak 9), methyl palmitoleate (peak 10), methyl heptadecanoate (peak 13), methyl stereate (peak 14), methyl oleate/elaidate (peak 15), methyl linoleate/linoelaidate (peak 17), and methyl linolenate (peak 18).

The chromatogram represented in Figure 16 shows a higher presence of palmitic acid (C16:0, peak 9), oleic and elaidate acid (C18:1 *cis* and *trans*, peak 15), as well as linoleic and linolelaidic (C18:2 *cis* and *trans*, peak 17) methyl esters. Unfortunately, C18:1 and C18:2 *cis* and *trans* isomers present in the standard mixture were not separable and must be considered together. However, peak 11 and peak 16 are very close to C18:1 and C18:2, respectively and were not able to be identified. There is a possibility that those peaks represent the *trans* configurations of the FAMEs mentioned and some unidentified factor in the extraction matrix assisted in the separation of these compounds, however no evidence can be presented for this speculation. On the other hand, it is also possible they are the conformations of the same fatty acid with their double bonds in positions of the hydrocarbon chain.

The relative areas of the peaks in the chromatograms can represent an estimation of the fatty acid profile of this strain of *Chlorella sorokiniana*. The means for the relative area of the peaks represented in Figure 16 was calculated from quadruplicates. These are represented in Table 9 along with the identified FAMEs, respective FA from which they derivate, FA abbreviation and mean standard deviation.

Table 9 - Identified fatty acid methyl esters (FAMEs) corresponding to the peaks of the chromatogram represented in figure 16, respective common fatty acid names, abbreviation, average relative area (%; n=4) and standard deviation (SD).

Peak nr.	Identified FAME:	Common Fatty Acid Name:	Abbreviation:	Average Relative Area (%)	SD
1	Methyl Tridecanoate	Tridecylic Acid	C13:0	0.72	0.04
2	Unidentified	Unidentified		0.65	0.06
3	Unidentified	Unidentified		0.52	0.20
4	Methyl Myristate	Myristic Acid	C14:0	1.13	0.18
5	Methyl Myristoleate	cis-10 Heptadecenoic Acid	C14:1	1.48	0.11
6	Unidentified	Unidentified		0.66	0.16
7	Unidentified	Unidentified		3.23	0.28
8	Unidentified	Unidentified		0.75	0.35
9	Methyl Palmitate	Palmitic Acid	C16:0	40.48	2.15
10	Methyl Palmitoleate	Palmitoleic Acid	C16:1	8.10	0.21
11	Unidentified	Unidentified		1.38	0.55
12	Unidentified	Unidentified		8.01	0.56
13	Methyl Heptadecenoate	Myristoleic Acid	C17:1	1.57	0.13
14	Methyl Stereate	Stearic Acid	C18:0	1.23	0.11
15	Methyl Oleate/Elaidate	Oleic Acid/Elaidic Acid*	C18:1	10.65	0.43
16	Unidentified	Unidentified		1.13	0.39
17	Methyl Linoleate/Linoelaidate	Linoleic Acid/ Linolelaidic acid*	C18:2	15.53	0.65
18	Methyl Linolenate	α-Linolenic	C18:3	2.81	0.36

* - *cis* and *trans* isomers

The results obtained help describe the fatty acid profile as composed of 43.56% saturated FAs, 21.80% monounsaturated FAs, and 18.34% polyunsaturated FAs. Assuming the identified FAs are composed in their majority of the most common and stable configurations it could be argued that the FAs profile of the biomass used in this study were composed of 2.8% ω-3 FAs, and 15.5% ω-6 FAs.

As it was previously stated in the introduction of this work, the reviewed literature shows that the fatty acid profile found in *C. sorokiniana* can differ with the culture growth conditions. Nonetheless, the fatty acid profiles reported are mostly consistent with the results obtained in the present work. Ngangkham *et al.* (2012) testing different treatments for *C. sorokiniana* report a composition from 29.1 to 43.6% palmitic acid, from 5.1 to 9.8% oleic acid, from 20.2 to 25.0% linoleic acid, and from 8.1 to 17.1% α-linolenic acid. Furthermore, they report a presence of hexadecadienoic acid (C16:2) between 9.7 and 10.6% which appears to fit with unidentified peak no. 12. Bazarnova *et al.* (2019) report results that despite some differences are still similar to those obtained here. For instance, palmitic acid was reported to make up 17.8% of the FA composition, and oleic acid made up 18.4%, while linoleic acid made up 14.8%

of FAs. Table 6 in section 1.4.3. contains further cited FA profile compositions of the microalga *Chlorella sorokiniana.*

3.2.2. Sequential extraction of Pigments and Fatty Acids

The results for the two-step sequential extractability of fatty acids from the same biomass after the extraction of pigments with different solvents for the valorization of biomass is described and discussed in this section. The possibility of the sequential extraction of FAs in a separated step would increase the value of the biomass. Pigment pre-extraction was carried out with methanol, ethanol, acetone, isopropyl acetate and ethyl acetate. Chloroform-methanol was not chosen for the pre-extraction of pigments due to its redundancy since the method used for the extraction of FAs is carried out by chloroform-methanol as well and total extraction of FAs was assumed to occur during pigment extraction. Due to an initial misinterpretation of the data obtained from the pigment extraction assay, the assessment of the sequential extractability of FAs from biomass pre-extracted with methyl acetate was not performed. The remaining solvents were discarded from this assay for their low pigment extractability performance.

The extraction yields of fatty acids were calculated based on the weight of total FAs extracted from the biomass after pigment pre-extraction and are represented in Table 10, along with the yield relative of total FA from whole biomass. Lower FA extraction values in Table 10 represent FAs that were extracted by the solvent in pigment extraction. It is possible to observe that similarly to the extraction of pigments, the water present in fresh biomass assists in the extraction of fatty acids, possibly due to their non-polar nature which will ease their transition to a medium of higher affinity.

Methanol was revealed to be one of the most efficient solvents for the extraction of pigments however, despite its polar nature it appears to leave no lipids from fresh biomass and only 13.7 remain in freeze-dried biomass after extracting pigments. Ethanol displayed a pigment extraction performance inferior to that of methanol and is also a polar solvent. However it seems to have removed a big portion of lipids as well from fresh biomass. Approximately 77.5% of lipids were recovered from the freeze-dried biomass after pre-extraction of pigments with ethanol. After methanol, acetone appears to have removed the most FAs from both fresh and freeze-dried biomass during pigment extraction. Isopropyl acetate and ethyl acetate displayed very similar behavior. Neither extracted a large amount of lipids from both fresh and freeze-dried biomass and both displayed average extraction yields for pigments for fresh biomass.

Table 10 - Fatty acid extraction yield (mg/g of biomass) for both fresh and freeze-dried biomass, including mean, standard deviation (SD), number of replicates (n), and yield extraction of the means relative to the means of direct extraction for samples pre-extracted with the different solvents.

Pigment pre-extraction	**Fresh Biomass**				**Freeze-Dried Biomass**			
	Fatty Acids (mg/g Biomass)				**Fatty Acids (mg/g Biomass)**			
	Mean	**SD**	**n**	**Yield (%)**	**Mean**	**SD**	**n**	**Yield (%)**
Direct extraction (no pre-extraction)	118.08	9.49	4	100.0	57.94	27.56	3	100
Methanol	0.00	0.00	3	0.0	7.96	1.70	3	13.7
Ethanol	32.19	10.30	4	27.3	44.89	12.78	4	77.5
Acetone	3.83	3.90	4	3.2	35.09	3.58	4	60.6
Isopropyl Acetate	96.06	12.92	3	81.4	46.90	3.52	4	81.0
Ethyl Acetate	90.82	5.59	4	76.9	48.09	4.32	4	83.0

The most efficient solvent and biomass conditions for pigment extraction also assisted in the extraction of the majority of fatty acids from the biomass. Although no assays were performed to verify the presence of the same FAs in the resulting solutions from the pigment extraction, it is likely they were retained in the solvent and should be further evaluated. The co-extractability of both pigments and FAs in the same solvent would probably increase the costs of the separation of both metabolites. On the other hand, using freeze-dried biomass for pigment extraction with methanol would allow an almost equally high extraction of pigments to facilitate a minor extraction of FAs. The results obtained in this assay could also suggest the use of methanol for the extraction of FAs instead of the chloroform-methanol mixture in the Bligh & Dyer (1959) method.

Alternatively, it would be possible to sacrifice a portion of pigments extracted for the subsequential of a higher amount of FAs from fresh biomass using a solvent such as an ethyl acetate on the first step. According to the results of the experiments carried out in the development of this thesis, approximately 8.71 ± 0.25 mg lutein / g biomass, 14.42 ± 1.69 mg Chl *b* / g biomass, and 0.63 ± 0.03 mg β-carotene / g biomass could be obtained as well as 90.82 ± 5.59 mg FAs / g biomass using fresh biomass and ethyl acetate for pigment extraction followed by a FA extraction.

No literature on the separate sequential extraction of both pigments and fatty acids was found that could contribute to ensure the consistency of the results. Furthermore, it should be stated that further research should be conducted to assess the extractability of FAs from biomass pre-extracted with methyl acetate.

3.3. Industrial Sustainability

A simple initial sustainability assessment of the sequential extraction of both pigments and fatty acids from *Chlorella sorokiniana* biomass was performed with SuperPro Designer software. For this, the results reported in the previous sections were used for balancing the biomass, solvents and final products through the plant stream flow. Estimations considered the extraction procedure on an amount of 100 kilograms of biomass per batch. Ethyl acetate was chosen as the designated solvent for pigment extraction despite its average performance concerning other solvents due to its lower removal rate for fatty acids compared to other solvents, and for being considered a green solvent since the study at hand consists of the sequential extraction of both in a more environmentally sustainable way. In this section, the economic evaluation of the whole process is reported and discussed.

The number of yearly batches processed by the simulated plant was estimated by SuperPro as 2436, meaning 243.6 thousand kilograms of biomass extracted, producing 2920.3 kilograms of pigment mixture, 21.3 thousand kilograms of fatty acid mixture and 177.5 thousand kilograms of spent biomass per year. The operating equipment and infrastructure were designed to work at maximum capacity to avoid expense on superfluous instruments, still the capital investment for such a plant was estimated to reach 71.8 million (M) euros (values for 2022). The minimum number of operators for the well-functioning of the facility was estimated to receive the average specialized operator wages for Portugal, 2022. The annual facility-dependent and quality control/quality assurance operating costs were estimated to reach 11.4 M euros. Operator expenses were estimated to reach 3.46 M euros/year. Waste disposal and utilities cost reached half a million euros/year, however the highest annual operating cost was the cost of raw materials, closing in 117.3 M euros each year.

Different scenarios for the market value of the final products were tested for pigments and fatty acids with a retail price of 10,000 euros needed per kilogram of pigments and 15 euros per kilogram of fatty acids being the only values able create a positive revenue margin. Considering an almost full return on the solvents used and approximately 60% return on the biomass used, such values were necessary to overcome the remaining operating costs which were mainly the unrecovered raw material costs, labor and facility costs. The estimated net profit for the operation would be of 11.7 M euros/year with a return investment of 16.3% and a period of 6 years and two months necessary to pay off the initial investment. Considering 12.5 years of useful project lifetime, the estimated Net Present Value of the project at 7.0% depreciation rate was 16.62 M euros, and the Internal Return Rate after taxes was 10.59%.

Standard quality lutein (90%) at face value can be sold for the equivalent of 260 thousand euros per kilogram in small quantities (Thermo Fisher Scientific, USA) while standard quality chlorophyll *b* (90%) would be approximately 268 M euros per kilogram (Sigma-Aldrich, USA). Assuming a 98% decrease in value for lutein and 99% for chlorophyll *b* for bulk retail prices and purity degree that would still give values of 5.2 thousand euros for kilogram of lutein and 2.68 M euros/kg for chlorophyll *b*. On the other hand 100% marigold flower extract advertised for their lutein content can be sold for 0.0025 euros/kg (minimum amount of 10 tons) (Awell Ingredients Co., Ltd., China). Marigold has been found to contain from 0.01 to 0.02% lutein, meaning a value of lutein in those extracts is actually 8 euros/kg not accounting for the impurity of the product (Hojnik, 2008; Cheng, 2019). No products sold for their chlorophyll *b* in bulk with less purity were found, however from the elevated difference in the pure products it is likely that such product would achieve higher prices than lutein.

The pigment product resulting from the present simulated industrial process would be a pigment mixture of high purity degree composed of approximately 58% chlorophyll *b*, 38% lutein and 4% β-carotene with the possibility of further purification for an increased selling value. The assumed value of 10 thousand euros per kilogram might be relatively low for the product obtained.

The value for vegetable oil in bulk can reach 2.5 euros per kg (more than 500 tons purchase) (Kerry Oils & Grains (Qingdao) Ltd., China) and fish oil can reach 1 euro per kg (minimum amount 1 ton) (AnHui Chempro Biochemical Limited, China) which seems very low compared to the value proposed for the fatty acids. Although no price was found in bulk for microalgae oil, algae oil was found with a price of 30 euros/kg (minimum 10 kg) (Qingdao Haosail Science Co., Ltd., China). The estimated value of 15 euros might be somewhat elevated for microalgae oil, however there is a growing interest for unsaturated fatty acids and the product was found to be composed of only approximately 43% saturated fatty acids.

As previously stated, this assay is only an initial assessment of the process and a more thorough evaluation should be performed, considering product purification, biomass production, solvent distillation, as well as further uses for the biomass, such as example fermentation or digestion for production of other materials. Initially, this process was idealized by the higher extractability of pigments by methanol since pigments have a much higher value than fatty acids and the polarity of methanol which was assumed to have a lower impact on the removal of fatty acids. Due to the lower pigment extraction capacity of ethyl acetate, it is likely that a similar processing plant dedicated only to the extraction of pigments would be far more lucrative.

4. Conclusions

The work performed in this thesis was directed toward the characterization of pigment and fatty acid composition of the non-axenic microalga *Chlorella sorokiniana*, evaluation of solvent extractability of pigments for the *Chlorella* biomass and the sequential extractability of fatty acids from the same biomass after pigment extraction with the same solvents. After these tasks were done, a very preliminary assessment of the industrial sustainability of the sequential extractability process was carried out.

Methanol showed to be the best solvent for both fresh and freeze-dried biomass regarding all the pigments quantified. Compared to methanol all other solvents with exception of chloroform-methanol (2:1, v/v) were substantially inferior to the results presented by methanol. It is possible that a step involving the evaporation of the solvent containing the pigments and their resuspension in methanol might be the cause for such low results. The main pigments present in *Chlorella sorokiniana* appear to be lutein and both chlorophylls *a* and *b*.

On the other hand, methanol appeared to also remove most of the fatty acids present in the biomass and as such sequential extraction of fatty acids from the same biomass is not possible. Ethyl acetate and Isopropyl acetate displayed the best behavior on sequential extraction of both metabolites with ethyl acetate being chosen for the industrial sustainability assessment of the process. Palmitic, palmitoleic, oleic and linoleic acids were shown to be present in higher concentrations in this microalga.

For both pigment and fatty acid extractions fresh biomass showed higher extractability which allows direct processing of the biomass without the additional costs of freeze-drying.

The economic assessment of the industrial process showed only feasibility at a very high product selling price which might be adequate due to the purity of the final product. However, a more thorough assessment of the whole process should be performed, considering other solvents or only pigment extraction with methanol.

Further studies should also be performed in this line of investigation, including but not limited to, the sequential extractability of fatty acids after the pigment extraction with methyl acetate and the co-extraction of other metabolites.

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