

Identification of microalgae strains producing high- value compounds

Ana Rita Limão Vieira

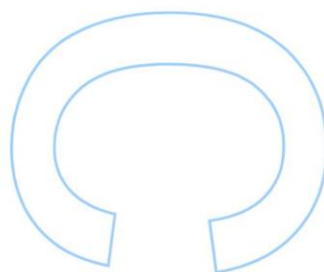
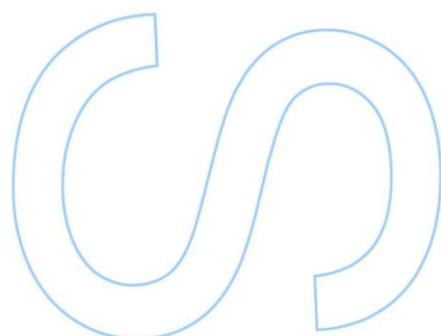
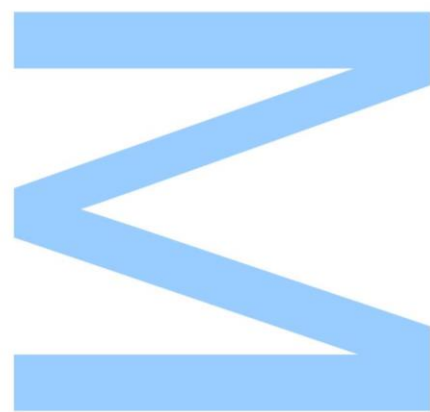
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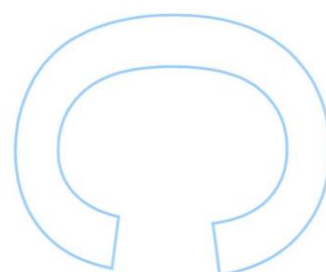
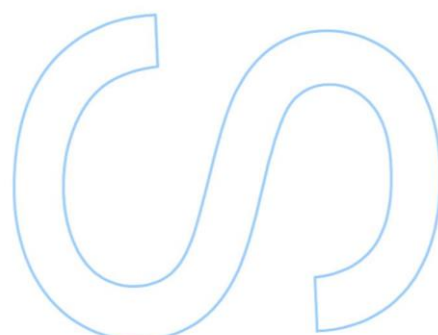
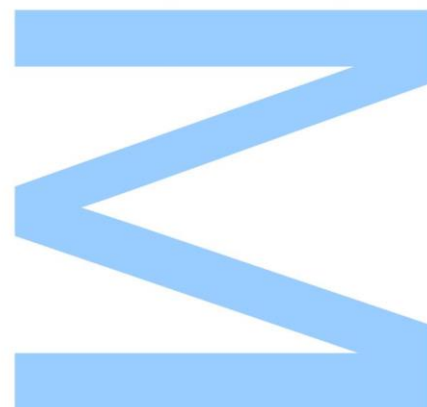
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Porto, 25th September of 2022

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Resumo

Produtos à base de microalgas apresentam uma elevada quimiodiversidade, oferecendo várias vantagens quando comparados a fontes convencionais sobre exploradas. Deste modo, estão progressivamente a desempenhar um papel importante na sociedade e na economia. As microalgas representam atualmente uma fonte valiosa de compostos de valor acrescentado (HVC) com um potencial biotecnológico crescente.

Este trabalho teve como objetivo a identificação das estirpes de microalgas produtoras de HVC, para o mercado nutracêutico e cosmético. Após análise de literatura e de mercado, foram selecionadas 22 microalgas, da Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC). Após cultura de microalgas, recolha e extração de biomassa, foram investigados diferentes HVCs: (A) fenóis (PC) com o ensaio de Folin-Ciocalteu; (B) pigmentos (astaxantina, cantaxantina, clorofila-a, equinenona, luteína, licopeno, neoxantina, violaxantina, zeaxantina e β -caroteno) por cromatografia líquida de alta eficiência; (C) ácidos gordos (com foco nos ácidos gordos polinsaturados, PUFA) por cromatografia gasosa acoplada a espectrometria de massa; e (D) polissacáridos por coloração com azul de Alcian e ensaio de Dubois. Paralelamente à identificação de HVC, foram obtidas curvas de crescimento para todas as estirpes cocóides.

Os resultados confirmaram o potencial das microalgas testadas para HVC. *Eustigmatos* sp. FB05M revelou potencial global para a produção de PC ($11.7 \text{ mg GAE g}^{-1}$), violaxantina (21.7 mg g^{-1}), EPA (39.0 mg g^{-1}) e polissacáridos (1.9 mg g^{-1}). *Chlamydocapsa* sp. FB06M demonstrou alto conteúdo em carotenoides (78.7 mg g^{-1}), PC (10.4 mg g^{-1}) e PUFA (74.8 mg g^{-1}). O género *Euglena* também revelou alto teor em PC e PUFA.

Em adição ao trabalho de investigação e desenvolvimento, foram também desenvolvidas atividades empresariais com a equipa de nutracêuticos da Fykia Biotech (Ralph Urbatzka PhD, Pedro Leão PhD, Ana Rita Vieira BSc, Filipe Henriques BSc e Hugo Amaral BSc). A equipa participou no programa *hands-on* de aceleração HiTech, com o projeto "*hpa, a compound to fight obesity*". Foi desenvolvido um pitch e um plano de negócios para um produto conceptual, como resultado da formação e mentoria nos temas de comercialização de tecnologia, avaliação de mercado e finanças, desenvolvimento de negócios e propriedade intelectual. Simultaneamente, a gestão da rede social da página LinkedIn da Fykia Biotech fez também parte das atividades empresariais desenvolvidas.

Palavras-chave: microalgas, compostos de valor acrescentado, carotenoides, clorofilas, fenóis, ácidos gordos, polissacáridos, empreendedorismo

Abstract

Microalgae-based products present high chemodiversity offering several advantages when compared with conventional overexplored sources. Therefore, they are progressively playing an important role in society and economy. Microalgae currently represent a valuable source of high-value compounds (HVC) with increasing biotechnology potential.

The target of this research was the identification of HVC-producing microalgae strains targeted for the nutraceutical and cosmetic market. After literature and market analysis, twenty-two microalgae strains were selected, from Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC). After microalgae cultivation, followed by biomass harvest and extraction, different HVCs were screened: (A) phenols (PC) by Folin-Ciocalteu assay; (B) pigments (astaxanthin, canthaxanthin, chlorophyll-a, echinenone, lutein, lycopene, neoxanthin, violaxanthin, zeaxanthin and β -carotene) by high-performance liquid chromatography; (C) fatty acids (with a focus on polyunsaturated fatty acids, PUFAs) by gas chromatography-mass spectrometry; and (D) polysaccharides by Alcian Blue staining and Dubois assay. In parallel with the HVC screening, growth curves were obtained for all microalgae coccoid strains.

Results confirmed the potential of the tested microalgae to produce HVC. *Eustigmatos* sp. FB05M revealed an overall potential for the production of PC (11.7 mg GAE g⁻¹), violaxanthin (21.7 mg g⁻¹), EPA (39.0 mg g⁻¹) and polysaccharides (1.92 mg g⁻¹). *Chlamydocapsa* sp. FB06M confirmed an overall high content in carotenoids (78.7 mg g⁻¹), PC (10.4 mg g⁻¹) and PUFAs (74.8 mg g⁻¹). The genus *Euglena* also showed high PC and PUFA contents.

In addition to research and development work, as part of Fykia Biotech's nutraceutical team (Ralph Urbatzka PhD, Pedro Leão PhD, Ana Rita Vieira BSc, Filipe Henriques BSc and Hugo Amaral BSc), entrepreneurial activities were also developed in the course of this work. The team was enrolled in the hands-on acceleration program HiTech, with the project "hpa, a compound to fight obesity". The program outcome was the development of a pitch and a business case for a concept product, as a result of training and mentoring in the subjects of technology commercialization, market and finance assessment, business development and intellectual property. Simultaneously, under the scope of this internship in business context, the social network management of Fykia Biotech's LinkedIn page was also part of the entrepreneurial activities developed.

Keywords: microalgae, high-value compounds, carotenoids, chlorophylls, phenols, fatty acids, PUFAs, polysaccharides, entrepreneurship

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Abbreviation List

AB	Alcian Blue, $C_{56}H_{68}Cl_4CuN_{16}S_4$
AI	Atherogenicity index
CAGR	Compound annual growth rate
Chl	Chlorophyll
CIIMAR	Interdisciplinary Centre of Marine and Environmental Research
DCM	Dichloromethane, CH_2Cl_2
DMSO	Dimethyl sulfoxide, $(CH_3)_2SO$
EFSA	European Food Safety Authority
EPS	Exopolysaccharides
EU	European Union
FA	Fatty acid
FAME	Fatty acid methyl ester
FDA	U.S. Food and Drug Administration
GA	Gallic acid, $C_6H_2(OH)_3CO_2H$
GAE	Gallic acid equivalents
GC-MS	Gas chromatography – mass spectrometry
H₂O	Water
Hex	<i>n</i> -hexane, $CH_3(CH_2)_4CH_3$
h/H	hypcholesterolemic/hypercholesterolemic ratio
hpa	13 ² -hydroxypheophytin a
HPLC-PDA	High-performance liquid chromatography with photo diode-array
HVC	High-value compound
LOD	Limit of detection
LOQ	Limit of quantification
MeOH	Methanol, CH_3OH

MUFA	Monounsaturated fatty acid
N₂	Nitrogen
OD	Optical density
PBP	Phycobiliprotein
PCA	Principal component analysis
PC	Phenolic compounds
PUFA	Polyunsaturated fatty acid
RA	Relative abundance
RT	Retention time
RT	Room temperature
SFA	Saturated fatty acid
TI	Thrombogenicity index
TPC	Total phenolic content
USA	United States of America
USD	United States dollar

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Chapter 1

Screening of microalgae strains for high-value compounds intended for nutraceutical and cosmetic purposes

I. Introduction

1. Microalgae, nature's "green gold"

Algae have been thriving over the last 2.45 billion years, their ability to survive and adapt to the most diverse environments led to unique biochemical profiles. These organisms have important roles in the environment and pose great potential for biotechnology applications [1-6]. These photosynthetic organisms are, in fact, the most genetically diverse group on the planet. Algae can assume micro- and macroscopic shapes and can either be prokaryotic or eukaryotic [7]. Of all species of algae known, microalgae represent a total of 70% [5].

Bioeconomy is defined by the innovative use of sustainable biological resources that can cover the growing demand in several industrial sectors such as food and energy [8, 9]. For over two thousand years algae have been used as a source for food and treatment in countries such as China, Japan, Taiwan, and Australia [6, 7, 10-15]. However, microalgal biotechnology only started to be explored in the early 1950s, after the increase in the world's population and the need to find new protein sources [13, 15]. Of thousands of different species identified, only a small percentage is industrially cultivated, opening a window of opportunity for unravelling the potential of these organisms [13].

Microalgae are versatile and can be applied in a plethora of fields such as bioremediation processes, biofuel production, high-value compounds, foods and feeds, bioplastics, cosmetics and biostimulants [5, 10, 16-18]. Microalgae are fast-growing organisms that can achieve high biomass productivities [19]. Microalgae-based products thus represent several advantages when compared with conventional biological sources (high photosynthetic efficiency – up to 10%, ability to recycle nutrients) and therefore are progressively playing an important role in the economy [5, 10].

1.1. Morphological Aspects

Microalgal cell ultrastructure results from 3.5 billion years of evolution, presenting highly diversified morphological, physiological, and structural forms with a remarkable adaptative capacity [5, 11]. Microalgae are present in diverse ecosystems such as saline, estuarine, freshwaters, deserts, soils, and rocks among others [11]. As mentioned, these unicellular or pluricellular microorganisms can either be prokaryotic (cyanobacteria) or eukaryotic; and can be produced autotrophically, the most conventional method, heterotrophically or mixotrophically [19-21]. Concerning size, they can range between 0.5 and 200 μm . In single-

celled stalks, cells can be flagellated or not. In colonial forms, physical aggregation of cells by mucilage is often seen [5, 22].

Currently, 16 classes of these microorganisms are considered [20]. The major microalgae phylum are cyanobacteria, chlorophyta, euglenophyta, haptophyta, dinophyceae, eustigmatophyceae and rhodophyta [5, 7].

In this work, the attention will be directly focused on eukaryotic microalgae. The ultrastructure of eukaryotic cells is more complex, comprising organelles such as nucleus, mitochondria, chloroplasts, endoplasmic reticulum, Golgi apparatus and lysosomes [5, 7]. Eukaryotic microalgae have higher intracellular product storage than cyanobacteria – an advantage for the production of some molecules [5, 23].

1.2. Biotechnology applications: a market view

Bioactive molecules can be described as compounds that can interact and modulate the physiological or cellular activities of humans and animals [6, 24].

As mentioned, microalgae comprise a set of unique features that enable the generation of metabolites with numerous biotechnology applications [25]. These characteristics make these organisms interesting targets for bioprospecting and potential exploitation - from bulk mass in feed to purified high-value compounds (HVC) [7, 24, 26]. Microalgae have a sustainable, vegan and environmentally friendly status, representing alternatives for the current animal- and petrochemical-sourced compounds.

Such biotechnological applications include wastewater bioremediation, atmospheric CO₂ mitigation, dietary supplements for nutrition, cosmeceuticals, antimicrobials, antioxidants, anti-inflammatory, and anti-obesity agents among others [2, 6, 10, 12, 25, 27].

The use of these chemically diverse compounds is subjected to a strict regulatory process that can vary from country to country, adding an overall cost to product production [26, 28].

1.2.1. Nutraceuticals

Nutraceuticals describe any food product/compound that can simultaneously provide health benefits and nutritional value to the body [14, 26]. A nutraceutical can either be a whole food product or, a dietary supplement with a concentrated nutraceutical compound that holds a health benefit claim [14].

As described, microalgae have been used as a source of food and feed since ancient times. Around 75% of all microalgae production is dedicated to food and nutraceutical products [29]. Microalgae can enhance the nutritional value of conventional foods and thus positively

affect human and animal health [10, 12, 13]. The commercialization of “superfoods” obtained from microalgae - whole-biomass products - has been gaining recognition due high content in protein, nutrients and health benefits [7]. However, some microalgae can cause serious side effects raising some doubts about the commercialization of these organisms. Accordingly, it is important to detect harmful metabolites and optimize extraction methods for targeted compounds, instead of using whole biomass [7]. To date, *Chlorella*, *Arthrospira*, *Spirulina*, *Dunaliella*, *Scenedesmus*, and *Porphyridium* are the most prevalent microalgae present in nutraceuticals [2, 9, 17, 30].

In the United States of America (USA), a GRAS (Generally Recognized as Safe) status by U.S. Food and Drug Administration (FDA) unlocks the exploitation of microalgae as cell biofactories. This status allows the commercialization of microalgae-derived products, helping the cost reduction in downstream purification of compounds. In Europe, foods that contain pure compounds or are produced from microorganisms, fungi or algae are classified as novel foods – being subjected to European regulatory and legislation frameworks ((EU) 2015/2283, Regulation (EC) No 258/97) [12]. A novel food is any food that was not used for human consumption significantly in the European Union (EU) before 1997. As novel food, the microalgae-derived product must not present any risk related to hazardous compounds intrinsic to the biomass or, acquired in the downstream process [12]. The novel food approval process is time- and resource-consuming, however, the Notification of a traditional food from a third country may be a shortcut for legislation purposes [12, 31].

According to the Grand View Research report, in 2021, the global nutraceutical market size was evaluated at USD 455 billion and is expected to have a Compound Annual Growth Rate (CAGR) of 9.0% from 2021 to 2030.

1.2.2. Cosmetics and Cosmeceuticals

Skin is the largest human organ, that acts as the primary barrier against desiccation and penetration of ultraviolet (UV) radiation, therefore keeping organism homeostasis [32-34].

Cosmetics can be described as products used to improve structure, morphology, and skin appearance. According to European Commission ((EC) No.1223/2009), cosmetics can be applied to the external parts of the human body with the exclusive or principal objective to clean, perfume, protect or change their appearance [22, 35, 36]. Cosmeceuticals, also known as nutricosmetics, are characterized as cosmetics with bioactive ingredients that can provide therapeutic benefits (drug-like) to humans [15, 22, 32, 37]. The current demand for environmentally sustainable cosmetic products has created a vast market opportunity for the commercialization of products derived from natural sources [34, 38].

Microalgae and derived products comprise a set of unique chemical and biological properties that can be exploited for cosmetics and cosmeceutical applications [19, 35, 39]. These organisms are valuable producers of antioxidants; water binding, anti-ageing, UV protection, whitening and thickening agents; colourants, among others that can be easily incorporated into cosmetic formulations [2, 10, 15, 29]. *Chlorella*, *Arthrospira* and *Spirulina*, are the predominant genus in the skin care industry, commonly used in antiaging and anti-irritating preparations [2, 13, 14, 19, 35].

Cosmetic and cosmeceutical ingredients from whole microalgae or extracts require a range of regulations in order to be commercialized. According to EU legislation, algae are classified as botanical extracts. There is no clear legislation concerning the highest concentrated dose in microalgae-derived products, in general, in cosmetic formulations the content is less than 10% [40]. In March 2022, the addendum (EU) 2022/677 to the (EC) No 1223/2009 regulation was released, which contains the already approved microalgal extracts for cosmetic application as ingredients.

In 2021, the global cosmeceutical market was valued at USD 50.8 billion, and it is projected to grow to 96.2 billion by 2023 with a CAGR of 8.4% - data obtained from Fortune Business Insights forecast (2022-2029).

2. High-value compounds from microalgae

When in sufficient concentration, pure molecules (polar and non-polar) can be extracted from microalgae that can lead to valuable products [13, 26]. Microalgae are prolific producers of molecules with value to our health and well-being. These metabolites include carbohydrates, lipids, proteins, essential vitamins, and minerals [7, 10, 41-43] (**Figure 1**). The cellular content of microalgae varies with different strains and with physiological responses to biotic and abiotic factors (light intensity, photoperiod, temperature, and nutrients among others) [7, 10, 42].

In general, microalgae can have around 50% of protein content, 20 to 30% of lipids, up to 40% in polysaccharides, 8 to 14% in carotenes and high vitamin concentration [6, 29, 31]. Microalgae can exceed the productivity of higher plants, as they present less seasonal variation and can be easily extracted [10, 35, 39].

The search for these high-value chemicals has recently regained interest as spin-off research of microalgae as a source of biofuels [26]. Recent economic viability studies have

in fact proved that the production of renewable biofuels, microalgae-sourced, is only cost-effective if the process is coupled with HVC commercialization [7, 26].

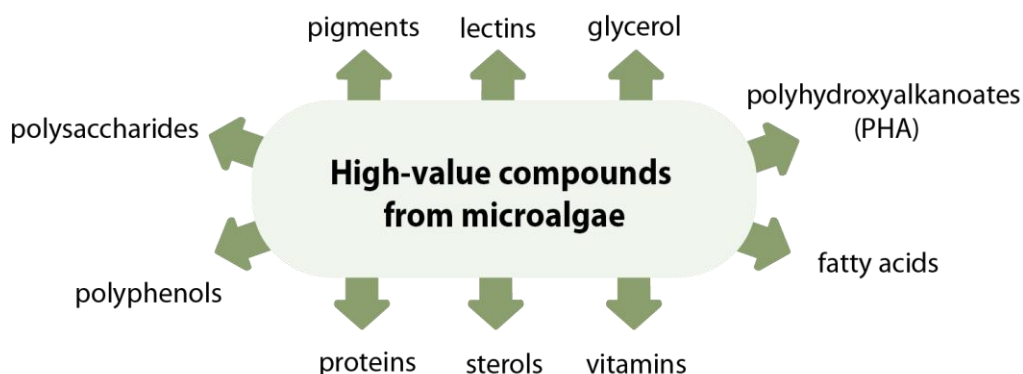


Figure 1. High-value compounds (HVC) produced by microalgae.

For the purpose of this work, the focus will be directed at phenolic compounds, pigments (carotenoids and chlorophyll derivatives), fatty acids (in particular long-chain polyunsaturated fatty acids) and polysaccharides.

2.1. Phenolic compounds

Phenolic compounds (PCs) are composed of at least two hydroxyl groups bonded to an aromatic ring in the case of phenols or connected to many aromatic rings - polyphenols (**Figure 2**). PCs are secondary metabolites that participate in defence mechanisms against biotic and abiotic stress factors, nutrition uptake and in the modulation of inflammatory stress [14, 38, 44, 45] - **Table 1**. These compounds are radical scavengers, that can donate hydrogen atoms or electrons [46, 47]. With regard to the chemodiversity (**Figure 2**), PCs are classified as flavonoids, tannins, phenolic acids, stilbenes and lignans [44].

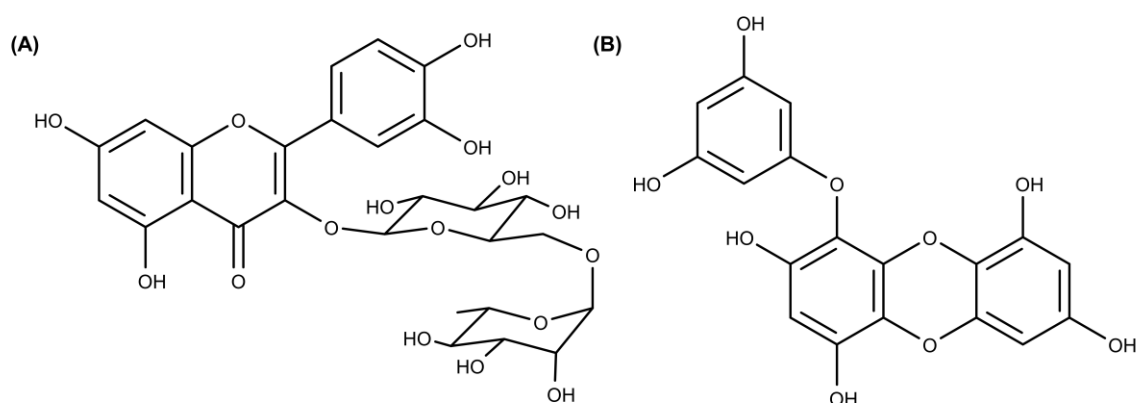


Figure 2. Chemical structure of some phenolic compounds present in microalgae: (A) rutin (flavonoid), (B) eckol (tannin).

By preventing oxidative stress, PC are desirable antioxidants. The associated toxicity risks of the use of synthetic antioxidants has opened a window for the search for new and natural oxidation inhibitors [6].

In blue biotechnology, research efforts have been focused on total phenolic (TPC) determination – the biochemistry study of single PC in microalgae is still very limited and heterogeneous [44].

In addition to PC, tocopherols, carotenoids, and phycobiliproteins are also excellent examples of microalgae-derived compounds that present antioxidant activity.

Table 1. Microalgae strains as producers of phenolic compounds (PC) and corresponding biotechnology applications.

Phenolic compound	Microalgae Source	Applications	Reference
Phenolic acids	<i>Chlorella</i> , <i>Desmodesmus</i> , <i>Dunaliella</i> , <i>Haematococcus</i> , <i>Scenedesmus</i> , <i>Tetraselmis</i> , <i>Porphyridium</i> , <i>Euglena</i> , <i>Nannochloropsis</i>	Antioxidant, antiviral, antiproliferative	[14, 44, 45, 47]
Flavonoids	<i>Chlorella</i> , <i>Dunaliella</i> , <i>Haematococcus</i> , <i>Scenedesmus</i> , <i>Tetraselmis</i> , <i>Porphyridium</i> , <i>Euglena</i>		[14, 44, 45]
Tannins	<i>Eckolnia</i> sp., <i>Eisenia</i> sp.	Antioxidant, anti-inflammatory, anti-allergic, anti-obesity, hepatoprotective	[44]

2.2. Pigments

Pigments are well known for their vital role in photosynthesis – light harvesting and energy transfer to reaction centres [24, 48, 49]. In microalgae, three classes of pigments can be identified: chlorophylls (chl), phycobilins and carotenoids lodged in the thylakoid membranes [24, 48]. In **Figure 3** the chemical structure of some pigments present in microalgae is represented.

Chlorophylls are porphyrins formed by four pyrrole rings with a centrally chelated magnesium ion; to the fourth pyrrole ring, a molecule of propionic acid is esterified by a long chain acyclic alcohol (phytol) [5, 24]. All photosynthetic organisms have chl *a*. Chl *b*, *c* or *d* can be present as accessory pigments [48, 50]. Although in industry, chlorophylls are primarily known as colourants, these pigments can play an important role in the health industry acting as antioxidants, antimutagenics, chemoprotective agents, anti-inflammatory

and antimicrobials [14, 24]. Chlorophylls can be further oxidized and form derivatives with a wide range of interesting activities [24, 51].

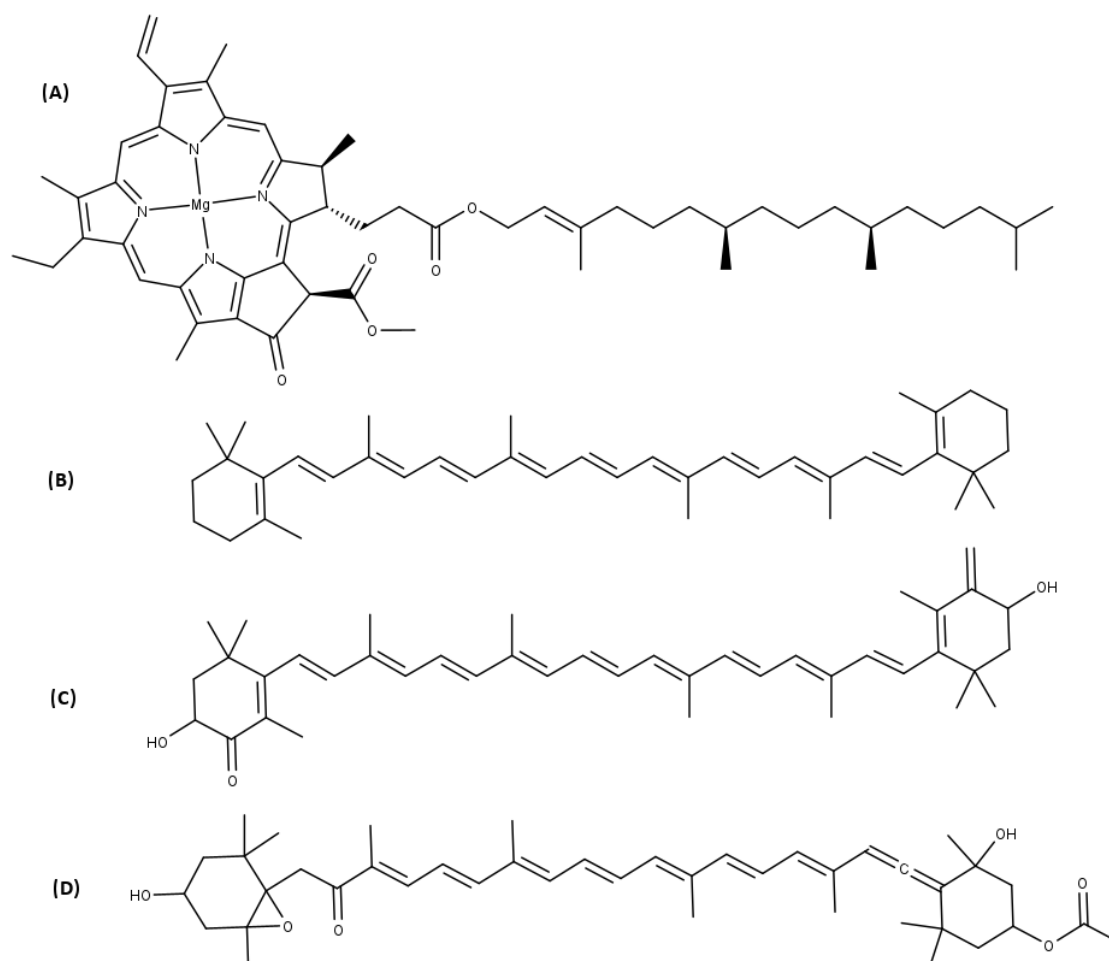


Figure 3. Chemical structure of some pigment compounds present in microalgae. (A) chlorophyll-a, (B) β-carotene (carotene), (C) astaxanthin (xanthophyll), and (D) fucoxanthin (xanthophyll).

Phycobilins are water-soluble pigments composed of a linear tetrapyrrole unit, linked to a cysteinyl residue of a protein – phycobiliprotein (PBP). PBPs are the major class of pigments-protein complexes exclusively present in cyanobacteria and algae (*Rhodophytes*, *Cryptomonads* and *Glaucocystophytes*) [24-26]. These hydrophilic pigments can be classified according to their chromophore content: phycocyanin (most common in cyanobacteria), phycoerythrin (most common in red algae), phycoerythrocyanin, and allophycocyanin [48, 50]. PBPs have intrinsic bioactive properties - antioxidant, anti-inflammatory, antiviral, antifungal, neuroprotective, anticancer - and can be used in the molecular biology and cytology fields as fluorescent markers [24, 25].

Carotenoids are a large group of lipophilic terpenoids vital for photosynthetic organisms – responsible for red, orange and yellow pigmentation [14, 24, 41, 50, 52]. Carotenoids

contain a 40-carbon methyl branched hydrocarbon backbone and polyene chains that are conjugated with double bonds [5, 50, 53, 54]. The extension of the conjugated double bond is directly proportional to the antioxidant capacity [55]. Carotenoids comprise two compound classes: carotenes and xanthophylls. Carotenes (α and β -carotene) are composed of carbon and hydrogen, while xanthophylls (e.g. violaxanthin, zeaxanthin, neoxanthin, lutein) are oxygenated derivatives with functional groups: hydroxyl-, oxi- or both [48, 50, 52, 56]. Carotenoids can be also classified as primary (crucial for survival) or secondary (protection mechanisms) taking into account their role in the cell [5]. Besides colourants and powerful antioxidants, microalgae-derived carotenoids can be employed in the treatment of neurodegenerative, cardiovascular, and inflammatory diseases as well as cell ageing [24, 49, 52, 57].

In general, adding up to their biological role, pigments have applications in food, feed, pharmaceutical, nutraceutical, aquaculture and cosmetic industries due to their colouring, antioxidant, anti-inflammatory, neuroprotective and hepatoprotective properties [48, 50]. The microalgae strains that are well documented in literature as pigment producers (chlorophylls, carotenoids and phycobilins) are displayed in **Table 2**.

Table 2. Microalgae strains as producers of pigments and respective biotechnology applications.

Pigment	Microalgae Source	Applications	Reference
Chlorophylls & derivatives	All	Food colourant (E140), antioxidant, antimutagenic, chemoprotective, anti-inflammatory and antimicrobial	[48, 50]
β-carotene	<i>Dunaliella salina</i> , <i>Arthrospira maxima</i> , <i>Chlorella vulgaris</i> , <i>C. saccharophila</i>	Antioxidant, provitamin A, AMD, liver fibrosis, anti-inflammatory, food colourant	[4, 6, 13, 14, 26, 29, 54, 58-63]
Astaxanthin	<i>Haematococcus pluvialis</i> , <i>Chlorella zofigiensis</i> , <i>Chlorella vulgaris</i> , <i>Chlorococcum</i> sp., <i>Scenedesmus</i> sp., <i>Xanthophyllomyces dendrorhous</i>	Pigment (aquaculture feed), antioxidant (able to span the lipid bilayers and provide superior antioxidant defence), anti-inflammatory, anticancer, eye health	[4, 6, 14, 26, 29, 53, 54, 58, 61, 64-71]
Lutein	<i>Dunaliella salina</i> , <i>Chlorella pyrenoidosa</i> , <i>Chlorella protothecoids</i> , <i>Scenedesmus</i> spp., <i>Muriellopsis</i> sp., <i>Dunaliella Salina</i>	Pigment, food, cosmetics, AMD, atherosclerosis, retinal neural damage, anticancer	[4, 14, 26, 29, 39, 53, 58, 59, 61, 72]
Zeaxanthin	<i>Porphyridium cruentum</i> , <i>C. protothecoids</i> , <i>C. saccharophila</i> , <i>Scenedesmus almeriensis</i> ,	Antioxidant, maculopathy, cataracts, anti-inflammatory	[4, 29, 54, 58, 61]

	<i>Nannochloropsis oculata</i> , <i>Dunaliella tertiolecta</i> , <i>Pavlova</i> <i>lutheri salina</i> & sp., <i>Synechocystis</i>		
Violaxanthin	<i>Chlorella ellipsoidea</i> , <i>Phaeodactylum tricornutum</i>	Food colouring, Anti-inflammatory, anti-cancer	[4, 26, 58]
Fucoxanthin	<i>Chlorella</i> spp., <i>Phaeodactylum tricornutum</i> , <i>Lobosphaera insicia</i>	Antioxidant, anti-inflammatory, anti-cancer, anti-obesity	[26, 59, 73, 74]
Canthaxanthin	<i>Coelastrella</i> , <i>Chlorella ellipsoidea</i>	Food colouring agent, potent lipid-soluble antioxidant, immunomodulatory	[14, 15, 29]
Lycopene	<i>Anabaena vaginicola</i>	Antioxidant (cosmetics), anti-carcinogenic, antiatherogenic	[15]
Phycocyanin	<i>Arthrospira platensis</i> , <i>Arthronema africanum</i> , <i>Nostoc</i> sp., <i>Phormidium valderuanum</i> , <i>Galdiera sulphuraria</i>	Immunological markers, dyeing agents, antioxidants, vitamin precursors, immune activators, ant-inflammatory, antiviral, antifungal, antihyperglycemic, neuroprotective, hepatoprotective	[24, 26, 53, 60, 61, 75]

The market for microalgae pigments is dominated by carotenoids. With the increasing demand for the use of these pigments - grown from natural sources, the carotenoid market is expected to have a CAGR of 3.5% reaching USD 1.6 billion in 2023 [29].

2.3. Polyunsaturated Fatty Acids (PUFAs)

Fatty acids (FA) are aliphatic hydrocarbon chains (with an even number of carbon atoms) saturated or unsaturated, with a carboxyl group at one end and a methyl group in the omega (ω) end.

FAs can be classified according to the double covalent bonds: saturated (SFAs), monounsaturated (MUFAs) and polyunsaturated (PUFAs) [24, 25]. In **Figure 4** the chemical structure of different fatty acids is illustrated.

PUFAs, can be classified according to the position of the first double bond from the ω end. Omega-3 (ω -3) - such as α -linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) - are considered essential fatty acids once they cannot be produced by the human organism [10, 14, 24]. Likewise, omega-6 (ω -6) – for example, linoleic acid (LA), γ -linolenic acid (GLA) and arachidonic acid (AA) – are also considered essential, acquired only through diet [10, 13, 76]. Fish oil is the conventional source of ω -3 FAs, nevertheless, these animals obtain ω 3 through diet (marine microalgae). Fish consumption has several disadvantages (mercury contaminants, decrease in fish stock) when compared to microalgae [61]. These compounds, especially the long chain PUFAs,

have recently earned a good deal of attention once they are fundamental for normal brain development and cardiovascular health – physiological function regulation [10, 14, 26, 54].

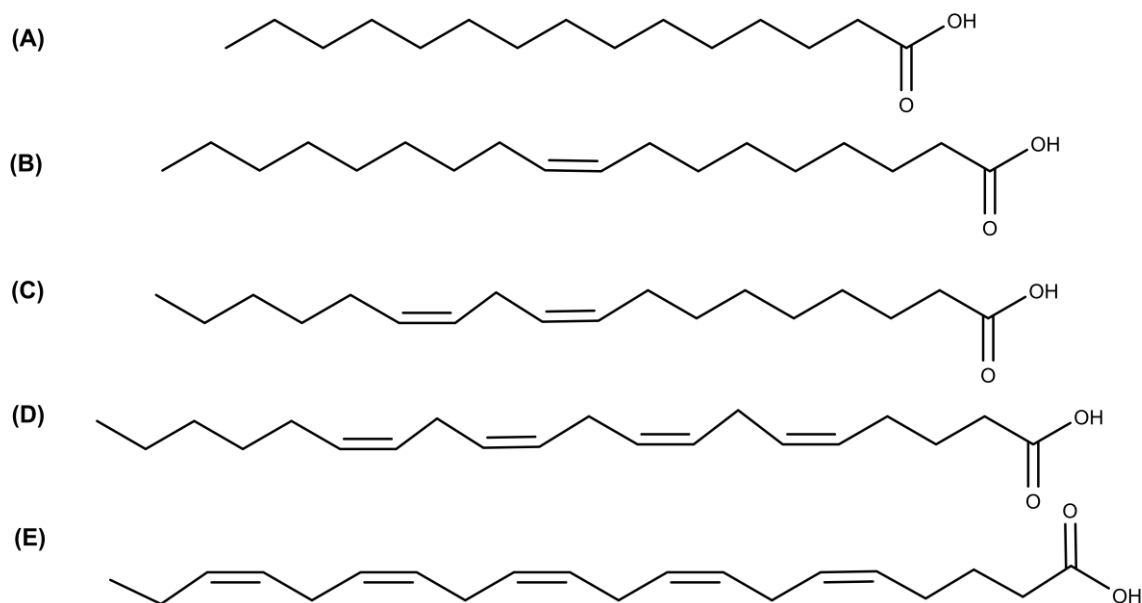


Figure 4. Chemical structure of some fatty acids present in microalgae. (A) palmitic acid (SFA, C16:0), (B) oleic acid (MUFA, C18:1 ω -9), (C) linoleic acid (PUFA, C18:2 ω -6), (D) arachidonic acid (PUFA, C20:4 ω -6) and, (E) eicosapentaenoic acid (PUFA, C20:5 ω -3).

Microalgae are keen producers of these fatty acids, with reports showing that in some strains, 30%–45% of the total fatty acids are composed of EPA [10, 24]. PUFAs have a plethora of health applications (**Table 3**): cardiovascular diseases, diabetes, inflammatory disease, atherosclerosis, cancer and age-related diseases [10, 24, 26, 59]. Studies have shown that high ratios of ω -6/ ω -3 promote cardiovascular diseases, cancer, and inflammatory and autoimmune diseases – on the other hand, lower ratios appear to have suppressive effects [24, 77].

Table 3. Microalgae strains as producers of polyunsaturated fatty acids (ω 3 and ω 6) and respective biotechnology applications.

Fatty Acid	Microalgae Source	Applications	Reference
α-linolenic acid (ALA, C18:3 ω -3)	<i>Nannochloropsis</i> spp. <i>Arthrospira</i>	Instant formula for full-term infants, nutritional supplements	[13, 58, 61]
Eicosapentaenoic acid (EPA, C20:5 ω -3)	<i>Phaedactylum tricornutum</i> , <i>Nitzschia</i> , <i>Schizochytrium</i> , <i>Porphyridium cruentum</i> , <i>Cryptocodinium cohnii</i> , <i>Nannochloropsis</i>	Reduces the occurrence of chronic diseases, lowers cholesterol triglycerides, instant formulas for infants,	[13, 14, 20, 26, 58, 59, 61, 78]

Docosahexaenoic acid (DHA, C22:6 ω -3)	<i>Schizochytrium</i> spp., <i>Ulkenia</i> spp., <i>Cryptocodinium</i> , <i>Monodus subterraneus</i> , <i>Thraustochytrium</i> sp., <i>Nannochloropsis</i> , <i>Phaedactylum</i> , <i>Nitzschia</i>	aquaculture, antineoplastic (haematological tumours), anti-inflammatory	[6, 13, 14, 26, 58, 59, 61, 78-80]
Linoleic acid (LA, C18:2 ω -6)	<i>Porphyridium cruentum</i> and <i>purpureum</i> , <i>H. pluvialis</i>	Emollient and moisturiser (cosmetic formulations), feed and food supplementation, antioxidant, cholesterol reduction	[6, 14, 20]
Arachidonic acid (ARA, C20:4 ω -6)	<i>Parietochloris incisa</i> , <i>Arthrospira platensis</i> , <i>Porphyridium</i>	Infant formulas for full- term/preterm infants, nutritional supplements	[6, 13, 26, 42]
γ-linoleic (GLA, C18:3 ω -6)	<i>Arthrospira</i>	Infant formulas for full-term infants, nutritional supplements	[6, 42]
Oleic acid (OA, C18:1 ω -9)	<i>Porphyridium cruentum</i>	Cholesterol reduction, lowers blood pressure, reduces risk of cardiovascular diseases, body weight regulation	[14]

The rising attention towards PUFAs and their respective health benefits allowed a huge market development. In 2019 the market share was USD 4.7 billion and a CAGR of 12.1% that would reach USD 8.5 billion by 2025, according to Markets and Market (2019) [29].

2.4. Polysaccharides

Polysaccharides are biopolymers present in all algae, formed by two or more monosaccharides monomers – composed of the same or different units [24, 61, 81, 82]. These highly diverse molecules have important functions in microalgae: storage, signalling, structure, and protection from stress factors [83-86].

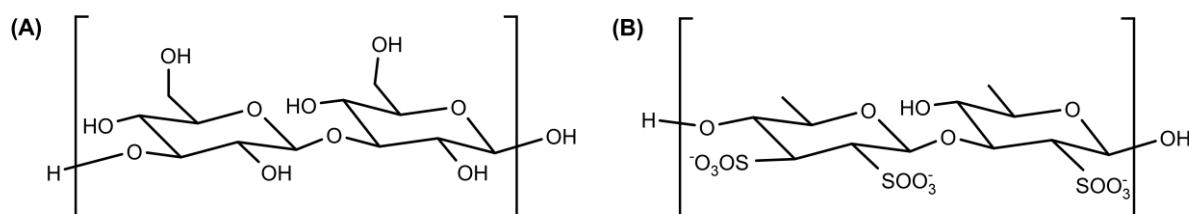


Figure 5. Chemical structure of some polysaccharide compounds present in microalgae. (A) paramylon (β-glucan) and, (B) fucoidan (sulfated polysaccharide).

Microalgae polysaccharides can be divided into three main groups: intracellular, structural and extracellular glycans [82, 87, 88] - in **Figure 5** the chemical structures of some of these polymers are represented.

Exopolysaccharides are water-soluble molecules secreted to the cell surroundings, having a role in biofilm assembly and other physiological processes [34, 82, 89, 90]. EPS are composed of sugars and uronic acid [85, 91]. In turn, sulfated EPS, with carboxyl and sulfate groups, present high molecular weights and anionic and acidic properties [24]. β -glucans are another type of polysaccharide, composed of D-glucose monomers joined by β -glycosidic bonds. They can either be water-soluble or insoluble [24].

Polysaccharides, in special sulfated exopolysaccharides, are described as anti-inflammatory, immunomodulators, anti-viral, antioxidants and neuroprotectors [14, 15, 24, 32, 82, 92]. In cosmetics formulations, polysaccharides can be used as gelling, thickener, and moisturizing agents. β -glucans have a special role in preventing external ageing once they are free-radical collectors and immunostimulants; as they also have anti-inflammatory activity, they can be applied to formulations for sensitive and reactive skin types (**Table 4**) [15, 35, 36, 40].

Table 4. Microalgae strains as producers of polysaccharides and respective biotechnology applications.

Polysaccharide	Microalgae Source	Applications	Reference
Exopolysaccharides (EPS)	<i>Gleocapsa</i> sp., <i>Chlorella</i> sp., <i>Chlamydomonas mexicana</i>	Water retention capacity, texturing agents, thickening, gelling, stabilizing, emulsifiers, antioxidant, antiviral, Antifungal	[42, 53, 58]
Sulfated EPS	<i>Rhodella</i> , <i>Scenedesmus ovalternus</i> , <i>Porphyridium</i> spp.	Inhibition of PMN (polymorphonuclear leukocytes) migrating toward chemoattracting molecules	[14, 24, 26, 58]
Fucoidan (sulfated EPS)	<i>Synurophyceae</i> , <i>Prymnesiophyceae</i>	Anticancer, antihypertensive, anti-inflammatory, and anti-obesity effects	[31, 93]
β-glucans	<i>Porphyridium purpureum</i> , <i>Porphyrium</i> sp.	Immuno-stimulating, hepatoprotective, antiproliferative, antioxidative, and antiviral effects	[20, 24, 58]
Paramylon (β -glucan)	<i>Euglena gracilis</i>	Immunostimulant, antimicrobial, lower cholesterol level, antidiabetic, antihypoglycemic, hepatoprotective	[24, 94-96]

3. Microalgae to product: general workflow

The journey for the commercialization of microalgae or microalgae-derived products begins with a strain screening for HVC potential. Following the cultivation process, the biomass needs to be harvested and processed. The biomass can be incorporated in products as - whole biomass, extract, or pure compound format. The last two formats mentioned require an extraction and purification downstream process. After the matrix transformation, a final product can be commercialized once the legislation to market entry is cleared. The overview of the industrial HVC production process from microalgae is schematized in **Figure 6**.

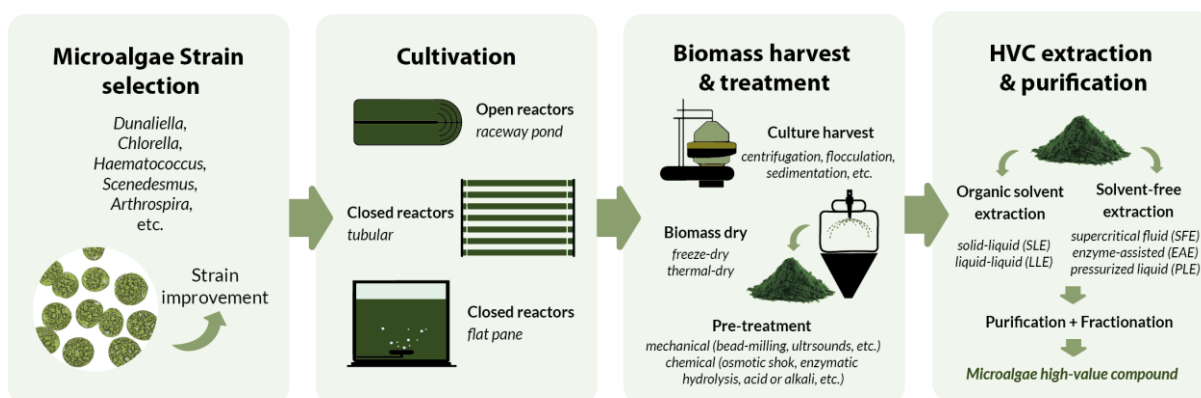


Figure 6. Overview of the industrial workflow of microalgae production of high-value compounds (HVC).

3.1. Improvement of microalgae strains

In order to enhance the synthesis of HVC, the first step that should be taken is the bioprospecting and screening of microalgae strains that have the potential for selective biomolecule production [29]. However, the future feasibility of the production of some compounds depends on the optimization of microalgae growth or the introduction of genetic or metabolic modifications that can influence HVC synthesis. There is a need to develop novel strains that can grow faster and have higher yields with less demanding resources, such as light and temperature [10, 25, 27].

3.2. Microalgal biomass production

In the early 1960s, the first large-scale cultures of microalgae for commercialization were being launched with *Chlorella*. By 1980, algae production facilities were already established in Asia, India, USA, Israel and Australia [23]. Since then, biomass production has been increasing worldwide [25]. In near the future, microalgae industrial scale cultivation will play an important role in the growth of sustainable industries with cost-effective HVC production [27].

According to the European Marine Observation and Data Network (EMODnet), in Europe (2019), there were 126 algae-producing companies, 42% of which focused on microalgae (**Figure 7**) [97]. This emerging sector is aligned with European Union's Green Deal priorities, which declare that the biomass demand must be environmental, social and economically sustainable.

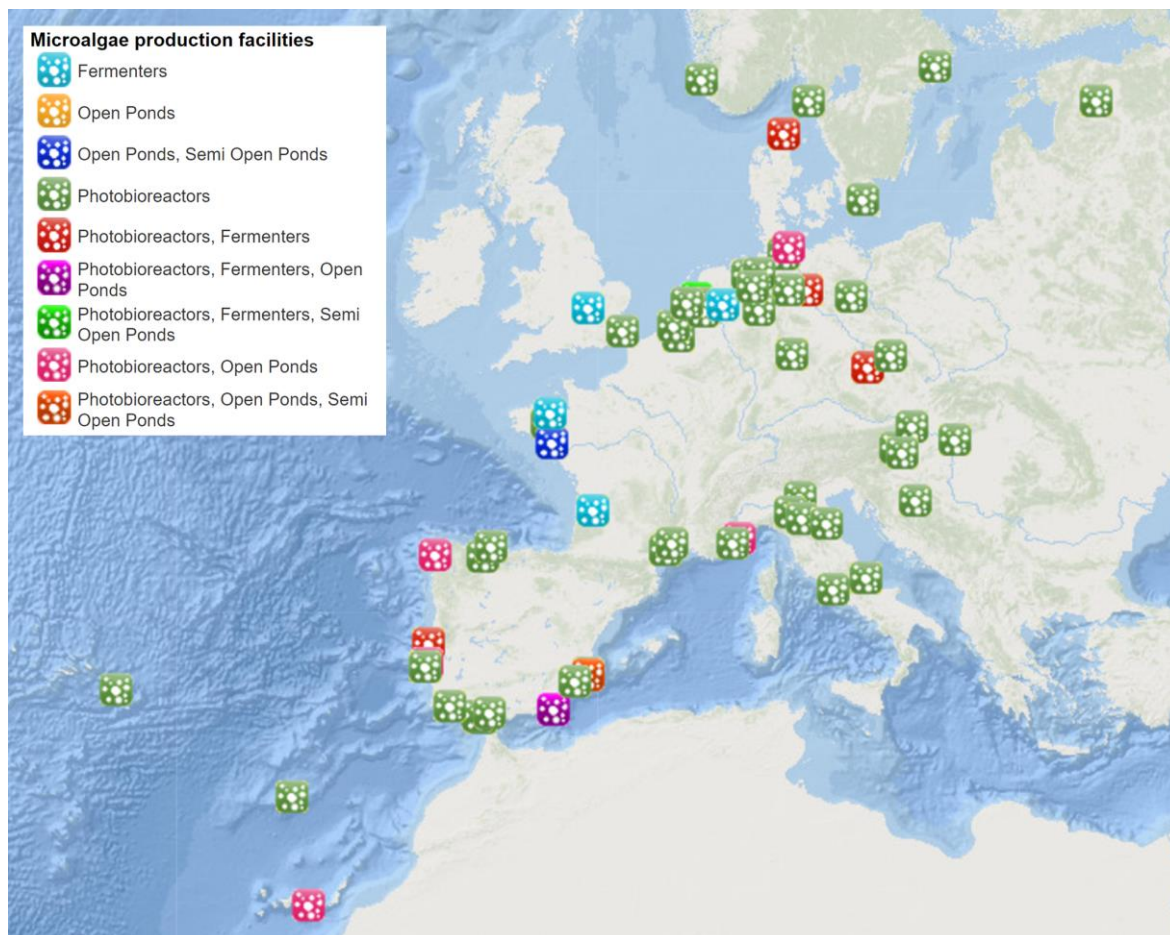


Figure 7. Microalgae production facilities in Europe, from European Marine Observation and Data Network (EMODnet), 2020.

Microalgal production systems need special conditions to operate in a sustainable mode: efficient design to maximize sunlight exposure (limiting factor) and use of low-impact materials and nutrients. For this to happen, culture conditions must be adapted and optimized for each strain being produced [19].

Microalgae can be grown in open ponds or closed systems such as fermenters or photobioreactors [28, 98]. In open reactors, like raceway ponds, the culture is in direct contact with the atmosphere thus exposed to sunlight and possible contaminations. Although culture conditions cannot be reliably controlled, open ponds represent more than 90% of the global autotrophic production of microalgae, once they require minimal amounts

of energy and materials that can help reduce biomass production costs, with sale values up to 2 euros per kg [13, 19, 99]. In closed systems (e.g. fermenters, reactors) growth conditions can be manipulated to manage compound production, quality and traceability [24], thus a greater number of resources is needed to operate these reactors, increasing biomass production costs (at least 20 euros per kg). These production systems are therefore mostly applied for the production of high and medium-value biomass [19].

3.3. Biomass harvest and treatment

After microalgae growth, the biomass needs to be harvested and processed. Harvesting processes include sedimentation, flocculation, centrifugation, or filtration [10, 98].

Often a drying step is needed, this can be accomplished with either thermal or freeze-drying [27, 98]. Prior to extraction, the biomass can be pre-treated to increase efficiency. A cell disruption step can be implemented – mechanical (bead-milling, high-pressure, ultrasounds, etc.), and non-mechanical methods (osmotic shocks, ionic liquids, enzymatic hydrolysis, etc.) [29, 50, 98, 100].

3.4. HVC purification, downstream process

Once the biomass is harvested, the bioactive compounds can be extracted with several different techniques. The extraction is the limiting step, being the most expensive in the process [98]. These methods directly influence the yield and activity of the extracted compounds, thus, there is a need for method optimization that takes into consideration: solvents, pH, temperature, and pressure among others [6].

Conventionally, microalgae HVC are extracted with classic solvent extraction and recovery methods, such as solid-liquid extraction (SLE) and liquid-liquid extraction (LLE) [6, 10, 50]. These methods often make use of high volumes of organic solvents (with different polarities) to penetrate cell membranes (e.g. hexane, dichloromethane, isopropanol, acetone, methanol). Solvent-dependent methods have several environmental concerns due to potential toxicity, they can present challenges in separation, and they can be very time-consuming (multiple extractions and purification steps) [6, 52, 98, 101]. In the past few years, efforts have been put into place to optimize solvent-free extraction methods such as pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), enzyme-assisted extraction (EAE) among others [29, 52, 98, 101]. These environmentally friendly methods present higher extraction yields, faster extraction times and they preserve HVC without loss of activity. However, purity and economic efficiency is still a limiting step [6, 10]. Further purification steps can include degumming, caustic refining,

deodorization, filtration and chromatographic methods. In regard to fractionation, membranes and resins are often used [22].

3.5. Microalgae market

After extraction and purification, the HVC or microalgae extracts are ready for commercialization. These products can enter the pharmaceutical, feed, food, nutraceutical, cosmetic and cosmeceutical markets as ingredients or additives. The value of microalgae-derived products relies on different variables: composition, purity level, application, and formulation [10].

Microalgae are sustainable and biochemical diverse organisms, that have earned status as producers of important and innovative resources to society with many different applications. In the past few decades, a lot of investment has been put in place to fund the screening of bioactive metabolites from microalgae [7].

According to the Data Bridge Market Research and Transparency Market Research reports, the microalgae market reached USD 950 million in 2021, and it is projected to reach USD 1.5 billion by 2029, with an expected CARG of 6.20%. Moreover, there is a great market opportunity for the development of microalgae-derived products. To accomplish a cost-efficient production, there is still a need for continuous research and enhancement of each step of the industrial workflow in microalgae biotechnology.

4. Objectives

The main goal set for research and development (R&D) work was the bioprospection of eukaryotic microalgae strains capable of producing HVC with application in nutraceutical and cosmetic industries, two of the main interest areas of Fykia Biotech company.

Twenty-two microalgae strains were selected from the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC). Microalgae cultures were grown under standard conditions, the biomass was harvested and dried for extraction. For coccoid strains, a growth curve was implemented with the interest of accessing the optimal growth for biomass harvest and HVC production. All strains were extracted with organic solvents: ethanol (70%) and dichloromethane:methanol (2:1, v/v) - according to HVC polarities and classical extraction methods.

All strains were screened for the presence of phenolic compounds (PC), pigments (carotenoids and chlorophylls), fatty acids and polysaccharides. Phenolic compound quantification was conducted with ethanolic extracts in a colourimetric assay with the Folin-Ciocalteu reagent. Carotenoids and chlorophylls were identified and quantified by high-

performance liquid chromatography (HPLC) coupled to a photodiode array detection system (PDA) – with ethanolic extracts. Fatty acids screening was achieved with dichloromethane:methanol (2:1, v/v) extracts, after a derivatization step to form fatty acid methyl esters (FAMES). FAMES were identified and quantified by gas-chromatography mass-spectrometry (GC-MS). Finally, polysaccharides were first qualitatively analyzed with Alcian blue dye - the content of most promising microalgae strains was then analysed by the Dubois assay - with ethanolic extracts.

II. Materials and Methods

1. Microalgae strains

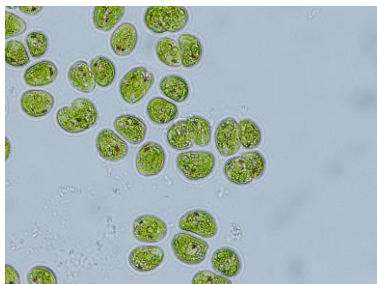
After literature and legislation research, twenty-two fresh-water microalgae strains (**Table 5** and **Figure 8**) were selected from the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC) [102] – a Fykia Biotech (FB) code was given to each microalgae strain. The strains were morphological identified, according to AlgaeBase classification [103]. The selection was based on the family and genus of promising microalga described for HVC production and/or with GRAS status granted by the FDA and/or approved for consumption/use by the European Food Safety Authority (EFSA).

Table 5. Selected microalgae strains for HVC screening.

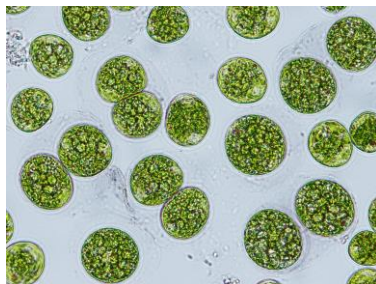
Strain Code	Genus	High-Value Compounds (HVC)	Reference
FB01M	<i>Euglena</i>		
FB02M	<i>Euglena</i>	β-carotene, L-ascorbic acid, PUFAs	[104]
FB03M	<i>Euglena</i>		
FB04M	<i>Tribonema</i>	Eicosapentaenoic acid	[105]
FB05M	<i>Eustigmatos</i>	Palmitoleic acid, Violaxanthin, Eicosapentaenoic acid	[106-108]
FB06M	<i>Chlamydocapsa</i>	Lutein, canthaxanthin and astaxanthin	[76]
FB07M	<i>Haematococcus</i>	Astaxanthin, Arachidonic Acid (AA), α-tocopherol	[4, 6, 14, 26, 29, 54, 61]
FB09M	<i>Haematococcus</i>		
FB10M	<i>Pandorina</i>	nf	-
FB12M	<i>Coelastrella</i>	Canthaxanthin, neoxanthin, pheophytin a, astaxanthin, lutein, violaxanthin, PUFAs	[29, 109, 110]
FB13M	<i>Oocystis</i>	Linolenic acid, Eicosapentaenoic acid	[111]
FB14M	<i>Chlorella</i>	β-carotene, Astaxanthin, Lutein, Zeaxanthin, Violaxanthin, Fucoxanthin, Canthaxanthin, Vitamin B9, Vitamin C, Exopolysaccharides (EPS)	[4, 6, 14, 26, 29, 54, 58, 61, 112]
FB15M	<i>Chlorella</i>		
FB08M	<i>Scenedesmus</i>		
FB11M	<i>Scenedesmus</i>		
FB16M	<i>Scenedesmus</i>	Astaxanthin, Lutein, Zeaxanthin, α-tocopherol, Sulphated	[24, 26, 53, 58, 59, 64, 113]
FB17M	<i>Scenedesmus</i>	EPS/polysaccharides (SPS), Linoleic Acid	
FB18M	<i>Scenedesmus</i>		
FB19M	<i>Scenedesmus</i>		
FB20M	<i>Microthamnion</i>	nf	-
FB21M	<i>Actinastrum</i>	nf	-
FB22M	<i>Sphaerocystis</i>	nf	-

nf: not found in literature

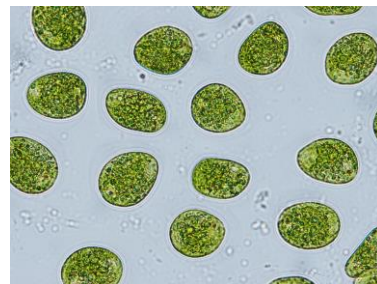
In **Figure 8** the microalgae morphology is shown through microscopic images captured from all strains selected for HVC screening. The microalgae strains were not axenic throughout all culture growth.



FB01M *Euglena* sp.



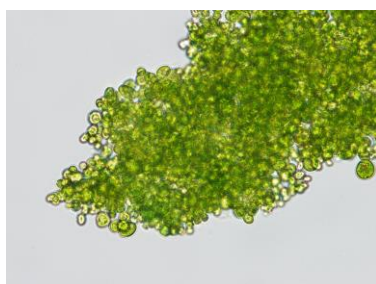
FB02M *Euglena* sp.



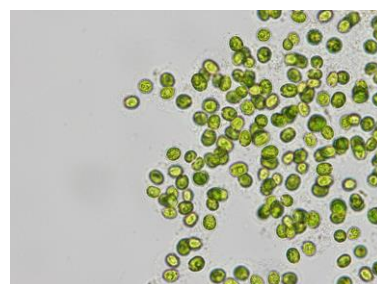
FB03M *Euglena* sp.



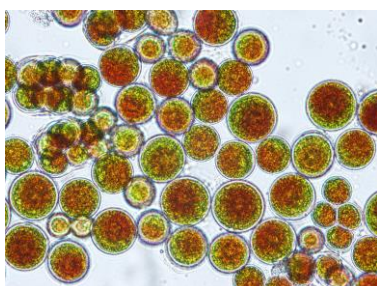
FB04M *Tribonema* sp.



FB05M *Eustigmatos* sp.



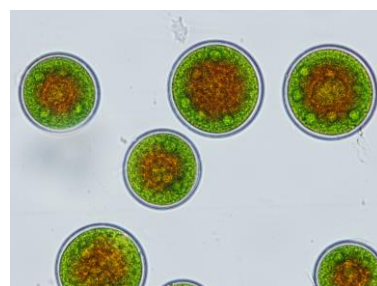
FB06M *Chlamydocapsa* sp.



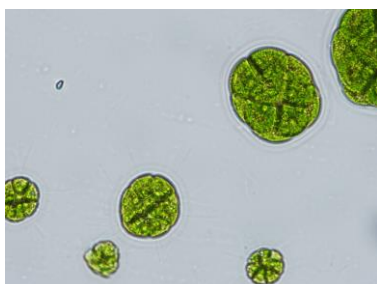
FB07M *Haematococcus* sp.



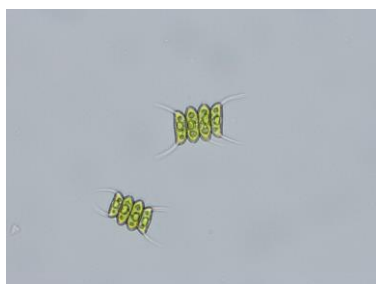
FB08M *Scenedesmus* sp.



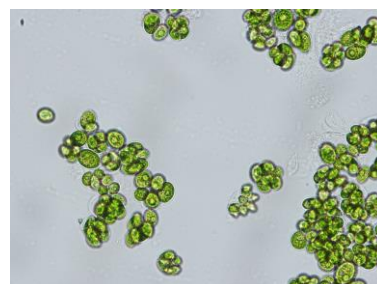
FB09M *Haematococcus* sp.



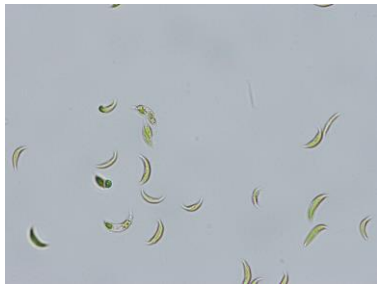
FB10M *Pandorina* sp.



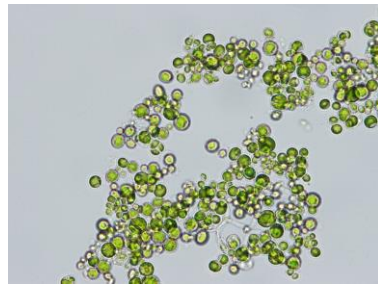
FB11M *Scenedesmus* sp.



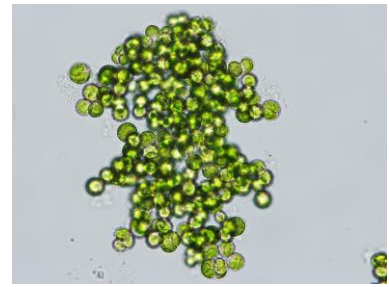
FB12M *Coelastrrella* sp.



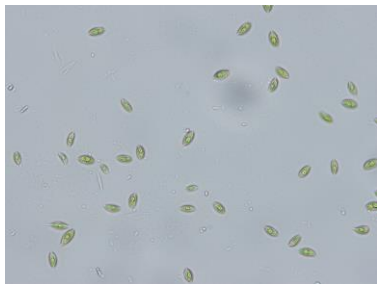
FB13M *Oocystis* sp.



FB14M *Chlorella* sp.



FB15M *Chlorella* sp.



FB16M *Scenedesmus* sp.



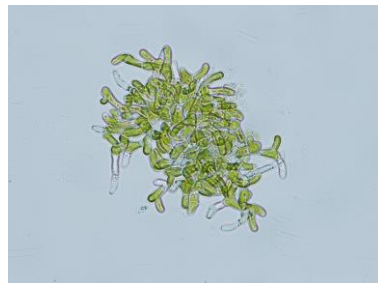
FB17M *Scenedesmus* sp.



FB18M *Scenedesmus* sp.



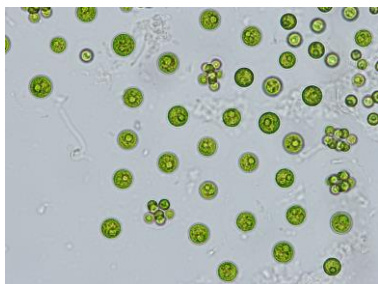
FB19M *Scenedesmus* sp.



FB20M *Microthamnion* sp.



FB21M *Actinastrum* sp.



FB22M *Sphaerocystis* sp.

Figure 8. Microscopic view of selected microalgae strains and respective classification at the genus level.

1.1. Standard growth conditions and biomass harvest

A standard culture growth (**Figure 9**) was carried out to obtain enough biomass to proceed with the extraction, identification, and quantification of HVC produced by the selected microalgae strains.

All microalgae cultures were grown up to 400 mL in Z8 medium (Kotai, 1972) at 25 ± 2 °C, under a 16:8 h light/dark cycle and constant aeration. Genus *Euglena* strains (FB01M, FB02M and FB03M) were also grown in M7 medium (potassium nitrate, KNO_3 (10 mL L^{-1}); magnesium sulfate heptahydrate, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (10 mL L^{-1}); diammonium hydrogen phosphate, $(\text{NH}_4)_2\text{HPO}_4$ (5 mL L^{-1}); calcium sulfate, CaSO_4 (10 mL L^{-1}), soil extract (100 mg L^{-1}); vitamin B12 (1 mL L^{-1}); and micronutrients solution (5 mL L^{-1})) in the same temperature and light conditions.

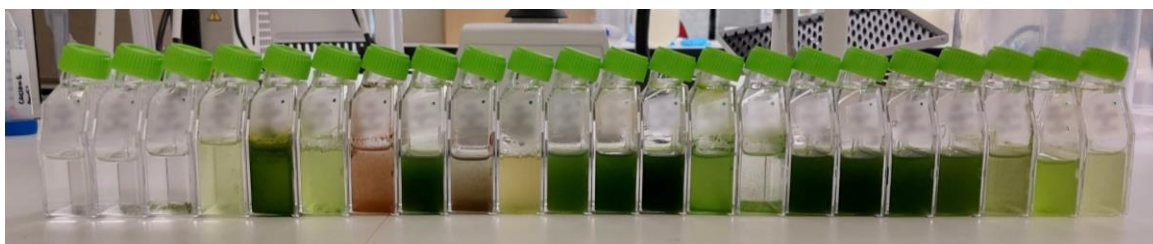


Figure 9. Microalgae strains in 40 mL of Z8 medium for standard culture growth.

When optimal density (750 nm) was reached, at the exponential growth phase, microalgae biomasses were harvested by centrifugation (8000 G for 10 min at 4 °C) or filtration for the filamentous strain, FB04M. After freeze-dried, the biomasses were stored at room temperature (RT) until further use. The biomass harvest process was repeated as many times as needed for each strain, according to the quantity needed for further HVC screening.

1.2. Growth curve

The growth rate of microalgae strains was accessed by turbidimetric determination, an indirect measure of biomass concentration in pigmented cells.

Optic density was read for the coccoid strains that could form an even suspension: FB05M, FB06M, FB08M, FB10M, FB11M, FB12M, FB13M, FB14M, FB16M, FB17M, FB18M, FB19M, FB21M and FB22M. The coccoid microalgae strains were grown in 40 mL of Z8 medium (Kotai, 1972) at 20 ± 2 °C, under a 14:10h light/dark cycle and maintained for at least 110 days until they reach the stationary phase.

For at least 15 weeks, the optical density (OD) of 1 mL of culture of the selected strains was read three times at 750 nm in a spectrophotometer, on Mondays, Wednesdays and Fridays.

The wavelength was chosen outside of the range of maximal absorbance by the major pigments to reduce reading errors [114].

When required, sequential dilutions were applied, and the OD (750 nm) units were then corrected taking into consideration the dilution factor. A growth curve was assembled for each of the coccoid strains to later determine the optimal OD for biomass harvesting.

2. Extractions for HVC screening

After harvested and freeze-dried, the biomass from all strains was extracted with organic solvents for further HVC screening through different methodologies.

2.1. Ethanolic extraction

An ethanolic extraction (GRAS solvent) was conducted for all microalga strains in order to determine total phenolic content (TPC), pigments (carotenoids and chlorophylls) and polysaccharides.

An established quantity of freeze-dried biomass of each strain was suspended in Ethanol (70%, C₂H₅OH) and vortexed for a few seconds for homogenization. Cells were disrupted by sonication (Ultrasonic probe sonicator, 60% Am for 3 min, pulse on for 30 s and off for 5 s) on ice - cell debris was removed by centrifugation (8000 G for 5 min at 4 °C) and the extracted content collected after cotton filtration. The extraction process was repeated at least 4 more times, to a final volume of ~0.6 mL of solvent per 1 mg of freeze-dried biomass.

Combined extracts were evaporated with the help of a rotary evaporator under reduced pressure (recirculating chiller at -8 °C and heating bath at 30 °C) and a CentriVac Vacuum Concentrator (SpeedVac). Dried content was weighed and stored in vials at -20 °C until further use (**Figure 10**). For HVC content screening different aliquots were created for the different assays.



Figure 10. Ethanolic extracts (70%) of all microalgae strains for HVC screening.

2.2. Dichloromethane:methanol extraction (Folch Method)

The total lipid content of all microalgae strains (FB01M - FB22M) was extracted by an adaption of the Folch method [115, 116], replacing the use of chloroform with dichloromethane (DCM). Lipids were extracted from 25 mg of freeze-dried biomass using 2 mL of dichloromethane:methanol (2:1, v/v) (DCM, CH₂Cl₂; MeOH, CH₃OH) mixture, the suspension was vortexed for 2 min and phase separation was achieved by centrifugation (2000 rpm for 10 min at 4 °C), and the organic phase was collected. This step was repeated at least four times (5 × 2 mL), and the combined organic phases (total extracts) were dried under nitrogen (N₂) stream, to prevent further oxidation.

Total extracts were subjected to a purification step to remove non-lipid contaminants. The initial extract was re-dissolved in 2 mL of DCM, 1 mL of MeOH and 0.75 mL of water (H₂O). The mixture was vortexed for 1 min followed by centrifugation (2000 rpm for 10 min at 4°C) to obtain phase separation. The organic phase was collected, and the aqueous phase was re-extracted two more times with 2 mL of DCM. Combined extracts were dried under a N₂ stream and stored in vials at -20°C after being weighted (**Figure 11**).

Each series of lipids extractions was performed three times. In order to prevent phthalate contamination, the material used for extraction was mainly composed of glass.



Figure 11. Dichloromethane:methanol (2:1, v/v) extracts of all microalgae strains for HVC screening.

3. Determination of total phenolic content (TPC)

The total phenolic content (TPC) of microalgae ethanolic extracts was determined by the Folin-Ciocalteu colourimetric assay [117]. The method is based on the reduction of the Folin-Ciocalteu reagent with phenolic compounds, in an alkaline state, which forms a blue chromophore that can be easily quantified [118].

3.1. Ethanolic extracts TPC quantification

All microalgae ethanolic extracts were resuspended in dimethyl sulfoxide (DMSO, (CH₃)₂SO) to a final concentration of 10 mg mL⁻¹. To 25 µL of extract were added 25 µL of Folin-Ciocalteu reagent, followed by 200 µL of sodium carbonate (Na₂CO₃, 75 g L⁻¹) and 500 µL of water. The mixture was vortexed and incubated at room temperature in the dark

for 1 h. In the blank samples, the Folin-Ciocalteu reagent was replaced by water. After incubation, the coloured samples were measured at 725 nm in a microplate reader (Synergy HT Multi-detection, Biotek) operated by GEN5 software.

3.2. Calibration curve with Gallic Acid

To quantify TPC in the ethanolic microalgae extracts, a calibration curve was obtained with seven different concentrations of Gallic Acid (GA, $C_6H_2(OH)_3CO_2H$), ranging between 0.025 to 0.5 mg mL⁻¹. The linear regression equation, R² value, limit of detection (LOD=3.3So/b) and the limit of quantification (LOQ=10So/b) are expressed in **Table 6**.

Table 6. Calibration curve of Gallic Acid standard used for total phenolic quantification (TPC).

Standard	Calibration Curve	R ²	LOD/ mg mL ⁻¹	LOQ/ mg mL ⁻¹
Gallic Acid	Abs = 2.6765 [GA] + 0.0846	0.9989	0.01166	0.0353

The total phenols concentration was expressed as mg GA equivalents (GAE) g⁻¹ of dry biomass.

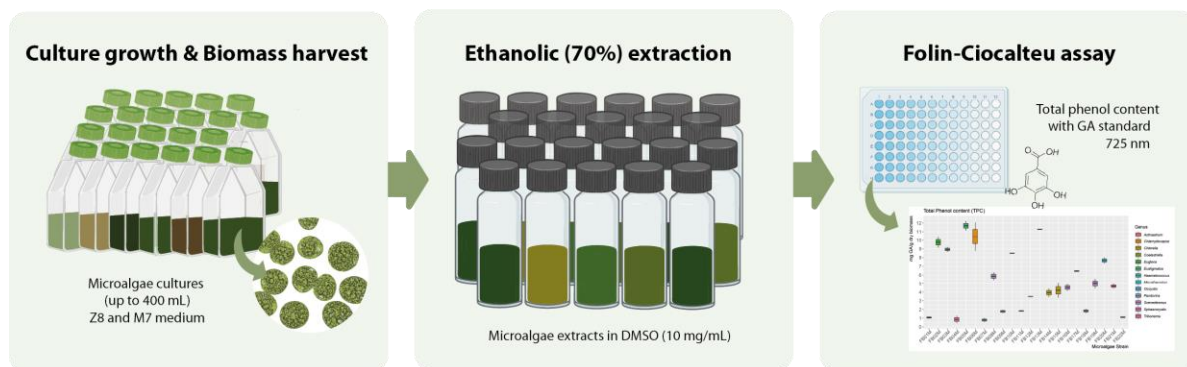


Figure 12. Workflow of Total Phenol Content (TPC) determination of microalgal strains.

4. Determination of carotenoid and chlorophyll profiles

The identification and quantification of carotenoid and chlorophyll pigments was achieved by high-performance liquid chromatography (HPLC) with a photodiode array detection system (PDA) – following a previously described method with small modifications [119-121].

4.1. High-performance liquid chromatography (HPLC) with photodiode array (PDA)

Ethanollic extracts from all microalgae strains were resuspended in MeOH (HPLC grade) to a final concentration of 10 mg mL⁻¹ and filtrated through a 0.2 µm pore membrane.

The microalgae extract analysis was conducted using HPLC Water e2695 Alliance Separations Modules (Water Corporation). The stationary phase used was YMC Carotenoid S - 5 µm (250 - 4.6 mm), kept at a constant temperature (25 °C) by a column heater (Waters Corporation). The mobile phase was composed of two solvents: methanol (A) and tert-butyl-methyl ether (C₅H₁₂O) (B), following the gradient: 95% A at 0 min, 70% A at 30 min, 50% A from 31 min to 40 min and 95% A from 41 to 50 min. All the solvents used in the analysis were previously filtrated with a 0.2 µm pore membrane disk (Pall Corporation). The flow rate was 0.9 ml min⁻¹ and the injection volume was 20 µL. The ethanollic extract (70%) from each strain was injected twice.

Data was processed by Empower 2 Software (Waters) and spectral data from all peaks collected in the 250-750 nm range.

4.2. Calibration curve with carotenoids and chlorophyll standards

In order to quantify the compounds, present in the microalgae strains under study, several calibration curves were conducted with authentic standards with concentrations varying between 0.003125 to 0.1 mg mL⁻¹. The standards were analyzed under the same conditions as the ethanollic extracts.

Astaxanthin, canthaxanthin, chlorophyll-a, echinenone, lutein, lycopene, neoxanthin, violaxanthin, zeaxanthin and β-carotene were identified and quantified with authentic standards (Sigma-Aldrich). Unidentified carotenoids were quantified as zeaxanthin; and chlorophyll-a derivatives and unidentified chlorophylls as chlorophyll-a. Linear regression equations, R², LOD and LOQ values are expressed in **Table 7**.

Table 7. Calibration curve of authentic standards used for carotenoid and chlorophyll quantification.

Standard	Calibration Curve	R ²	LOD/ mg mL ⁻¹	LOQ/ mg mL ⁻¹
Astaxanthin	y = 8206624x - 4922	0.9984	0.0030	0.0092
Canthaxanthin	y = 8878420x - 2170	0.9982	0.0026	0.0080
Chlorophyll-a	y = 81617017x - 692397	0.9630	0.0193	0.0584

Echinenone	$y = 968797602x - 3195691$	0.9942	0.0075	0.0227
Lutein	$y = 320052486x + 312545$	0.9740	0.0109	0.0331
Lycopene	$y = 348282258x - 1953735$	0.9942	0.006	0.0191
Neoxanthin	$y = 254947204x - 2200948$	0.9744	0.0160	0.0483
Violaxanthin	$y = 8323474x - 224$	0.9714	0.0161	0.0487
Zeaxanthin	$y = 553290855x - 767152$	0.9984	0.0020	0.0060
β -carotene	$y = 338714186x - 462602$	0.9976	0.0024	0.0072

4.3. Data Analysis

Carotenoids and chlorophylls were identified through a direct comparison of their retention time (RT) with those of authentic standards, and later quantified by the measurement of the absorbance recorded in the chromatograms relative to external standards at 450 nm. The results were expressed in mg of extract g⁻¹ of dry biomass and relative abundance (RA) (%).

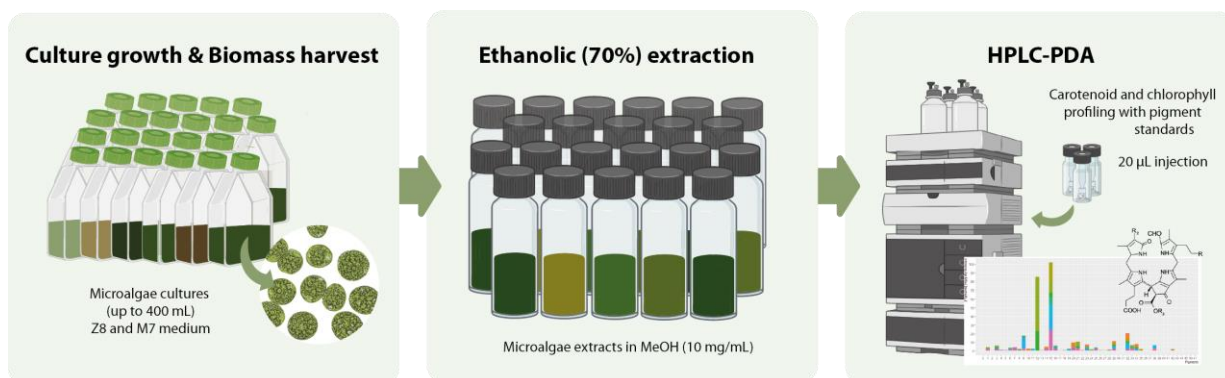


Figure 13. Workflow of carotenoid and chlorophyll identification and quantification of microalgal strains by HPLC-PDA.

5. Determination of fatty acid profile

Fatty acid identification and quantification of all strains was carried out by the analysis of DCM:MeOH (2:1, v/v) extracts by gas-chromatography mass-spectrometry (GC-MS). The chromatographic analysis was performed in the Lipidomics Laboratory (integrated in the Mass Spectrometry Centre at the Chemistry Department of the University of Aveiro) following a previously described method [113, 122].

5.1. Fatty Acid derivatization

The fatty acid methyl esters (FAMES) were obtained by a transesterification reaction of the total lipid extracts according to the previously described methodology [122]. The extract of each strain (30 µg) was dissolved in 1 mL of internal standard solution of a methyl ester fatty acid C19:0 (1.08 µg mL⁻¹ in *n*-hexane, Hex, CH₃(CH₂)₄CH₃) (Sigma). To the sample, 200 µL of a methanolic solution of potassium hydroxide (2.0 M, KOH) was added, followed by a 2 min vortex. After vigorously mixed, it was added 2 mL of sodium chloride saturated solution (0.01 g mL⁻¹, NaCl) and phase separation was obtained by centrifugation (2000 rpm for 5 min). The organic phase (600 µL) was collected and dried under a N₂ stream.

5.2. Gas-chromatography mass spectrometry (GC-MS)

Fatty acid analysis was carried out using a GC-MS (Agilent Technologies 8860 GC system) connected to an Agilent 6977B GC/Mass Selective Detector 8860, operated with electron impact ionization at 70 eV and a scanning range of *m/z* 50-550 (1 s cycle in a full scan mode). The column used was a DB-FFAP with 30 m length, 0.32 mm internal diameter and 0.25 µm film thickness from J&W Scientific. The conditions used were as follows: helium as carrier gas (1.4 mL min⁻¹ constant flow), inlet temperature of 220 °C and detector temperature of 230 °C. The following program conditions were used for the oven temperature: 58 °C for 2 min, 25 °C min⁻¹ to 160 °C, 2 °C min⁻¹ to 210 °C, and 30 °C min⁻¹ to 225 °C and maintained for 20 min.

The three independent replicates of 30 µg of methylated lipid extracts of each microalga were injected (splitless, extracts resuspended in Hex, 2.0 µL injection volume). Data was processed by GCMS 5977B/Enhanced MassHunter data acquisition software.

5.3. Data Analysis

Acquired data was analyzed with the Agilent MassHunter Qualitative Analysis 10.0 software. FAs were identified by retention time (RT) and comparison of the mass spectrum (MS) with the NIST (National Institute of standards and Technology) chemical database library and confirmed in the online database LIPID MAPS® (Lipidomics Gateway, comprehensive classification system for lipids) and literature reports [123].

Quantitative analysis of FA was determined from six-point calibration curves of several methyl esters of fatty acids obtained from a commercial FAME mixture (Supelco 37 Component FAME Mix). The standards were analyzed under the same conditions as the extracts, and the results were expressed in mg of extract g⁻¹ of dry biomass and relative abundance (RA) (%). Fatty acids with a RA equal or greater than 1% were analyzed.

Atherogenic (AI), thrombogenic (TI) and hypocholesterolemic/hypercholesterolemic (h/H) indexes were calculated using the following equations (1) – (3), according to [113, 124]:

$$AI = \frac{(4 + C12:0) \times (C14:0 + C16:0)}{\sum MUFA + \sum PUFA\omega6 + \sum PUFA\omega3} \quad (1)$$

$$TI = \frac{C14:0 + C16:0 + C18:0}{0.5 \times \sum MUFA + 0.5 \times \sum PUFA\omega6 + 3 \times \sum PUFA\omega3 + \frac{\sum PUFA\omega6}{\sum PUFA\omega3}} \quad (2)$$

$$\frac{h}{H} = \frac{C18:1\omega9 + C18:2\omega6 + C18:3\omega3 + C20:4\omega6 + C20:5\omega3}{C14:0 + C16:0} \quad (3)$$

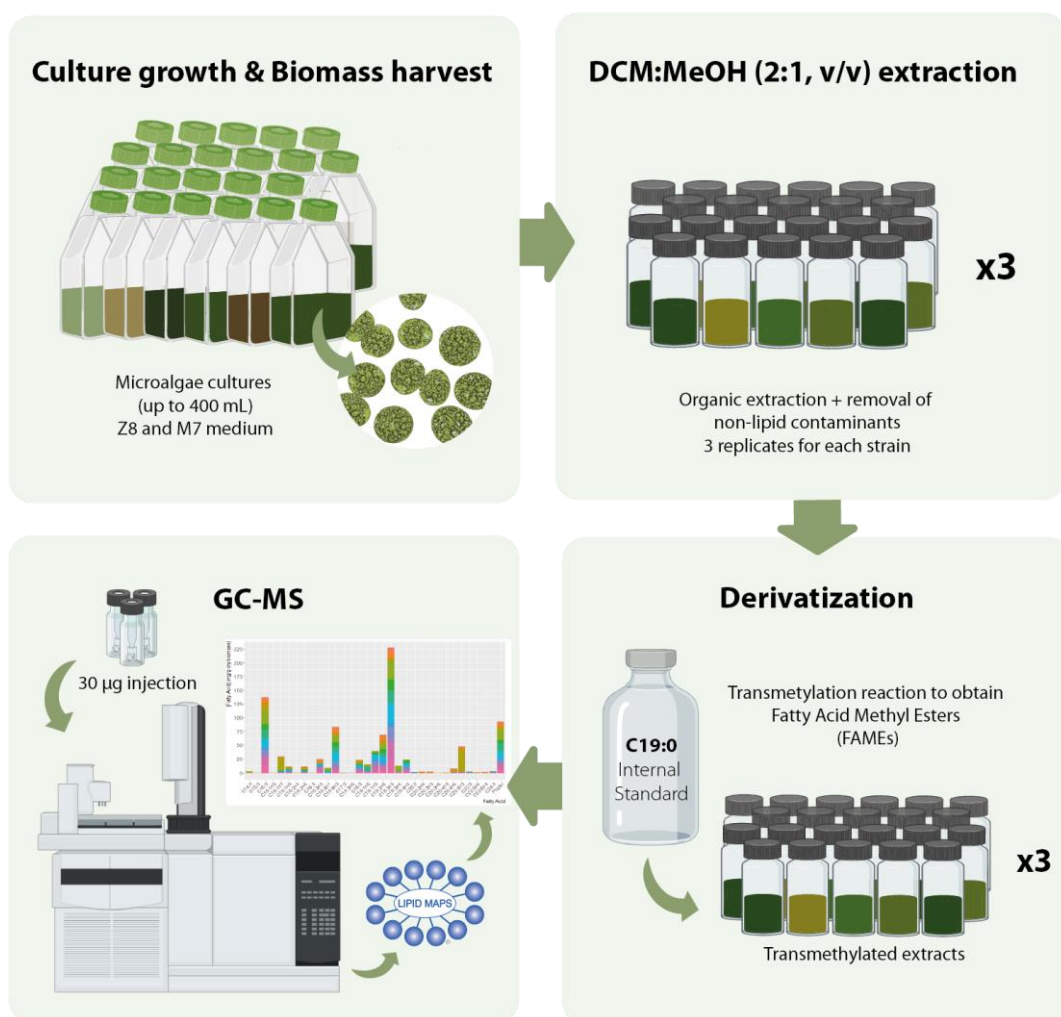


Figure 14. Workflow of fatty acid profiling of microalgal strains by GC-MS.

6. Polysaccharides screening

The evaluation of polysaccharide content produced by microalgae was conducted by a qualitative method with Alcian Blue (AB) staining [125, 126] that highlights the exopolysaccharides (EPS) and by Dubois colourimetric assay with slight modifications [127].

6.1. Alcian Blue staining

Alcian Blue ($C_{56}H_{68}Cl_4CuN_{16}S_4$) is a cationic copper phthalocyanine dye capable of staining and precipitating acid polysaccharides by complexing with the anionic carboxyl and half-ester sulfate groups of acidic algal polysaccharide. EPS are highlighted by a blue colour, due to the presence of copper, easily noticed in a microscopic cell evaluation.

To perform a microscopic visualization of the blue staining, to a lamina it was added: (A) 10 μ L of AB 0.5% in Ethanol (50%) to 10 μ L of microalgae culture - neutral polysaccharides; and (B) 10 μ L of AB 0.5% in Acetic acid (CH_3COOH) to 10 μ L microalgae culture for negative polysaccharides. The culture was mixed with the dye and visualized immediately on the microscope. All microalgae strains were analyzed with both solutions (A) and (B).

6.2. Determination of polysaccharide content by Dubois Assay

Dubois's colourimetric method is based on the principle that simple sugars, oligosaccharides, polysaccharides, and derivatives present an orange-yellow colour when treated with phenol and concentrated sulfuric acid.

After evaluating the polysaccharide content by qualitative analysis by Alcian Blue staining, the four more promising microalgae strains were selected to proceed with quantification: FB05M, FB06M, FB21M and FB22M.

6.2.1. Ethanolic extracts polysaccharide quantification

Firstly, 25 mL of supernatant from the selected microalgae strains was collected by centrifugation (6000 G for 10 min at 4 °C) and added to 75 mL of Ethanol (96%) (1:3 supernatant/solvent ratio) and incubated in -20 °C overnight, to form a precipitate rich in polysaccharides and other compounds. Once the precipitate did not form in enough quantity for further quantification, ethanolic extracts were used to carry on the determination.

Microalgae ethanolic extracts of FB05M, FB06M, FB21M and FB22M strains were resuspended in water to a final concentration of 2.5 mg mL⁻¹ (after optimization assays).

In duplicate, to the extract was added 110 μ L of phenol (C_6H_5OH) and 550 μ L of sulfuric acid (96%, H_2SO_4) and incubated for 10 min at room temperature. In the blank sample,

phenol was replaced by water. The samples were mixed 10 s with a vortex (3000 rpm). After homogenization samples were incubated for 15 min at RT followed by 30 min in a water bath at 35 °C. The coulometric product was read in a spectrophotometer at 483 nm.

6.2.2. Calibration curve with D-(+)-Glucose

To quantify the polysaccharide content in the ethanolic FB05M, FB06M, FB21M and FB22M microalgae extracts, a calibration curve was obtained with six different concentrations of D-(+)-glucose ($C_6H_{12}O_6$), ranging between 0.05 and 0.1 mg mL⁻¹. The linear regression equation, R² value, LOD and LOQ are represented in **Table 8**.

Table 8. Calibration curve of D-(+)-Glucose standard used for polysaccharide quantification.

Standard	Calibration Curve	R ²	LOD/ mg mL ⁻¹	LOQ/ mg mL ⁻¹
D-glucose	Abs = 9.2036[Gluc] - 0.0228	0.9918	0.0061	0.0186

The polysaccharide concentration was expressed as mg g⁻¹ of dry biomass.

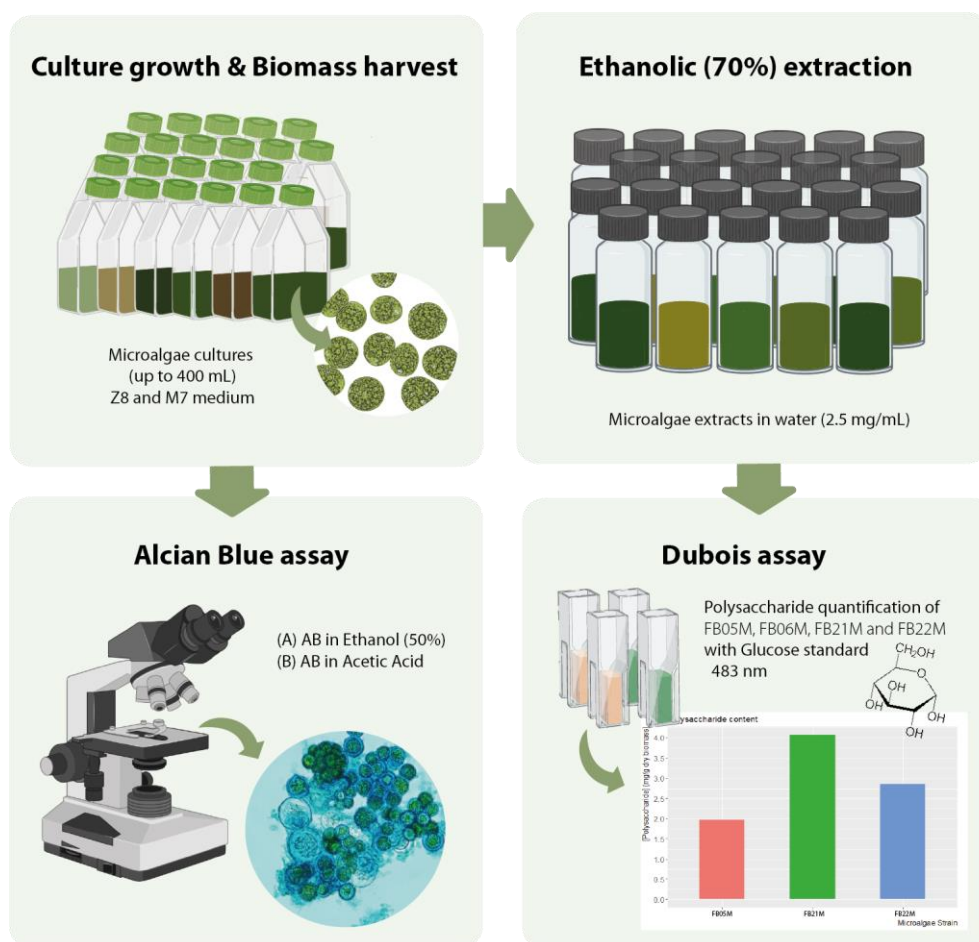


Figure 15. Workflow of polysaccharide content determination of microalgal strains.

7. Statistical Analysis

Data analysis was performed using R version 4.2.1 in RStudio [128]. All graphics and boxplots were created using the R package ggplot2 [129].

For fatty acid and pigment analysis, a principal component analysis (PCA) was conducted for exploratory data analysis, with an R built-in function. PCA and ellipses (level of 0.95) were created using R built-in function, ggplot2, FactoMineR [130] and Factoextra [131] packages. Other complementary R packages used for graphics assembly included dplyr, tidyverse and ggforce.

III. Results and Discussion

1. Growth curve of coccoid microalgae strains

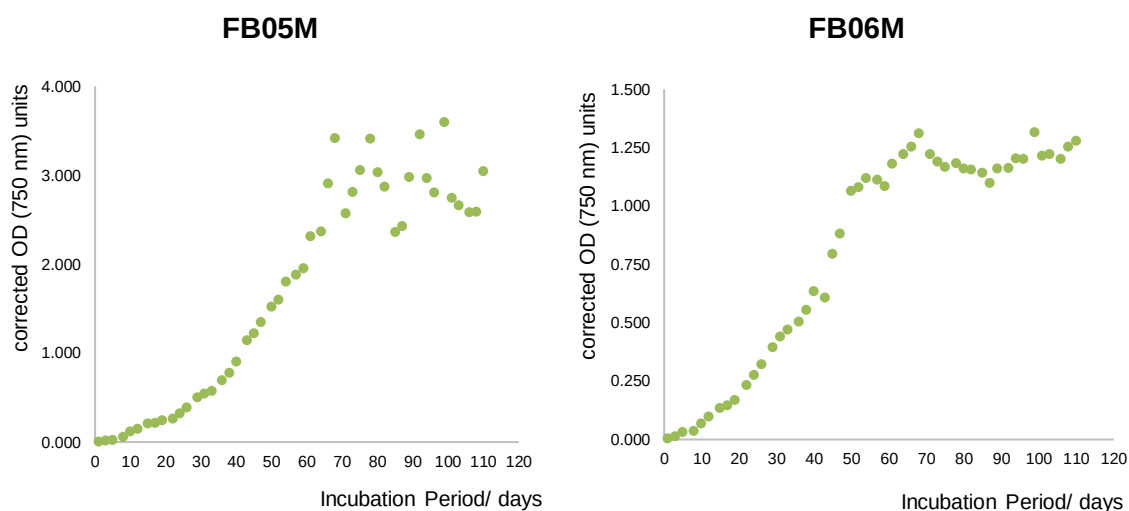
Growth curves obtained from the selected coccoid microalgae strains were expressed as OD versus time. **Figure 16** shows the graphic representations of the strains FB05M, FB06M, FB10M, FB12M, FB13M, FB14M, FB21M and FB22M. Growth curves for strains belonging to the genus *Scenedesmus* - FB08M, FB11M, FB16M, FB17M, FB18M and FB19M strains - are represented in **Figure 17**. Common to all strains, an exponential growth phase is observed – first section of the high slope - that leads, in most cases, to a stationary growth phase. Distinct growth patterns are observed with different microalgae genera.

Sphaerocystis sp. FB22M was the first strain to reach the stationary growth phase – around 50 days after incubation. This strain also presented the lowest OD measurements – which can represent a smaller culture density related to cell concentration, when compared to the other strains.

FB05M and FB06M strains share a similar growth model, reaching a stationary phase around 60 days after inoculation. FB05M however showed higher values of OD readings.

Both FB10M, FB12M, FB13M, FB14M and FB21M strains revealed a continuous increase in OD readings, with *Chlorella* sp. FB14M and *Actinastrum* sp. FB21M strains showing the highest values of OD measurements.

OD readings were conducted for at least 100 days. Hence the initial inoculum volume was only 40 mL, some of the microalgae cultures volume ended before a stationary phase could be observed by spectrometry.



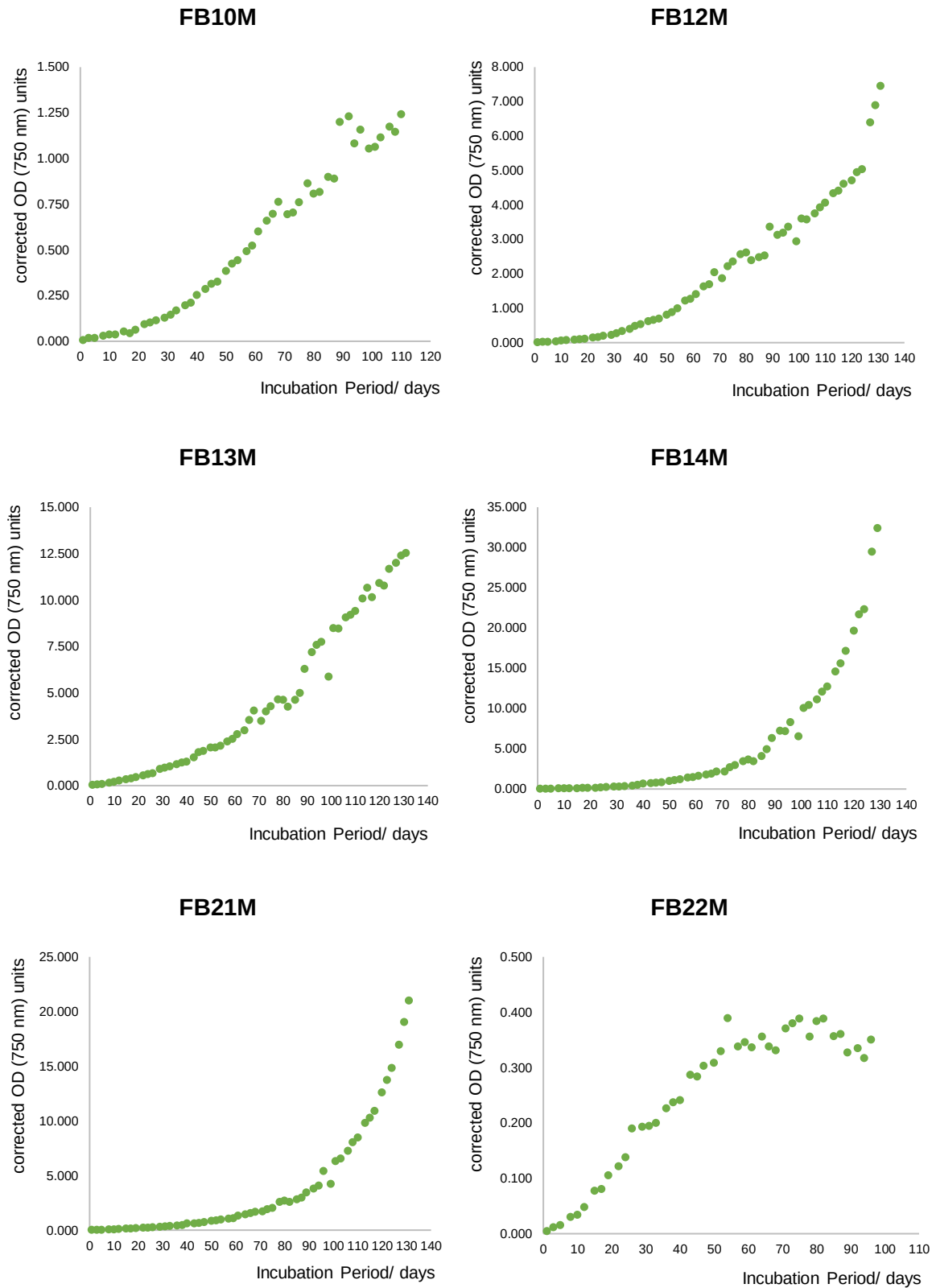


Figure 16. Growth curve of the microalgae strains *Eustigmatos* sp. FB05M, *Chlamydocapsa* sp. FB06M, *Pandorina* sp. FB10M, *Coelastrella* sp. FB12M, *Oocystis* sp. FB13M, *Chlorella* sp. FB14M, *Actinastrum* sp. FB21M and *Sphaerocystis* sp. FB22M.

For strains belonging to the genus *Scenedesmus* (**Figure 17**), a similar pattern was observed, with overlap curves in the first measurement days. The microalgae FB19M showed the highest OD values with the sharpest slope.

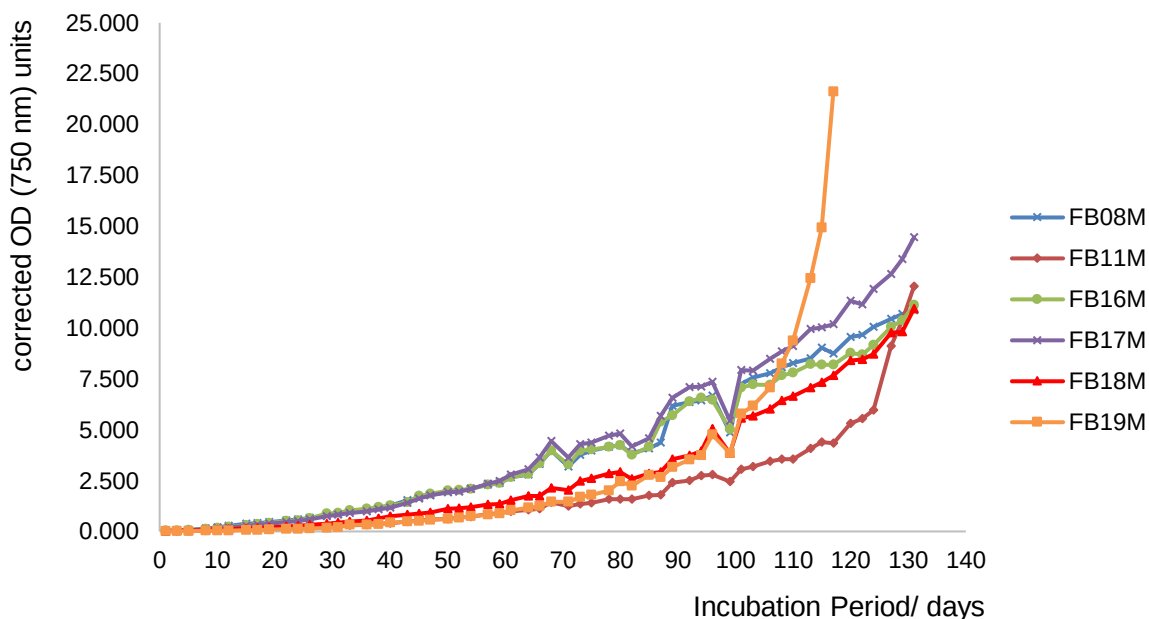


Figure 17. Growth curve of the genus *Scenedesmus* microalgae strains FB08M, FB11M, FB16M, FB17M, FB18M and FB19M.

When compared to literature reports of cyanobacteria growth [132], the eukaryotic microalgae take a longer period to achieve stationary growth and presented higher OD measurements. This data verifies that cyanobacteria growth rates are significantly lower than those of eukaryotic microalgae, as previously described [133].

After the assessment of the growth of the selected coccoid strains, *Sphaerocystis* sp. FB22M was the strain that revealed the lowest growth rate and *Chlorella* sp. FB14M the highest.

The growth curves were obtained by OD measurements of standard growth conditions, allowing to access the optimal growth for biomass harvest for further HVC screening. Culture growth and biomass production can be further optimized by manipulation of factors like temperature, light wavelength, photoperiod, pH and nutrients.

Spectrometry readings were conducted directly in 1 mL of culture aliquots. Microalgae growth can be also determined with OD measurements of chemically extracted pigments from microalgae. A dry weight measurement of harvested biomass from culture can be another potential method for growth and biomass productivity evaluation [60, 134].

2. Organic Extractions

After several months of growth culture, biomass was harvested in an exponential growth phase. Culture from all microalgae strains was collected several times to have enough biomass content for each organic extraction and further analysis.

2.1. Ethanolic extraction

Ethanol is a green, safe, and environmentally friendly solvent that has GRAS status by FDA and is EFSA-approved. This solvent allows the extraction of low water-soluble and water-soluble compounds from microalgae.

All microalgae strains were extracted with ethanol (70%) to conduct an HVC analysis of phenols, pigments (carotenoids and chlorophylls) and polysaccharides. Extraction yields are represented in **Table 9**.

Table 9. Ethanolic extraction yield (%) of all microalgae strains.

Strain	Extraction Yield / %	Strain	Extraction Yield / %
FB01M	12.9	FB12M	23.0
FB02M	42.0	FB13M	39.8
FB03M	29.1	FB14M	42.2
FB04M	24.3	FB15M	28.8
FB05M	39.1	FB16M	27.9
FB06M	32.8	FB17M	51.7
FB07M	11.3	FB18M	19.4
FB08M	27.8	FB19M	73.8
FB09M	19.0	FB20M	44.8
FB10M	24.6	FB21M	38.4
FB11M	17.1	FB22M	12.4

A yield superior to 40% of extract per dry biomass was found in the microalgae strains *Euglena* sp. FB02M, *Eustigmatos* sp. FB05M, *Oocystis* sp. FB13M, *Chlorella* sp. FB14M, *Scenedesmus* sp. FB17M, *Scenedesmus* sp. FB19M and *Microthamnion* sp. FB20M. FB19M strain had the highest yield obtained, 73.8%.

2.2. Dichloromethane:methanol extraction

Solvents polarity affects the lipid content in microalgae extracts [135]. Dichloromethane, a relatively non-polar solvent, non-water miscible, can extract hydrophobic lipids, and methanol, a polar solvent can solubilize membrane-bound lipids. Even though these

classical laboratory solvents can promote high extraction yields, they cannot be used in extraction industrial procedures for the production of food ingredients or nutraceuticals.

According to Folch's extraction method for fatty acid content analysis [115], all microalgae strains were extracted three times with DCM:MeOH (2:1, v/v), extraction yields are shown in **Table 10**.

Table 10. Dichloromethane:methanol (2:1, v/v) extraction yield (%) of all microalgae strains after a purification step. All strains were extracted three times.

Strain	Replicate	Extraction Yield / %	Strain	Replicate	Extraction Yield / %
FB01M	1	10.7	FB12M	1	2.6
	2	1.0		2	2.5
	3	1.9		3	2.6
FB02M	1	13.0	FB13M	1	19.3
	2	14.7		2	21.2
	3	10.5		3	20.5
FB03M	1	3.3	FB14M	1	1.9
	2	8.5		2	1.8
	3	9.2		3	2.7
FB04M	1	1.1	FB15M	1	4.3
	2	0.9		2	5.4
	3	0.9		3	5.2
FB05M	1	20.6	FB16M	1	8.2
	2	19.0		2	15.1
	3	16.4		3	10.8
FB06M	1	23.8	FB17M	1	3.9
	2	21.3		2	5.0
	3	21.0		3	5.4
FB07M	1	1.7	FB18M	1	4.1
	2	1.9		2	3.8
	3	1.6		3	4.0
FB08M	1	14.3	FB19M	1	2.6
	2	12.4		2	3.3
	3	12.6		3	2.9
FB09M	1	1.1	FB20M	1	19.1
	2	1.2		2	17.8
	3	0.7		3	17.8
FB10M	1	12.4	FB21M	1	14.5
	2	10.0		2	15.2
	3	13.9		3	11.4
FB11M	1	5.1	FB22M	1	2.3
	2	2.7		2	2.2
	3	3.5		3	2.3

Eustigmatos sp. FB05M, *Chlamydocapsa* sp. FB06M, *Oocystis* sp. FB13M and *Chlorella* sp. FB14M strains had the highest extraction yields, with more than 15% of extract per dry biomass. In a recent study, a chloroform/methanol lipid extraction of *Chlorella vulgaris* had a maximum yield of 22% [136], superior to one obtained for the extraction of the *Chlorella* sp. FB14M and 15M strains, of the same genus, with the method used in this work using DCM and MeOH.

Consecutive efforts have been made to enhance lipid extractions at an industrial scale with the use of greener alternatives. As recently reported, the combination of an ethanol (GRAS solvent) extraction with mechanical processes can maximize lipid recovery, especially of PUFAs, thus eliminating the use of chlorinated solvents [137]. Other current extraction methods include supercritical fluid extraction and microwave-assisted extraction [135, 136, 138].

3. Total Phenolic Content

Phenolic compounds have a key role in antioxidation with other associated bioactivities. In this work, phenols of all microalgae strains were quantified by Folin-Ciocalteu's colourimetric assay with ethanol extracts, expressed in gallic acid equivalents (GAEs) (Table 11 and Figure 18).

Table 11. Quantification of Total Phenolic Content (TPC) of microalgal ethanolic extracts.

Strain	$\mu\text{g GAE mg}^{-1} \text{ extract}$	$\text{mg GAE g}^{-1} \text{ dry biomass}$
FB01M	8.28 ± 1.43	1.07 ± 0.18
FB02M	23.23 ± 2.07	9.76 ± 0.87
FB03M	30.72 ± 1.33	8.94 ± 0.39
FB04M	3.53 ± 2.52	0.86 ± 0.61
FB05M	29.89 ± 1.99	11.69 ± 0.78
FB06M	31.76 ± 7.13	10.43 ± 2.34
FB07M	7.03 ± 2.92	0.79 ± 0.33
FB08M	21.09 ± 2.40	5.86 ± 0.67
FB09M	9.36 ± 1.40	1.78 ± 0.27
FB10M	34.57 ± 0.07	8.50 ± 0.02
FB11M	10.72 ± 0.63	1.83 ± 0.11
FB12M	15.21 ± 0.20	3.49 ± 0.05
FB13M	28.39 ± 0.22	11.29 ± 0.09
FB14M	9.32 ± 1.73	3.94 ± 0.73
FB15M	14.60 ± 4.20	4.20 ± 1.21

FB16M	16.29 ± 2.17	4.54 ± 0.60
FB17M	12.48 ± 0.26	6.45 ± 0.14
FB18M	9.48 ± 1.80	1.84 ± 0.35
FB19M	6.79 ± 1.08	5.01 ± 0.80
FB20M	17.14 ± 1.07	7.67 ± 0.48
FB21M	12.22 ± 1.04	4.69 ± 0.40
FB22M	8.97 ± 1.02	1.11 ± 0.13

The highest TPC was found in *Eustigmatos* sp. FB05M strain (11.7 mg GAE g⁻¹ dry biomass) followed by *Oocystis* sp. FB13M (11.3 mg GAE g⁻¹ dry biomass) and *Chlamydocapsa* sp. FB06M (10.4 mg GAE g⁻¹ dry biomass). *Haematococcus* sp. FB07M and *Tribonema* sp. FB04M were the strains with the lowest phenolic content – less than 1 mg GAE g⁻¹ dry biomass.

As visually represented in **Figure 18**, *Euglena*, *Eustigmatos*, *Chlamydocapsa* and *Oocystis* were the microalgae genera that showed the highest overall content in phenolic compounds. Within the same genus, microalgae strains show similar values of phenolic content.

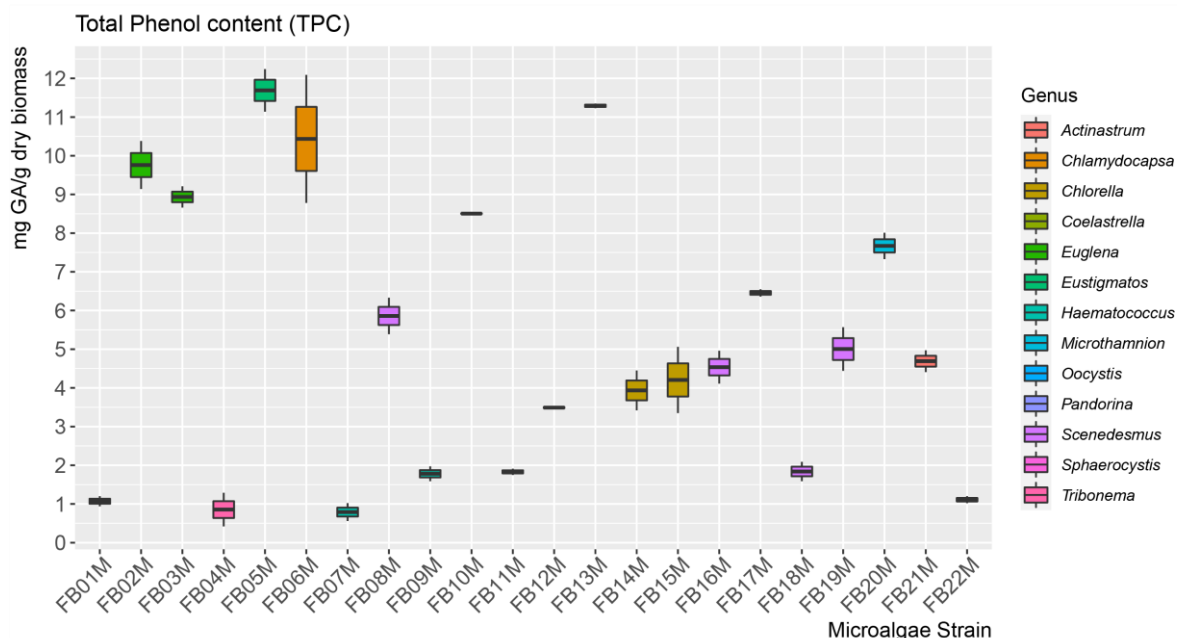


Figure 18. Total Phenol Content (TPC) distribution in microalgae strains with the different genera highlighted.

When compared to cyanobacteria and green macroalgae, the TPC in the microalgae under study (FB01M to FB22M) demonstrated an overall superior concentration per g of dry biomass, which usually ranges between 1.07 and 17.6 mg per g⁻¹ dry biomass [33, 46, 120, 139].

The Folin-Ciocalteu method is widely described in the literature for microalgae TPC assessment, the following study reports described use the same quantification methodology. Goiris et al., 2012 [45], reported that different ethanol/water extracts (3:1, v/v) from *Chlorella vulgaris* strain, had 0.75 to 2.21 mg GAE g⁻¹ of dry biomass. In another report by Maadane et al., 2015 [43], the phenolic content of an ethanol/water extract (1:1, v/v) of *Chlorella* sp. strain was quantified with 8.1 mg GAE g⁻¹. In this work, genus *Chlorella* strains FB14M and FB15M showed intermediate values among those reported (3.94 and 4.20 mg GAE g⁻¹, respectively), with quantification of the ethanol extracts (70% - ethanol/water (3:1, v/v)).

Goiris et al., 2012 also quantified the *Haematococcus pluvialis* strain, with TPC values varying between 0.54 and 1.23 mg GAE g⁻¹, similar to those found in *Haematococcus* sp. FB07M (0.79 mg GAE g⁻¹) and *Haematococcus* sp. FB09M (1.8 mg GAE g⁻¹) strains in the present study. In Chaudhuri et al., 2014 [44, 140], a methanolic extract (70%) of *Euglena tuba* strain is reported to have a TPC of 1.12 mg GAE g⁻¹. Martel et al., 2017 [46], reported that *Euglena cantabrica* had 5.87 in mg GAE g⁻¹ in TPC. Genus *Euglena* microalgae FB01M, FB02M and FB03 strains have shown overall higher values for phenolic content, varying between 1.1 to 9.8 mg GAE g⁻¹. Bulut et al, 2019 [141], reported that *Scenedesmus* sp. ethanol/water (3:1, v/v) extracts had 5.40 mg GAE g⁻¹ in TPC, whereas in Almendinger et al. 2021 [47], an aqueous extract of *Scenedesmus obliquus* was evaluated with 1.2 mg GAE g⁻¹. In this work, microalgae of the genus *Scenedesmus* (FB08M, FB11M, FB16M, FB17M, FB18M and FB19M) ranged from 1.8 to 6.5 mg GAE g⁻¹, values that are in the same order of magnitude of the ones previously described.

Other microalgae strains, reported with high values of total phenolic compounds, are *Isochrysis* sp. (4.57 – 13.4 mg GAE g⁻¹), *Phaedactylum tricornutum* (3.19 – 16.8 mg GAE g⁻¹), *Dunaliella* sp. (4.52 – 19.3 mg GAE g⁻¹) and *Tetraselmis* sp. (3.74 – 25.5 mg GAE g⁻¹) [44, 45].

Previous literature reports for phenol content were not found for the other microalgae strains under study, because they are still not commonly used in the biotech industry. *Eustigmatos* sp. FB05M, *Oocystis* sp. FB13M and *Chlamydocapsa* sp. FB06M (10.4 to 11.7 mg GAE g⁻¹) thus represent underexploited strains that can be very promising targets for PC exploitation.

Microalgae have been reported to have similar or even higher PC content when compared to vegetables and fruits; currently, the main producers are red berries.

The phenolic content evaluated in this work was assessed by extracts obtained from standard growth microalgae cultures. As the industry demand for these compounds increases, a lot of effort has been put in place to enhance PC production from microalgae. The induction of stress conditions in culture growth, like salt stress, has already proved to be an efficient method in the boost of antioxidant molecules production in microalgae strains [139].

4. Carotenoids and Chlorophylls

Carotenoids and chlorophylls are compounds that have a plethora of bioactive applications, therefore being labelled as HVC.

The chromatogram profiles of the twenty-two microalgae strains under study are illustrated in **Appendix 1**, with all pigments highlighted. It was not possible to optimize the chromatographic conditions in useful time, and thus a small overlap can be observed in some of the compound peaks, which does not allow an accurate compound quantification - and further comparison with literature reports. However, as the same method for the selection of the base peak was applied, a comparison between the different microalgae strains under study can be established.

The ethanolic extracts from all microalgae strains were analysed by HPLC-PDA for identification and quantification of pigments. From this analysis, 45 compounds were screened: 14 chlorophylls, 4 chlorophyll derivatives and 27 carotenoids - 6 of which were unidentified. The chlorophyll and carotenoid content was expressed in relative abundance (**Table 12**) and in mg of compound per g of dry biomass (**Appendix 2** and **Figure 20**).

Regarding carotenoids, the xanthophylls identified were violaxanthin (peak 15), neoxanthin (peak 16), astaxanthin (peak 27), lutein (peak 29), zeaxanthin (peak 31), canthaxanthin (peak 36) and lycopene (peak 44). In the carotenes group, β -carotene (peak 42) was also identified. Compounds with UV-Vis spectra similar to identified carotenoids but with different retention times (RT) were classified as carotenoid derivatives. Compounds with a carotenoid characteristic spectrum (shape) were classified as unidentified (peaks 9, 12, 25, 33, 35 and 38).

In the case of chlorophylls, chl a (peak 32) and chl a derivatives (peaks 6, 20, 22 and 34) were identified by the same process as carotenoid derivatives. The compounds with a chl typical spectrum were also classified as unidentified (peaks 1, 2, 3, 4, 7, 10, 13, 14, 19, 21, 23, 26 and 28).

Table 12. Carotenoids and chlorophyll content of all microalgae strains by HPLC-PDA. Pigments are expressed in relative abundance (%) $\pm$ standard deviation (SD).

Peak	Pigment	RT/ min	FB01M	FB02M	FB03M	FB04M	FB05M	FB06M	FB07M
1	Unidentified chlorophyll	3.4						2.43 $\pm$ 0.15	
2	Unidentified chlorophyll	3.7							
3	Unidentified chlorophyll	4.4						1.72 $\pm$ 0.06	
4	Unidentified chlorophyll	4.5							
5	Unidentified carotenoid	5.5							
6	Chlorophyll a derivative	5.9							
7	Unidentified chlorophyll	6.0							
8	Unidentified carotenoid	6.7						0.87 $\pm$ 0.03	
9	Violaxanthin derivative	7.1							
10	Unidentified chlorophyll	7.2		13.49 $\pm$ 2.70					
11	Lutein derivative	7.5							
12	Violaxanthin derivative	7.6					7.34 $\pm$ 0.36	68.21 $\pm$ 1.30	
13	Chlorophyll a derivative	7.7	17.90 $\pm$ 0.64						
14	Unidentified chlorophyll	7.8		11.63 $\pm$ 1.09					
15	Violaxanthin	8.1				3.86 $\pm$ 0.27	86.48 $\pm$ 0.48	14.36 $\pm$ 1.24	
16	Neoxanthin	8.7		2.95 $\pm$ 0.16				0.72 $\pm$ 0.02	20.70 $\pm$ 0.14
17	Lutein derivative	8.8				0.85 $\pm$ 0.03	1.02 $\pm$ 0.01		
18	Unidentified carotenoid	9.4							
19	Unidentified chlorophyll	10.4							20.95 $\pm$ 0.06
20	Chlorophyll a derivative	10.5		16.39 $\pm$ 1.44	17.73 $\pm$ 0.48				
21	Unidentified chlorophyll	10.6					3.14 $\pm$ 0.04	5.22 $\pm$ 0.08	
22	Chlorophyll a derivative	10.8	27.66 $\pm$ 0.77						
23	Unidentified chlorophyll	11.3		8.31 $\pm$ 2.33	12.42 $\pm$ 0.55				
24	Neoxanthin derivative	11.5					0.88 $\pm$ 0.02		
25	Violaxanthin derivative	11.9				18.87 $\pm$ 0.84			
26	Unidentified chlorophyll	12.0	7.27 $\pm$ 1.38					0.54 $\pm$ 0.00	
27	Astaxanthin	12.3							19.46 $\pm$ 0.26
28	Unidentified chlorophyll	12.5			6.38 $\pm$ 0.19				
29	Lutein	13.6		8.15 $\pm$ 0.69	11.52 $\pm$ 0.33	3.37 $\pm$ 0.09		1.33 $\pm$ 0.00	
30	Unidentified carotenoid	15.3	8.28 $\pm$ 0.34						
31	Zeaxanthin	16.0							
32	Chlorophyll a	16.2	13.90 $\pm$ 0.93	25.16 $\pm$ 2.17	31.13 $\pm$ 0.21	10.80 $\pm$ 0.12	0.61 $\pm$ 0.02		21.61 $\pm$ 0.03
33	Violaxanthin derivative	16.4						2.95 $\pm$ 0.00	
34	Chlorophyll a derivative	17.9	11.17 $\pm$ 0.54	11.92 $\pm$ 0.62	14.91 $\pm$ 0.54				
35	Violaxanthin derivative	18.0				62.43 $\pm$ 0.10			
36	Canthaxanthin	18.1							17.58 $\pm$ 0.87
37	Zeaxanthin derivative	18.9	2.27 $\pm$ 0.27		0.93 $\pm$ 0.04				
38	Violaxanthin derivative	19.0							
39	Lutein derivative	19.2		0.67 $\pm$ 0.05					
40	Zeaxanthin derivative	22.2	1.50 $\pm$ 0.07						
41	Lutein derivative	28.9							
42	β -Carotene	33.3	1.22 $\pm$ 0.53	0.84 $\pm$ 0.00	3.54 $\pm$ 3.27				
43	Unidentified carotenoid	37.9	2.27 $\pm$ 0.27						
44	Lycopene	38.6	3.71 $\pm$ 0.19						
45	Unidentified carotenoid	39.1	0.96 $\pm$ 0.31						
Total carotenoids (%)			20.22 $\pm$ 0.82	12.61 $\pm$ 0.71	16.49 $\pm$ 3.28	89.37 $\pm$ 0.89	96.15 $\pm$ 0.60	89.42 $\pm$ 1.84	57.75 $\pm$ 0.92
Total chlorophylls (%)			77.91 $\pm$ 2.02	86.90 $\pm$ 4.59	82.57 $\pm$ 0.95	10.80 $\pm$ 0.12	3.75 $\pm$ 0.04	10.38 $\pm$ 0.18	42.56 $\pm$ 0.07

Peak	Pigment	RT/ min	FB08M	FB09M	FB10M	FB11M	FB12M	FB13M	FB14M
1	Unidentified chlorophyll	3.4							
2	Unidentified chlorophyll	3.7							
3	Unidentified chlorophyll	4.4			10.92 ± 0.45	13.59 ± 0.07			
4	Unidentified chlorophyll	4.5							
5	Unidentified carotenoid	5.5							
6	Chlorophyll a derivative	5.9			7.00 ± 0.06				
7	Unidentified chlorophyll	6.0							
8	Unidentified carotenoid	6.7	1.79 ± 1.92					1.71 ± 0.02	
9	Violaxanthin derivative	7.1			12.89 ± 0.23	13.67 ± 1.62	23.73 ± 1.96		35.00 ± 0.10
10	Unidentified chlorophyll	7.2							
11	Lutein derivative	7.5				0.69 ± 0.01		6.76 ± 0.25	
12	Violaxanthin derivative	7.6	68.74 ± 1.56						
13	Chlorophyll a derivative	7.7							
14	Unidentified chlorophyll	7.8							
15	Violaxanthin	8.1	14.83 ± 1.65		44.08 ± 0.26	26.24 ± 0.99	19.02 ± 2.41	46.42 ± 2.83	40.13 ± 0.01
16	Neoxanthin	8.7	0.96 ± 0.10		1.82 ± 0.01	5.43 ± 0.05	7.50 ± 0.15	1.81 ± 0.02	1.80 ± 0.07
17	Lutein derivative	8.8							
18	Unidentified carotenoid	9.4							
19	Unidentified chlorophyll	10.4			2.36 ± 0.01	12.73 ± 0.15		5.01 ± 0.27	
20	Chlorophyll a derivative	10.5					9.54 ± 0.69		
21	Unidentified chlorophyll	10.6	5.61 ± 0.70			6.44 ± 0.07			
22	Chlorophyll a derivative	10.8							
23	Unidentified chlorophyll	11.3		33.97 ± 0.10				5.10 ± 0.31	4.16 ± 0.01
24	Neoxanthin derivative	11.5	0.85 ± 0.11		1.35 ± 0.01	5.14 ± 0.02			
25	Violaxanthin derivative	11.9							3.63 ± 0.16
26	Unidentified chlorophyll	12.0							
27	Astaxanthin	12.3					13.28 ± 5.27		
28	Unidentified chlorophyll	12.5		32.26 ± 0.01					
29	Lutein	13.6	2.30 ± 0.29		6.97 ± 0.07	8.35 ± 0.04	4.70 ± 0.00	3.59 ± 0.21	2.79 ± 0.03
30	Unidentified carotenoid	15.3							
31	Zeaxanthin	16.0							
32	Chlorophyll a	16.2	3.27 ± 0.42	33.67 ± 0.05	2.00 ± 0.01	7.41 ± 0.07	12.02 ± 0.41	15.32 ± 0.89	4.60 ± 0.04
33	Violaxanthin derivative	16.4			7.99 ± 0.14				
34	Chlorophyll a derivative	17.9	1.14 ± 0.01		1.37 ± 0.00		7.96 ± 0.04	4.91 ± 0.30	1.82 ± 0.00
35	Violaxanthin derivative	18.0							
36	Canthaxanthin	18.1							
37	Zeaxanthin derivative	18.9							
38	Violaxanthin derivative	19.0						8.59 ± 0.49	5.46 ± 0.05
39	Lutein derivative	19.2							
40	Zeaxanthin derivative	22.2							
41	Lutein derivative	28.9							
42	β-Carotene	33.3					1.53 ± 0.03		
43	Unidentified carotenoid	37.9							
44	Lycopene	38.6							
45	Unidentified carotenoid	39.1							
Total carotenoids (%)			89.85 ± 2.99	0.00 ± 0.00	76.18 ± 0.38	59.52 ± 1.90	69.75 ± 6.12	69.64 ± 2.89	89.44 ± 0.21
Total chlorophylls (%)			10.02 ± 0.81	99.90 ± 0.11	23.65 ± 0.45	40.16 ± 0.20	29.52 ± 0.81	30.33 ± 1.03	10.59 ± 0.05

Peak	Pigment	RT/ min	FB15M	FB16M	FB17M	FB18M	FB19M	FB20M	FB21M	FB22M
1	Unidentified chlorophyll	3.4						5.95 ± 1.12		
2	Unidentified chlorophyll	3.7								27.64 ± 0.47
3	Unidentified chlorophyll	4.4	10.79 ± 0.27					5.07 ± 0.39		15.68 ± 0.06
4	Unidentified chlorophyll	4.5			9.70 ± 0.36					
5	Unidentified carotenoid	5.5						2.52 ± 0.23		
6	Chlorophyll a derivative	5.9		8.47 ± 0.43	15.04 ± 0.12					20.69 ± 0.49
7	Unidentified chlorophyll	6						12.12 ± 3.05		
8	Unidentified carotenoid	6.7							0.74 ± 0.07	
9	Violaxanthin derivative	7.1							13.53 ± 1.52	
10	Unidentified chlorophyll	7.2								
11	Lutein derivative	7.5	1.95 ± 0.22		3.99 ± 0.09	2.10 ± 0.25				5.22 ± 0.02
12	Violaxanthin derivative	7.6								
13	Chlorophyll a derivative	7.7								
14	Unidentified chlorophyll	7.8		16.71 ± 1.41			29.11 ± 1.18	2.62 ± 0.27		
15	Violaxanthin	8.1	46.13 ± 0.15	21.72 ± 6.17	18.05 ± 3.42	44.38 ± 0.33	15.16 ± 2.97	56.02 ± 1.66	14.44 ± 1.10	
16	Neoxanthin	8.7	6.27 ± 0.09	2.63 ± 0.32	4.11 ± 0.00	12.12 ± 0.58	13.83 ± 0.53	2.29 ± 0.04	2.43 ± 0.09	10.79 ± 0.00
17	Lutein derivative	8.8								
18	Unidentified carotenoid	9.4	0.99 ± 0.02	0.45 ± 0.01	0.80 ± 0.04	1.91 ± 0.06				
19	Unidentified chlorophyll	10.4								
20	Chlorophyll a derivative	10.5					18.74 ± 0.50	10.22 ± 0.24		
21	Unidentified chlorophyll	10.6	12.01 ± 0.05		4.29 ± 0.00	14.81 ± 0.33			16.66 ± 0.68	
22	Chlorophyll a derivative	10.8		6.37 ± 0.26						
23	Unidentified chlorophyll	11.3		6.38 ± 0.47	9.72 ± 0.00					
24	Neoxanthin derivative	11.5							2.50 ± 0.11	
25	Violaxanthin derivative	11.9			5.77 ± 0.50				9.51 ± 0.69	
26	Unidentified chlorophyll	12		2.93 ± 0.21						
27	Astaxanthin	12.3								
28	Unidentified chlorophyll	12.5		3.30 ± 0.41	5.36 ± 0.00					
29	Lutein	13.6	3.85 ± 0.00	6.11 ± 0.49	5.12 ± 0.03	7.01 ± 0.20	3.23 ± 0.01	1.87 ± 0.03	6.39 ± 0.20	5.29 ± 0.04
30	Unidentified carotenoid	15.3								
31	Zeaxanthin	16							0.49 ± 0.02	
32	Chlorophyll a	16.2	7.12 ± 0.02	17.45 ± 1.50	11.95 ± 0.44		19.86 ± 0.65			15.58 ± 1.19
33	Violaxanthin derivative	16.4	9.57 ± 0.12			17.29 ± 1.12			15.21 ± 0.37	
34	Chlorophyll a derivative	17.9		6.51 ± 0.57	6.80 ± 0.06					
35	Violaxanthin derivative	18								
36	Canthaxanthin	18.1								
37	Zeaxanthin derivative	18.9								
38	Violaxanthin derivative	19							17.01 ± 0.48	
39	Lutein derivative	19.2								
40	Zeaxanthin derivative	22.2								
41	Lutein derivative	28.9						0.27 ± 0.02		
42	β-Carotene	33.3	1.31 ± 0.01	0.82 ± 0.01	1.05 ± 0.39				0.63 ± 0.01	
43	Unidentified carotenoid	37.9								
44	Lycopene	38.6								
45	Unidentified carotenoid	39.1								
Total carotenoids (%)			70.07 ± 0.31	31.80 ± 6.20	38.88 ± 3.48	84.82 ± 1.35	32.21 ± 3.02	64.00 ± 1.68	83.31 ± 2.10	21.31 ± 0.05
Total chlorophylls (%)			29.92 ± 0.28	68.12 ± 2.29	62.86 ± 0.58	14.81 ± 0.33	67.70 ± 1.43	35.98 ± 3.29	16.66 ± 0.68	79.58 ± 1.38

The highest relative abundance (RA) of total chlorophylls was found in *Haematococcus* sp. FB09M and the highest RA of carotenoids was found in *Eustigmatos* sp. FB05M. Regarding the total concentration of chlorophylls, *Euglena* sp. FB02M was the strain with the highest concentration and *Chlamydocapsa* sp. FB06M was the strain that revealed the highest concentration of total carotenoids (**Figure 19**).

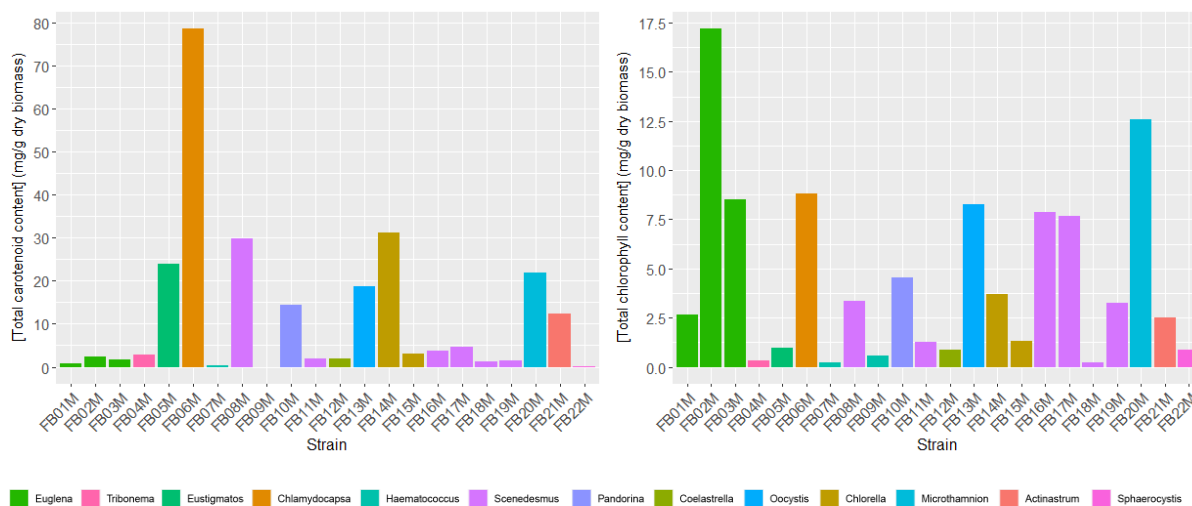


Figure 19. Carotenoid and chlorophyll total content distribution in microalgae strains with the different genera highlighted.

As illustrated in **Figure 20**, the carotenoid violaxanthin (peak 15) showed the highest total concentration in all species, followed by a violaxanthin derivative (peak 12) and chlorophyll a (peak 32).

As expected, chlorophyll a (peak 32) was identified in all microalgae strains, although in some strains was not possible to quantify. *Euglena* sp. FB02M was the strain with the highest RA of chl a (25.2 %) with 4.94 mg g^{-1} dry biomass.

Violaxanthin (peak 15) was quantified in 16 of the 22 microalgae strains analysed. The highest violaxanthin RA value was observed in *Eustigmatos* sp. FB05M (86.5 %) with 21.7 mg g^{-1} dry biomass, which also corresponds to the highest concentration found in all strains under study, as previously described by Wang et al., 2018 [107]. Neoxanthin (peak 16) was quantified in 17 of the 22 strains with RA between 0.7 to 20.7%, with the highest abundance value reported in *Haematococcus* sp. FB07M (0.1 mg g^{-1}). The maximum neoxanthin concentration per dry biomass was registered in *Microthamnion* sp. FB20M with 0.8 mg g^{-1} .

Astaxanthin (peak 27) was only quantified in the *Haematococcus* sp. FB07M (0.09 mg g^{-1}) and *Coelastrella* sp. FB12M (0.4 mg g^{-1}) strains. This pigment was also identified in *Haematococcus* sp. FB09M although it was not quantifiable. Astaxanthin is a pigment characteristic of the genus *Haematococcus* - the low concentration observed in FB07M and

FB09M strains, can be explained by an early biomass harvest, where the optimal pigment concentration had not yet been reached.



Figure 20. Distribution of carotenoids and chlorophylls content (mg g^{-1} dry biomass) in all microalgae strains.

Lutein (peak 29) was quantified in 18 of the 22 strains, with RA from 1.3 to 11.5%. The highest RA value was registered in *Euglena* sp. FB03M, with 1.17 mg g^{-1} . Zeaxanthin (peak 31) was only quantified in *Actinastrum* sp. FB21M with an abundance of 0.49% and a concentration of 0.07 mg g^{-1} . Canthaxanthin (peak 36) was found only in *Haematococcus* sp. FB07M with 17.6% of RA and 0.08 mg g^{-1} of concentration. β -carotene (peak 42) was

quantified in 15 of the 22 microalgae under study with RA between 0.18 and 3.54%. The highest abundance was found in *Euglena* sp. FB03M strain which also corresponds to the highest concentration found in the strains under study (0.4 mg g^{-1}). Lycopene (peak 44) and the unidentified carotenoids (peaks 43 and 45) were only identified in *Euglena* sp. FB01M.

The pigment profile varied with different genera and species. Genus *Euglena* (FB01M, FB02M and FB03M) showed similar percentages of overall chlorophyll (around 80%) and carotenoid (around 20%) content. However, *Euglena* sp. FB01M revealed a slightly different pigment pattern when compared to *Euglena* sp. FB02M and FB03M. In *Haematococcus*, and *Scenedesmus*, pigment profile within species from the same genus is very similar, but RA of total carotenoids and total chlorophylls are variable. *Chlorella* sp. FB14M and FB15M share the same pigment profile, but the FB14M strain has a much higher concentration for each pigment.

Literature reports allowed the characterization of the pigment profile of several microalgae strains. Banskota et al. 2019 and Goiris et al. 2012, reported total carotenoid contents of several microalgae between 0.25 and 29.5 mg g^{-1} dry biomass [45, 142]. For instance, according to Mehariya et al. 2021, the xanthophyll lutein can be found in *Chlorella* sp. with a concentration of $2.3 - 5.9 \text{ mg g}^{-1}$ dry biomass, and in *Coelastrella* sp. with 6.5 mg g^{-1} [29]. Soares et al. 2019, reported values of $0.14 - 0.40 \text{ mg g}^{-1}$ of β -carotene in freshwater microalgae strains [56].

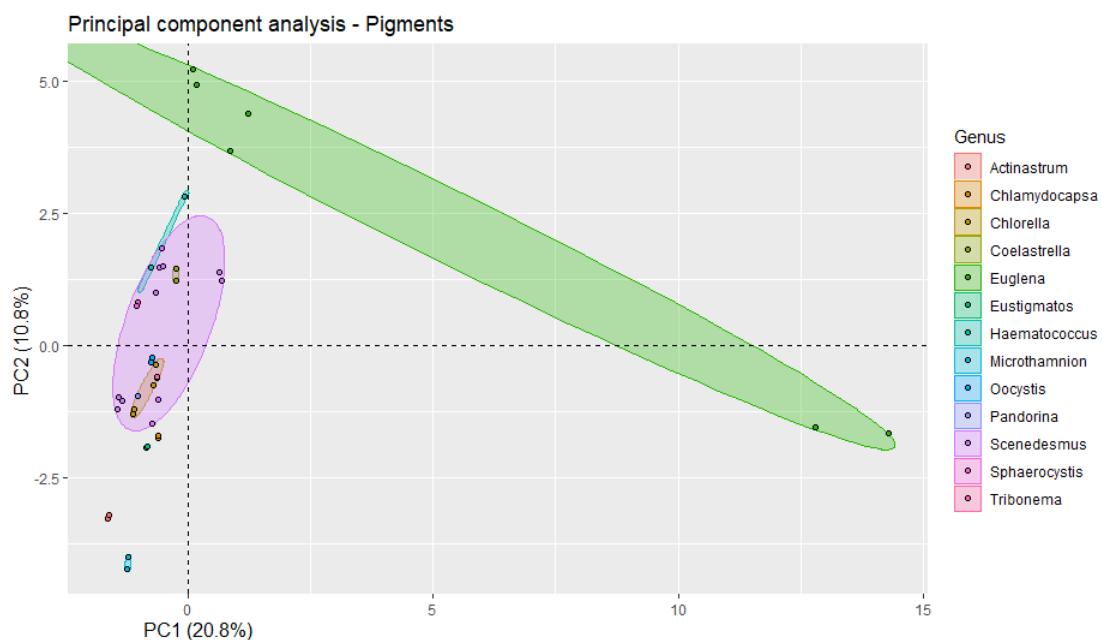


Figure 21. Principal component analysis (PCA) scores plot of carotenoid and chlorophyll analysis.

Principal component analysis (PCA) showed that the first two principal components are responsible for 31.6% of the total variance (PC1 20.8%, PC2 10.8%) (**Appendix 3**). The six major contributors to discrimination are the unidentified carotenoids (peak 30 and 43), 2 chlorophyll a derivatives (peak 13 and 22), a zeaxanthin derivative (peak 40) and lycopene (peak 44) – (**Appendix 4** and **Appendix 5**).

In standard conditions, carotenoids can represent 0.1 to 0.2% of the microalgae dry biomass, reaching 12% under optimal conditions [58]. In order to optimize pigment production in microalgae strains, several growth modulations have been explored: light intensity, nitrogen deficiency, high temperatures and salt stress. However, reports have shown distinct outcomes with different microalgae species [53].

5. Fatty Acids

Several microalgae strains have been reported as great sources of PUFAs, currently playing key roles in the food and feed industries. In this study, FA profiles (FAMES), from 22 selected microalga, were identified by GC-MS from total lipid extracts, after transesterification reaction – results are summarized in **Table 13**. Chromatogram profiles of all microalgae strains under study are represented in **Appendix 6**.

The fatty acid screen allowed the identification of 28 fatty acids and phytol (acyclic diterpene alcohol). Of all FA identified, 6 of those were saturated FA (C14:0, C16:0, C18:0, C20:0, C22:0 and C24:0), 5 monounsaturated FA (C16:1 Δ^{11} (ω -5), C16:1 Δ^9 (ω -7) and C16:1 Δ^7 (ω -9), C18:1 Δ^{13} (ω -5) and C18:1 Δ^9 (ω -9)) and 17 polyunsaturated FA. Within the identified PUFAs, 8 were omega-3 (C16:3 $\Delta^{7,10,13}$ (ω -3), C16:4 $\Delta^{4,7,10,13}$ (ω -3), C18:3 $\Delta^{9,12,15}$ (ω -3), C18:4 $\Delta^{6,9,12,15}$ (ω -3), C20:3 $\Delta^{11,14,17}$ (ω -3), C20:4 $\Delta^{5,11,14,17}$ (ω -3), C20:5 $\Delta^{5,8,11,14,17}$ (ω -3) and C22:6 $\Delta^{4,7,10,13,16,19}$ (ω -3)) and, 6 were omega-6 (C16:2 $\Delta^{7,10}$ (ω -6), C18:2 $\Delta^{9,12}$ (ω -6), C18:3 $\Delta^{6,9,12}$ (ω -6), C20:2 $\Delta^{11,14}$ (ω -6), C20:4 $\Delta^{5,8,11,14}$ (ω -6) and C22:5 $\Delta^{4,7,10,13,16}$ (ω -6)). The fatty acid content was expressed in relative abundance (**Table 13**) and in mg of compound per g of dry biomass (**Appendix 7** and **Figure 23**).

SFAs presented values of 12.5 – 39% of RA, with concentrations within 1.3 and 18.5 mg g⁻¹ dry biomass. MUFAs showed the overall lowest abundance (3.2 – 36.5%) and concentration (0.2 – 22.2 mg g⁻¹) - **Figure 22**.

Overall, the microalgae strains under study showed high PUFA content, with RA values ranging between 24.2% and 84.3%, and concentrations within 0.7 and 74.8 mg g⁻¹ dry biomass. Conde et al. 2021, after accessing the fatty acid profile of six commercial strains, reported total PUFAs values of 39.7 – 64.6% of RA [113].

Table 13. Fatty acid content of microalgae strains by GC-MS. Fatty acids are expressed in relative abundance (%), values are the means of three analytical samples (n = 3) ± standard deviation (SD).

Peak	Fatty Acid	FB01M	FB02M	FB03M	FB04M	FB05M	FB06M	FB07M	FB08M	FB09M	FB010M	FB11M
1	C14:0				3.30 ± 1.71	3.51 ± 0.17						
2	C16:0	10.10 ± 1.33	12.72 ± 0.43	19.53 ± 2.17	26.30 ± 3.44	14.09 ± 0.28	20.85 ± 1.18	21.94 ± 1.16	17.64 ± 1.17	20.33 ± 0.65	22.22 ± 0.46	13.35 ± 2.43
3	C16:1 Δ ¹¹ (ω-5)				1.81 ± 0.13							
4	C16:1 Δ ⁹ (ω-7)	2.07 ± 1.49	1.72 ± 0.15	2.69 ± 1.46	34.67 ± 4.61	26.57 ± 0.13					5.19 ± 4.10	0.98 ± 0.33
5	C16:1 Δ ⁷ (ω-9)		2.17 ± 0.04	1.04 ± 0.24		1.62 ± 0.14	1.26 ± 0.16				1.79 ± 0.71	1.10 ± 0.32
6	C16:2 Δ ^{9,12} (ω-4)				1.26 ± 0.16							
7	C16:2 Δ ^{7,10} (ω-6)	2.95 ± 0.71	2.50 ± 0.42	4.27 ± 1.84			1.74 ± 0.14	1.25 ± 0.13	1.32 ± 0.10		1.67 ± 0.53	
8	C16:3 Δ ^{7,10,13} (ω-3)	7.04 ± 1.20	7.39 ± 0.47	4.25 ± 0.52			1.88 ± 0.18	2.40 ± 0.16	1.22 ± 0.11			
9	C16:3 Δ ^{6,9,12} (ω-4)				1.69 ± 0.12							
10	C16:4 Δ ^{6,9,12,15} (ω-1)						3.45 ± 0.39				2.74 ± 0.53	
11	C16:4 Δ ^{4,7,10,13} (ω-3)	12.38 ± 3.02	14.94 ± 0.68	12.72 ± 0.92			13.65 ± 0.36	10.93 ± 0.69	15.57 ± 1.08	5.92 ± 1.77	13.09 ± 1.07	19.02 ± 3.71
12	C18:0	2.40 ± 0.05	1.47 ± 0.07	6.35 ± 2.68	9.64 ± 3.99	2.10 ± 0.33	1.46 ± 0.19	4.61 ± 1.07	3.25 ± 0.78	7.39 ± 2.73	2.27 ± 0.39	7.74 ± 2.04
13	C18:1 Δ ¹³ (ω-5)						2.65 ± 0.07	5.88 ± 0.09		3.31 ± 0.44	8.15 ± 1.17	7.37 ± 3.21
14	C18:1 Δ ⁹ (ω-9)	1.10 ± 0.30	1.07 ± 0.20	1.18 ± 0.96					6.21 ± 0.33	1.07 ± 0.34		3.07 ± 0.98
15	C18:2 Δ ^{9,12} (ω-6)	8.21 ± 1.72	15.83 ± 1.28	16.80 ± 4.12	3.61 ± 0.29		9.67 ± 1.08	10.88 ± 0.40	12.04 ± 0.34	6.87 ± 0.86	9.15 ± 3.37	2.45 ± 0.08
16	C18:3 Δ ^{9,12,15} (ω-3)	24.11 ± 7.13	22.77 ± 1.37	17.57 ± 1.14			34.18 ± 1.19	30.80 ± 1.24	34.47 ± 0.46	13.86 ± 4.02	29.90 ± 1.77	37.67 ± 4.16
17	C18:3 Δ ^{6,9,12} (ω-6)						8.06 ± 1.08	1.41 ± 0.02				
18	C18:4 Δ ^{6,9,12,15} (ω-3)						1.16 ± 0.11		5.68 ± 0.19	7.02 ± 1.41	3.84 ± 0.73	5.58 ± 1.01
19	C20:0							1.30 ± 0.05		7.94 ± 1.03		
20	C20:2 Δ ^{11,14} (ω-6)	3.02 ± 1.41	2.33 ± 0.12	2.33 ± 0.21								
21	C20:3 Δ ^{11,14,17} (ω-3)	2.41 ± 1.07	1.79 ± 0.07	1.20 ± 0.49								
22	C20:4 Δ ^{5,11,14,17} (ω-3)	2.02 ± 0.48	1.07 ± 0.01									
23	C20:4 Δ ^{5,8,11,14} (ω-6)	6.54 ± 2.38	3.22 ± 0.03	4.22 ± 0.79	8.21 ± 0.80	2.64 ± 0.04		2.40 ± 0.13		1.21 ± 0.22		
24	C20:5 Δ ^{5,8,11,14,17} (ω-3)	6.81 ± 3.26	5.40 ± 0.07	5.85 ± 0.46	9.40 ± 0.60	49.48 ± 0.28		2.16 ± 0.10		1.44 ± 0.34		
25	C22:0							1.73 ± 0.18	1.00 ± 0.01	16.61 ± 2.36		1.65 ± 0.88
26	C22:5 Δ ^{4,7,10,13,16} (ω-6)	3.54 ± 0.39	1.16 ± 0.13									
27	C22:6 Δ ^{4,7,10,13,16,19} (ω-3)	5.31 ± 2.35	2.46 ± 0.06									
28	C24:0							2.31 ± 0.17	1.59 ± 0.07	7.03 ± 1.04		
	Σ SFA	12.50 ± 1.33	14.19 ± 0.44	25.88 ± 3.45	39.24 ± 5.54	19.70 ± 0.46	22.31 ± 1.20	31.89 ± 1.60	23.48 ± 1.41	59.30 ± 3.95	24.49 ± 0.60	22.74 ± 3.29
	Σ MUFA	3.17 ± 1.52	4.96 ± 0.25	4.91 ± 1.76	36.48 ± 4.61	28.19 ± 0.19	3.91 ± 0.17	5.88 ± 0.09	6.21 ± 0.33	4.38 ± 0.56	15.13 ± 4.32	12.52 ± 3.39
	Σ PUFA	84.34 ± 9.50	80.86 ± 2.10	69.21 ± 4.89	24.17 ± 1.06	52.12 ± 0.28	73.79 ± 2.02	62.23 ± 1.50	70.30 ± 1.25	36.32 ± 4.71	60.39 ± 4.09	64.72 ± 5.67
	Σ PUFAω-6	24.26 ± 3.36	25.04 ± 1.36	27.62 ± 4.59	11.82 ± 0.85	2.64 ± 0.04	19.47 ± 1.53	15.94 ± 0.44	13.36 ± 0.35	8.08 ± 0.89	10.82 ± 3.41	2.45 ± 0.08
	Σ PUFAω-3	60.08 ± 8.88	55.82 ± 1.60	41.59 ± 1.69	9.40 ± 0.60	49.48 ± 0.28	50.87 ± 1.26	46.29 ± 1.43	56.94 ± 1.19	28.24 ± 4.63	46.83 ± 2.19	62.27 ± 5.66
	ω-6/ω-3 ratio	0.40 ± 0.20	0.45 ± 0.06	0.66 ± 0.17	1.26 ± 0.10	0.05 ± 0.02	0.38 ± 0.08	0.34 ± 0.04	0.23 ± 0.03	0.29 ± 0.20	0.23 ± 0.32	0.04 ± 0.10
	AI	0.46 ± 0.17	0.59 ± 0.04	1.05 ± 0.13	2.05 ± 0.15	0.88 ± 0.02	1.12 ± 0.06	1.29 ± 0.06	0.92 ± 0.07	2.00 ± 0.12	1.22 ± 0.08	0.69 ± 0.20
	TI	0.01 ± 0.05	0.01 ± 0.05	0.04 ± 0.42	0.28 ± 0.31	0.03 ± 0.07	0.01 ± 0.13	0.03 ± 0.23	0.02 ± 0.24	0.08 ± 0.37	0.01 ± 0.18	0.04 ± 0.27
	(h/H)	4.63 ± 0.22	3.80 ± 0.05	2.34 ± 0.15	0.72 ± 0.14	2.96 ± 0.02	2.10 ± 0.07	2.11 ± 0.06	2.99 ± 0.07	1.20 ± 0.17	1.76 ± 0.10	3.24 ± 0.21

Peak	Fatty Acid	FB12M	FB13M	FB14M	FB15M	FB16M	FB17M	FB818M	FB19M	FB20M	FB21M	FB22M
1	C14:0											
2	C16:0	11.73 ± 0.22	15.18 ± 1.28	19.49 ± 2.11	18.42 ± 0.91	14.89 ± 0.94	14.15 ± 0.91	18.76 ± 1.93	20.55 ± 2.42	19.59 ± 1.93	21.70 ± 5.07	23.65 ± 1.08
3	C16:1 Δ ¹¹ (ω-5)											
4	C16:1 Δ ⁹ (ω-7)			1.99 ± 0.91	1.80 ± 0.26							2.58 ± 1.83
5	C16:1 Δ ⁷ (ω-9)		2.10 ± 0.13	2.80 ± 0.16		1.81 ± 0.39		1.15 ± 0.53			2.91 ± 0.81	
6	C16:2 Δ ^{9,12} (ω-4)											
7	C16:2 Δ ^{7,10} (ω-6)	4.11 ± 0.16		2.54 ± 0.18	1.69 ± 1.13					1.91 ± 0.18		
8	C16:3 Δ ^{7,10,13} (ω-3)	2.87 ± 0.09	1.27 ± 0.02	6.59 ± 0.27	10.41 ± 1.03		1.94 ± 0.04	0.95 ± 0.10		9.03 ± 1.33	3.67 ± 0.23	0.95 ± 0.23
9	C16:3 Δ ^{6,9,12} (ω-4)											
10	C16:4 Δ ^{6,9,12,15} (ω-1)		1.89 ± 0.54									
11	C16:4 Δ ^{4,7,10,13} (ω-3)	4.46 ± 0.18	20.41 ± 0.36		1.07 ± 0.28	15.94 ± 2.55	16.42 ± 0.01	17.37 ± 2.35	11.37 ± 0.93		11.96 ± 0.77	
12	C18:0	5.26 ± 0.85	2.14 ± 0.85	5.70 ± 1.75	3.54 ± 0.30	2.92 ± 0.74	3.53 ± 0.35	8.10 ± 2.60	8.83 ± 1.91	2.71 ± 1.23	4.43 ± 2.78	4.66 ± 1.49
13	C18:1 Δ ¹³ (ω-5)	1.76 ± 0.22		3.50 ± 0.91	3.60 ± 0.49	1.85 ± 0.77		1.94 ± 2.00	2.53 ± 1.01		1.87 ± 0.14	4.53 ± 2.47
14	C18:1 Δ ⁹ (ω-9)	28.60 ± 0.72	7.40 ± 0.32	4.17 ± 0.36	15.88 ± 5.77	8.06 ± 4.49	15.23 ± 0.61	6.94 ± 1.26	6.17 ± 0.41	9.40 ± 2.19	3.94 ± 0.47	26.42 ± 8.14
15	C18:2 Δ ^{9,12} (ω-6)	14.91 ± 0.11	7.50 ± 1.06	20.88 ± 1.24	10.69 ± 2.60	6.37 ± 2.07	6.37 ± 0.53	2.71 ± 0.29	3.58 ± 0.31	7.80 ± 0.24	7.11 ± 0.57	18.04 ± 0.37
16	C18:3 Δ ^{9,12,15} (ω-3)	18.04 ± 0.29	36.56 ± 2.49	29.34 ± 1.92	30.04 ± 4.68	35.46 ± 0.85	29.82 ± 3.05	31.05 ± 3.11	26.15 ± 1.38	44.21 ± 0.90	33.95 ± 5.20	11.49 ± 6.12
17	C18:3 Δ ^{6,9,12} (ω-6)	1.71 ± 0.05				0.98 ± 0.16			1.71 ± 0.10		2.63 ± 0.17	0.97 ± 0.20
18	C18:4 Δ ^{6,9,12,15} (ω-3)	5.41 ± 0.41	5.65 ± 0.65	3.00 ± 1.50	1.89 ± 0.89	7.57 ± 0.25	6.51 ± 0.06	7.46 ± 5.53	19.10 ± 3.41	1.01 ± 0.12	5.84 ± 0.55	6.71 ± 2.30
19	C20:0					1.41 ± 0.49	1.86 ± 0.62	1.73 ± 0.96				
20	C20:2 Δ ^{11,14} (ω-6)											
21	C20:3 Δ ^{11,14,17} (ω-3)											
22	C20:4 Δ ^{5,11,14,17} (ω-3)											
23	C20:4 Δ ^{5,8,11,14} (ω-6)											
24	C20:5 Δ ^{5,8,11,14,17} (ω-3)				0.96 ± 0.12					1.11 ± 0.04 2.16 ± 0.11		
25	C22:0					1.22 ± 0.43	2.04 ± 0.69	1.85 ± 0.99				
26	C22:5 Δ ^{4,7,10,13,16} (ω-6)											
27	C22:6 Δ ^{4,7,10,13,16,19} (ω-3)											
28	C24:0	1.14 ± 0.17				1.50 ± 0.53	2.12 ± 0.67			1.05 ± 0.25		
Σ SFA		18.13 ± 0.89	17.32 ± 1.54	25.19 ± 2.74	21.96 ± 0.96	21.94 ± 1.46	23.70 ± 1.50	30.44 ± 3.52	29.38 ± 3.08	23.35 ± 2.30	26.13 ± 5.78	28.31 ± 1.84
Σ MUFA		30.36 ± 0.75	9.50 ± 0.35	12.46 ± 1.35	21.28 ± 5.80	11.72 ± 4.57	15.23 ± 0.61	10.03 ± 2.42	8.70 ± 1.09	9.40 ± 2.19	8.72 ± 0.95	33.53 ± 8.70
Σ PUFA		51.51 ± 0.58	73.28 ± 2.86	62.35 ± 2.75	56.75 ± 5.65	66.32 ± 3.41	61.06 ± 3.10	59.54 ± 6.77	61.91 ± 3.81	67.23 ± 1.64	65.16 ± 5.32	38.16 ± 6.56
Σ PUFAω-6		20.73 ± 0.20	7.50 ± 1.06	23.42 ± 1.25	12.38 ± 2.83	7.35 ± 2.08	6.37 ± 0.53	2.71 ± 0.29	5.29 ± 0.33	10.82 ± 0.30	9.74 ± 0.59	19.01 ± 0.42
Σ PUFAω-3		30.78 ± 0.54	63.89 ± 2.60	38.93 ± 2.45	44.37 ± 4.88	58.97 ± 2.70	54.69 ± 3.05	56.83 ± 6.77	56.62 ± 3.79	56.41 ± 1.61	55.42 ± 5.29	19.15 ± 6.54
ω-6/ω-3 ratio		0.67 ± 0.02	0.12 ± 0.15	0.60 ± 0.08	0.28 ± 0.25	0.12 ± 0.29	0.12 ± 0.10	0.05 ± 0.16	0.09 ± 0.09	0.19 ± 0.04	0.18 ± 0.11	0.99 ± 0.34
AI		0.57 ± 0.02	0.75 ± 0.09	1.04 ± 0.12	0.94 ± 0.11	0.76 ± 0.10	0.74 ± 0.08	1.08 ± 0.15	1.16 ± 0.13	1.02 ± 0.10	1.17 ± 0.24	1.32 ± 0.16
TI		0.04 ± 0.16	0.01 ± 0.40	0.04 ± 0.31	0.02 ± 0.10	0.02 ± 0.26	0.02 ± 0.10	0.05 ± 0.32	0.05 ± 0.22	0.02 ± 0.45	0.03 ± 0.63	0.06 ± 0.34
(h/H)		5.25 ± 0.02	3.39 ± 0.10	2.79 ± 0.12	3.13 ± 0.15	3.35 ± 0.12	3.63 ± 0.09	2.17 ± 0.13	1.75 ± 0.12	3.30 ± 0.11	2.07 ± 0.26	2.37 ± 0.19

The highest RA of total PUFAs and ω -6 PUFAs was found in the genus *Euglena* (FB01M, FB02M and FB03M). The highest concentration of total PUFAs was observed in *Chlamydocapsa* sp. FB06M (74.8 mg g⁻¹) and *Oocystis* sp. FB13M (62.5 mg g⁻¹). FB06M strain also revealed the highest concentration of ω -6 PUFAs (19.8 mg g⁻¹). Regarding total ω -3 PUFAs, *Scenedesmus* sp. FB11M showed the highest relative abundance (62.3%), followed by *Euglena* sp. FB01M (60.1%). FB13M and FB06M, with 54.4 and 51.6 mg g⁻¹ respectively, presented the highest concentration of ω -3 PUFAs.

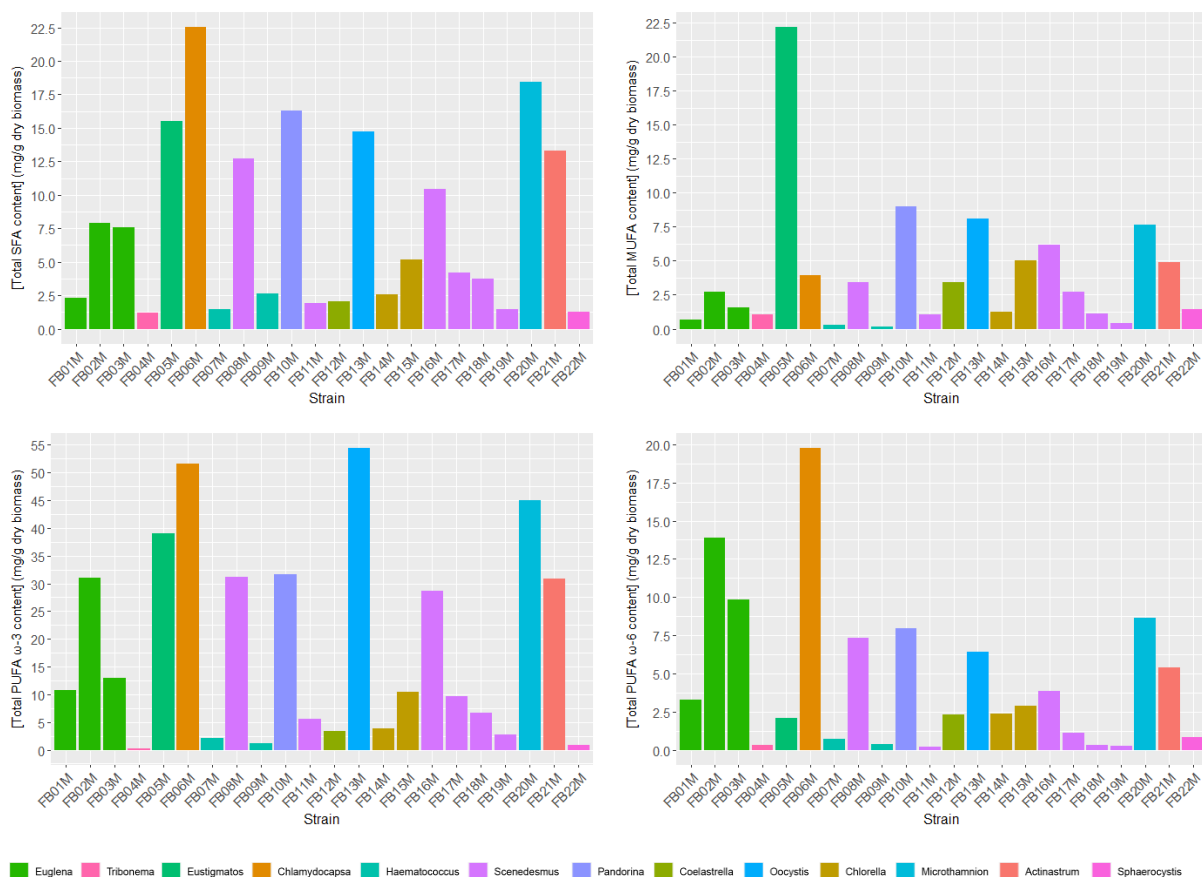


Figure 22. Saturated, monounsaturated, and polyunsaturated (ω -3 and ω -6) fatty acid total content distribution in microalgae strains with the different genera highlighted.

The combination of the FA concentration from all strains showed the α -linolenic acid (ALA, C18:3 $\Delta^{9,12,15}$ (ω -3)) as the most abundant, followed by the palmitic acid (C16:0) and C16:4 $\Delta^{4,7,10,13}$ (ω -3).

With regard to the ω -3 fatty acids, the α -linolenic acid (ALA, C18:3 $\Delta^{9,12,15}$ (ω -3)) was identified in all microalgae strains except *Tribonema* sp. FB04M and *Eustigmatos* sp. FB05M. The highest RA was found in *Microthamnion* sp. FB20M with 44.2% and 35.2 mg g⁻¹ dry biomass. *Chlamydocapsa* sp. FB06M and *Oocystis* sp. FB13M also presented a high content in ALA (34.7 and 31.2 mg g⁻¹ respectively). Morais et al., 2021 [138] and Conde et al. 2021 [113], reported ALA relative abundance content of 18.3 - 26.3% for *Chlorella*

vulgaris and 35.0 - 39.3% for *Scenedesmus* sp., which are similar to those found in this study for strains belonging to the same genus.



Figure 23. Distribution of fatty acid content (mg g⁻¹ dry biomass) in all microalgae strains.

Eicosapentaenoic acid (EPA, C20:5 $\Delta^{5,8,11,14,17}$ (ω -3)) was quantified in 9 strains, with the maximum abundance reported in *Eustigmator* sp. FB05M (49.5%) which translates to 39.0 mg g⁻¹, a very promising quantity for this essential omega-3 fatty acid. In literature reports so far, the highest EPA values documented were found in *Nannochloropsis* sp. (30.8% - 34.0% RA, 31.7 mg g⁻¹) and *Phaedactylum tricornutum* (26.1 - 27.3% RA, 28.5 mg g⁻¹) [14, 26, 113, 138, 142]. Docosahexaenoic acid (DHA, C22:6 $\Delta^{4,7,10,13,16,19}$ (ω -3)) was only identified in *Euglena* sp. FB01M and FB02M with small abundance values. *Cryptocodinium*, *Schizochytrium* and *Ulkenia* are the most common microalgae sources described for DHA production [58].

In ω -6 fatty acids, linoleic acid (LA, C18:2 $\Delta^{9,12}$ (ω -6)) was identified in all strains and quantified in 21 of them, with abundances between 2.5 and 20.9%. The highest LA content was found in *Chlamydocapsa* sp. FB11M (9.8 mg g⁻¹) followed by *Euglena* sp. FB02M (8.8 mg g⁻¹). Conde et al., 2021 [113] observed a 17.6% RA of LA in *Chlorella vulgaris*, which once again is in the order of magnitude of the strains under study from the same genus (FB14M and FB15M). γ -linoleic acid (GLA, C18:3 $\Delta^{6,9,12}$ (ω -6)) was quantified in 7 of the 22 analysed microalgae strains. The highest concentration was found in *Chlamydocapsa* sp. FB06M with an abundance of 8.1% (8.2 mg g⁻¹). Previous studies reported that the cyanobacteria *Spirulina* sp. is a good source of GLA (RA of 23.3%) [14, 113]. Arachidonic acid (ARA, C20:4 $\Delta^{5,8,11,14}$ (ω -6)) was quantified in 8 strains. *Tribonema* sp. FB04M showed the highest abundance (8.2%) in ARA, however, the highest concentration was found in *Eustigmator* sp. FB05M with 2.1 mg g⁻¹ dry biomass. *Porphyridium cruentum* is described in literature as one of the microalgae strains with the highest abundance in ARA (40%) [26].

At last, oleic acid (OA, C18:1 Δ^9 (ω -9)) was present in 18 of the 22 microalgae strains. The highest content was found in *Microthamnion* sp. FB20M (7.7 mg g⁻¹) followed by *Scenedesmus* sp. FB16M (4.6 mg g⁻¹) strain.

The fatty acid profile is similar between strains from the same genus; however, some concentration differences are noticeable. For instance, in the genus *Euglena*, the strain FB02M showed an overall higher FA concentration when compared with FB01M and FB03M. A similar pattern can be found within the genus *Chlorella*, where the FB15M strain revealed a higher FA content. In the genus *Scenedesmus*, FB08M and FB16M were the strains that showed a greater amount of FA.

PCA analysis demonstrated that PC1 (48.9%) and PC2 (15.4%) were responsible for 64.3% of the total variance (**Appendix 8**). The six major contributors for discrimination (**Appendix 9** and **Appendix 10**) are C18:3 $\Delta^{9,12,15}$ (ω -3), C20:5 $\Delta^{5,8,11,14,17}$ (ω -3), C16:1 Δ^9 (ω -7), C18:1 Δ^9 (ω -9), C18:4 $\Delta^{6,9,12,15}$ (ω -3) and C18:2 $\Delta^{9,12}$ (ω -6).

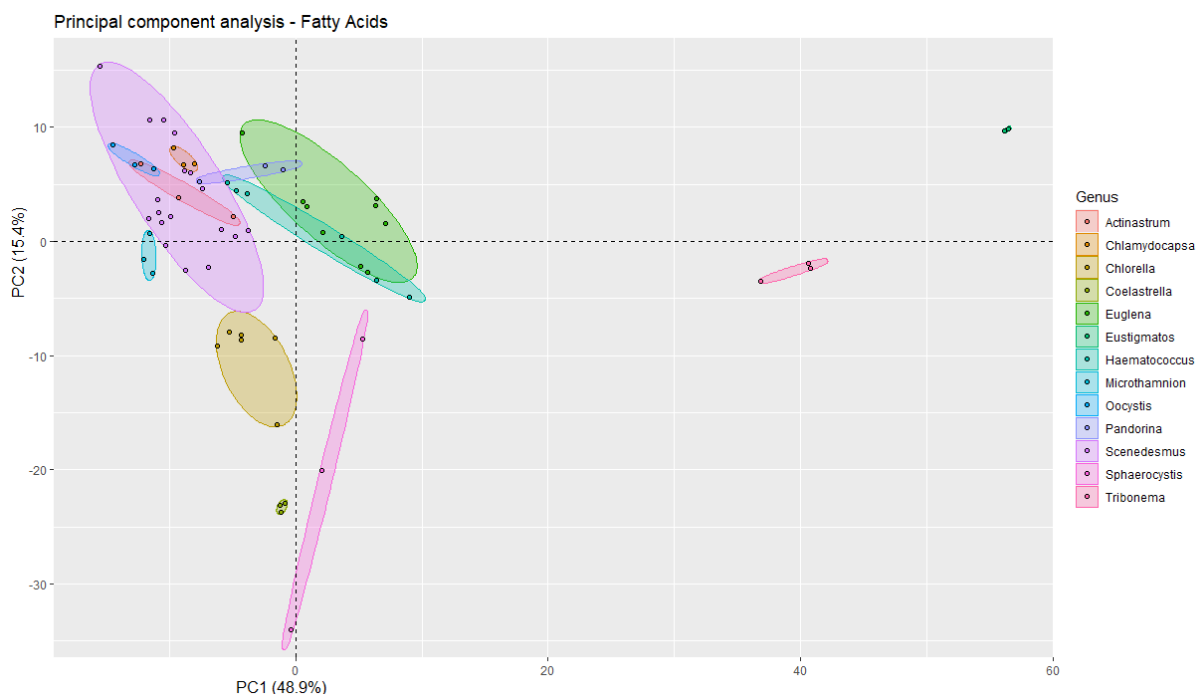


Figure 24. Principal component analysis (PCA) scores plot of fatty acids analysis.

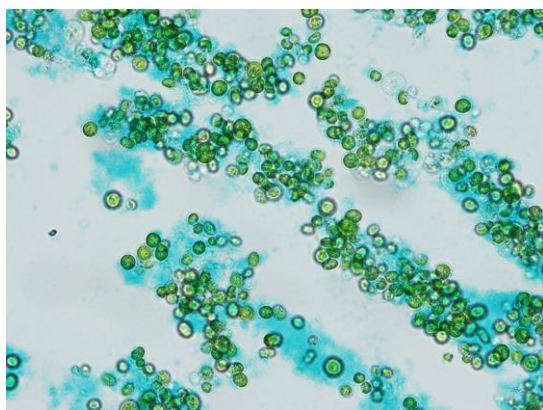
The nutritional index and associated health benefits of all 22 microalgae strains were assessed with the ω -6/ ω -3 ratio, thrombogenicity index (TI), atherogenicity index (AI) and the hypocholesterolemic/hypercholesterolemic (h/H) ratio (**Table 13**). The presence of a high ω -6/ ω -3 ratio in the human diet is correlated as one of the major causes of diseases like cardiovascular, atherosclerosis, cancer and inflammatory. A PUFA-rich diet is expected to increase the ω -3/ ω -6 ratio, thus preventing the risk for the previously mentioned diseases and possibly having a suppressive effect [77, 113, 124]. Regarding the ω -6/ ω -3 ratio calculated for the strains under study, *Scenedesmus* sp. FB11M (0.04), *Eustigmatos* sp. FB05M (0.05) and *Scenedesmus* sp. FB18M (0.05) indicated the lowest values. AI registered values between 0.46 and 2.00, with *Euglena* sp. F01M holding the lowest value. TI ranged between 0.01 and 0.28. Finally, the lowest (h/H) ratio was found in *Tribonema* sp. FB04M (0.72).

Conde et al. 2021, reported the nutritional indexes of seven commercial microalgae strains - ω -6/ ω -3 ratio with values ranging between 0.1 and 0.6, AI within 0.2 and 0.7, TI values from 0.1 to 1.6 and (h/H) ratio with 0.6 to 2.9. Thus, the index values determined in this work are within the described for microalgae strains.

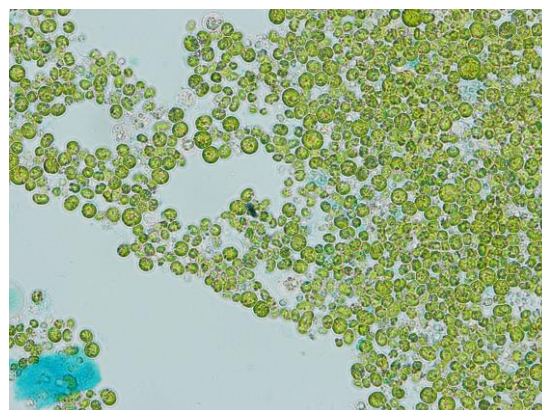
Strategies for productivity improvement of ω -3 and 6 have been developed. One of the most described methods is the induction of nitrogen stress by partially or complete depriving microalgae of N_2 . Heterotrophic growth has also proven to be a good strategy for higher lipid yields. However, these strategies can negatively affect biomass productivity, reducing growth rates [58].

6. Polysaccharides

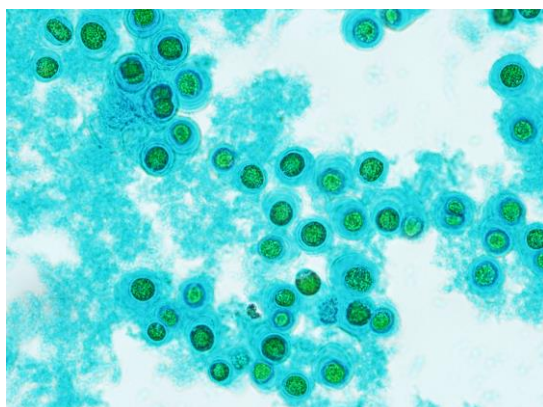
Whilst the microalgae cultures were manipulated for scale-up growth and growth curve measurements, a presence of viscous substances was detected in some of the microalgae culture flasks. A polysaccharide screening was then conducted to verify the presence of possible EPS polymers. In order to first assess the polysaccharide content, an Alcian Blue staining was performed in all microalgae strains. The qualitative results are shown in **Figure 25** (4 most promising strains) and **Appendix 11** (remaining 18 strains).



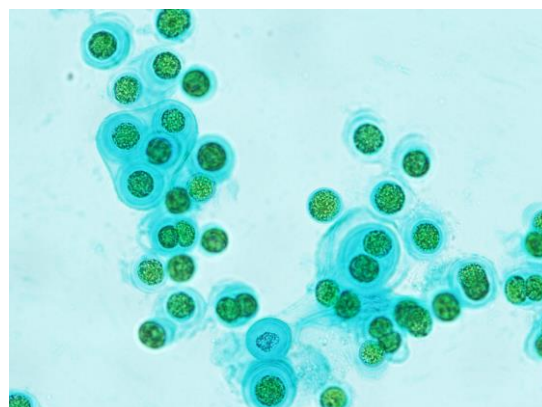
FB05M with AB 0.5% in Ethanol (50%)



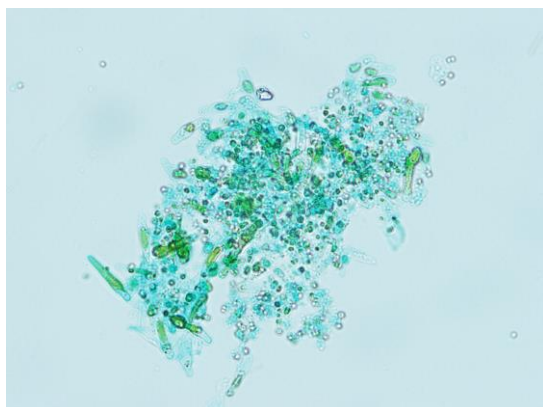
FB05M with AB 0.5% in Acetic acid



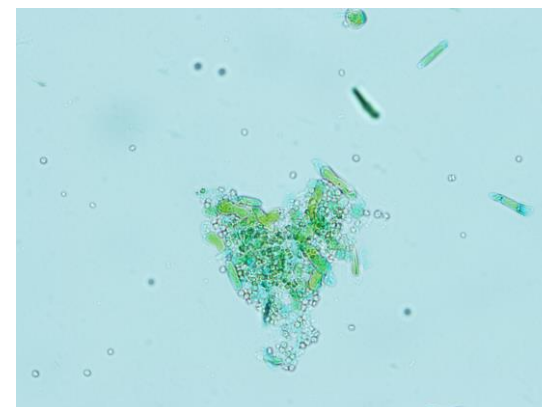
FB06M with AB 0.5% in Ethanol (50%)



FB06M with AB 0.5% in Acetic acid



FB21M with AB 0.5% in Ethanol (50%)



FB21M with AB 0.5% in Acetic acid

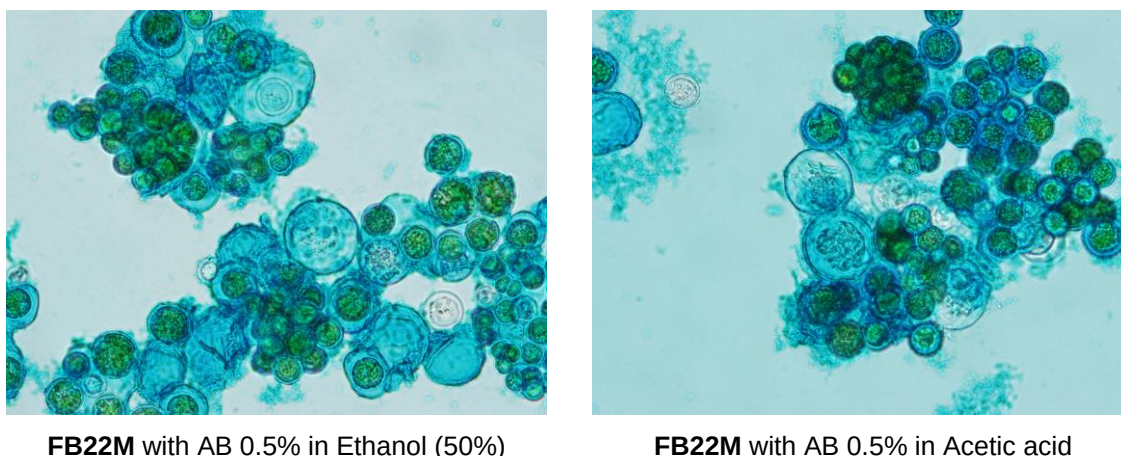


Figure 25. Microscopic visualization of microalgae strains FB05M, FB06M, FB21M and FB22M with Alcian Blue dye 0.5% in (A) ethanol (50%) and (B) acetic acid.

After visual observation of the presence of the blue stain, *Eustigmatos* sp. FB05M, *Chlamydocapsa* sp. FB06M, *Actinastrum* sp. FB21M and *Sphaerocystis* sp. FB22M strains were selected for further polysaccharide quantification. This evaluation corresponds to the first naked eye visualization of the polymers present in the culture flasks.

FB05M, FB06M, FB21M and FB22M ethanolic extracts (70%) were then quantified with the Dubois colourimetric method for polysaccharide quantification. The results are expressed in **Table 14**.

Table 14. Quantification of polysaccharide content of FB05M, FB06M, FB21M and FB22M microalgal ethanolic extracts.

Strain	$\mu\text{g mg}^{-1}$ extract*	mg g^{-1} dry biomass*
FB05M	5.04 ± 0.44	1.97 ± 0.17
FB06M	not quantifiable	
FB21M	12.39 ± 1.50	4.07 ± 0.49
FB22M	23.06 ± 0.51	2.85 ± 0.06

* results expressed in D-(+)-glucose units

The polysaccharide content of *Chlamydocapsa* sp. FB06M was not quantifiable. The highest polysaccharide content, obtained from a D-(+)-glucose calibration curve, was found in the FB21M strain, followed by FB22M and then FB05M. No literature studies reporting the polysaccharide content of these genera were found, until present moment.

According to the initial observation by the qualitative analysis, with Alcian blue staining, the strain FB22M revealed the highest polysaccharide content per extract ($23.1 \mu\text{g mg}^{-1}$ extract). However, the extraction yield of FB22M was only 12.4% (**Table 9**), which translated to a lower quantity per dry biomass (2.9 mg g^{-1}), when compared to FB21M strain (4.1 mg g^{-1}).

Polysaccharides from microalgae are a very desirable target for incorporation in cosmetic formulations and as a source of nutrients, but the research and development in this field is still at an early stage, with scarce information available. Recent studies report *Porphyridium* sp., *Chlorella* sp. (0.09 g g⁻¹ biomass), *Spirulina* sp. and *Nostoc* sp. as possible candidates for polysaccharide production [82, 88].

The genus *Euglena* is well-known for the HVC production of the polysaccharide paramylon [24, 94-96], a future exploitation of strains belonging to this genus would be interesting to pursue.

Current growth optimization methods are being tested for an increase in polysaccharide production in microalgae, for example, the continuous culture exposure to high-light intensity. Blue and red light have also been proved to be efficient in photosynthesis and polysaccharide content increase [53]. Extraction and purification techniques are also under the scope of the efforts made, however, a balance between cost-efficiency must be achieved.

IV. Conclusion

Microalgae are keen producers of high-value compounds with a plethora of biotechnological applications, especially in nutraceuticals and cosmetics/cosmeceuticals – that can confer health benefits for human lifestyles. These photosynthetic organisms are still an underexplored source of biotechnological potential due to an intrinsic high chemodiversity.

In this work, twenty-two fresh-water microalgae strains, non-axenic, were screened for HVC production - phenolic compounds, pigments, fatty acids, and polysaccharides. The microalgae strains under study belong to different genera – *Euglena*, *Tribonema*, *Eustigmatos*, *Chlamydocapsa*, *Haematococcus*, *Scenedesmus*, *Pandorina*, *Coelastrella*, *Oocystis*, *Chlorella*, *Microthamnion*, *Actinastrum* and *Sphaerocystis*.

The initial growth assessment of coccoid strains revealed that these microalgae, in standard culture conditions, needed at least 60 days to reach the stationary growth phase, where HVC production would reach the optimum stage.

Phenolic compound quantification of ethanolic extracts by the Folin-Ciocalteu method demonstrated that *Eustigmatos* sp. FB05M (11.7 mg GAE g⁻¹), *Oocystis* sp. FB13M (11.3 mg GAE g⁻¹) and *Chlamydocapsa* sp. FB06M (10.4 mg GAE g⁻¹) were the most promising strains, with TPC values similar to those described for the best PC-producing strains in the literature.

Pigment profiling of the ethanolic extracts by HPLC-PDA, revealed that *Eustigmatos* sp. FB05M had the highest total carotenoid relative abundance (95.7%) and *Chlamydocapsa* sp. FB06M the highest total carotenoid concentration (78.7 mg g⁻¹). Violaxanthin (peak 17) was the most abundant carotenoid found: 21.7 mg g⁻¹ dry biomass in FB05M. The violaxanthin derivative (peak 12) and chlorophyll a were the next most abundant pigments. Lutein was present in almost every strain, with *Euglena* sp. FB03M registering 1.17 mg g⁻¹.

Fatty acid screening of FAMES (DCM:MeOH derivatized extracts) by GC-MS indicated that α -linolenic acid was the most abundant FA, followed by palmitic acid (C16:0) and C16:4 $\Delta^{4,7,10,13}$ (ω -3). The highest relative abundance of total PUFA and ω -6 PUFA was found in the genus *Euglena*. *Eustigmatos* sp. FB05M presented a 49.5% content in Eicosapentaenoic acid - 39.0 mg g⁻¹, the highest concentration value in microalgae described so far in the literature. *Scenedesmus* sp. FB11M presented the highest linoleic acid content (9.8 mg g⁻¹). The highest content in γ -linoleic acid was found in *Chlamydocapsa* sp. FB06M with an abundance of 8.1% (8.2 mg g⁻¹), values in the same order as those reported in literature.

Pigment and fatty acid profiling allowed the separation of the strains into different groups, according to compound similarities.

In the cardiovascular disease risk predictors - ω -6/ ω -3 ratio, thrombogenicity index (TI), atherogenicity index (AI) and the hypocholesterolemic/hypercholesterolemic (h/H) ratio – allowed the nutritional evaluation of the incorporation of microalgae in human diets. *Scenedesmus* sp. FB11M (0.04), *Eustigmatos* sp. FB05M (0.05) and *Scenedesmus* sp. FB18M (0.05) indicated the lowest values of ω -6/ ω -3 ratio. *Euglena* sp. F01M registered the lowest value of AI (0.46) and the lowest (h/H) ratio was found in *Tribonema* sp. FB04M (0.72). All microalgae strains presented low TI values. These values are in the same range of those described in literature.

Polysaccharide evaluation by Alcian Blue staining allowed for the selection of four microalgae strains - *Eustigmatos* sp. FB05M, *Chlamydocapsa* sp. FB06M, *Actinastrum* sp. FB21M and *Sphaerocystis* sp. FB22M. The ethanolic extracts of these strains were then quantified by Dubois assay, with the highest polysaccharide content per dry biomass was found in FB21M (4.07 mg g⁻¹), followed by FB22M (2.85 mg g⁻¹) and FB05M (1.92 mg g⁻¹).

The overall analysis of HVC screening highlights different compositions within different microalgae genera. *Eustigmatos* FB05M was the most promising strain, with high phenolic, carotenoid (violaxanthin), PUFA (EPA) and polysaccharide content. FB05M also presented a high growth rate (**Figure 16**) and high extraction yields (**Table 9** and **Table 10**). To the best of our knowledge, few studies can be found regarding the potential of this microalgae strain, which is not yet approved for consumption and cosmetic application (EFSA). The genus *Euglena* recently approved for commercialization, also uncovered a lot of potential in HVC production. However, this strain had very low biomass productivity, creating several challenges for efficient culture growth in this work (growth attempt with both M7 medium and Z8 medium).

In conclusion, this study reinforced microalgae strains as high-value compound producers, emphasizing the still unrecognized potential of some strains that are yet to be regulated for their market entries.

V. Future perspectives

The purpose of this work was to conduct a first characterization of some HVC profiles in a pool of microalgae strains belonging to different genera.

It would be interesting to further optimize HVC production with manipulation of growth cultures – light, photoperiod, temperature, culture medium, pH – in order to boost HVC production. Recent efforts in heterotrophic and mixotrophic growth have shown interesting effects on compounds and biomass productivity [143]. Recent studies have also considered the possibility of industrial growth of different microalgae strains in co-culture, with some consortia already proven to enhance bioactive compound content, namely lipid productivity [144]. Other reports focused on microalgae-bacteria consortia, with promising results in agro-industrial waste-water bioremediation [16].

To complement these work findings, complementary assays can be performed to sustain the bioactive properties claimed to the identified HVC – namely cell culture and cytotoxicity analysis (e.g. MTT assay), antioxidant assays (e.g. ABTS^{•+} and DPPH[•] free radical scavenging activity, superoxide anion radical scavenging capacity, tyrosinase inhibitory assay), cell culture anti-inflammatory activity (e.g. evaluation of inflammatory mediators - tumour necrosis factor α (TNF- α) and interleukin-6 (IL-6), prostaglandin E2 (PGE2) and nitrous oxide (NO)).

Studies of bioavailability, bioaccessibility and synergistic interactions among HVC are also mandatory to better understand the physiological and therapeutic effect of these compounds in the human body.

Several microalgae-derived products are already well established in the market, and there is a clear opportunity for the introduction of new ones. However, at an industrial level, several limiting steps that affect product cost-efficiency still need to be overcome to have sustainable costs associated with microalgae production and downstream processes. Hence, there is a need for the bioprospection of new microalgae strains to broaden the spectrum of already commercialized and accepted microalgae, thus reshaping the current nutraceutical and cosmetic industries.

Chapter 2

Entrepreneurial activities in business
context

I. Introduction

1. Biotechnology Sector

The biotechnology industry can be categorized as an engine for science innovation and growth that makes use of living organisms and their properties for the production of knowledge, goods and services. Biotechnology is nowadays perceived as the future of sustainable and resilient economies.

According to the Grand View Research report, the global biotechnology market was estimated at USD 1024 billion in 2021, with an expected CAGR of 13.9% from 2022 to 2030. According to the Portugal Biotech report of June 2021, the Portuguese biotechnology sector Portugal, in 2019 - with 98 registered companies - had a turnover of 36.5 million euros. In Europe, the biotech sector is dominated by five countries: the United Kingdom, Germany, Netherlands, Belgium and France

Within this vast industry, microalgae biotechnology arises as a promising sector with a pool of different applications in nutraceutical, cosmetic, pharmaceutical, aquaculture, bioremediation, agriculture, and biofuel sectors.

2. Fykia Biotech Company

Fykia Biotech Lda is a young, microalgae biotechnology start-up, based in the metropolitan area of Porto (Portugal), a bustling hub for algal technology.

The company was launched in 2021 as a spin-off company of the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) by the hands of four researchers: João Morais, Research Technician and curator of the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE-CC); Vitor Vasconcelos, principal investigator (PI) of Blue Biotechnology and Ecotoxicology group; Pedro Leão, PI of Cyanobacterial Natural Products group; and Ralph Urbatzka, head of anti-obesity properties natural compounds research team.

Currently located in CIIMAR the company is a partner with different projects and networks - EnhanceMicroAlgae, REDEALGAE, Sherpa do Mar and Hi Seed Tech.

2.1. Company mission

As a research-intensive company, Fykia Biotech has focused on biologically active algae metabolites on three scientific areas: nutraceuticals, cosmetics, and agriculture (**Figure 26**).

The company wants to create innovative solutions powered by cyanobacteria and microalgae that can tackle the needs and demands of current society. In nutraceuticals,

Fykia Biotech aims to develop HVC from microalgae with a special interest in antiobesogenic nutraceuticals to treat the ongoing obesity epidemic. In cosmetics and cosmeceuticals, the company aspires to introduce new all-natural ingredients into the market, that can act as antioxidants, anti-inflammatory and anti-ageing agents. In agriculture, the focus is shifted to the development of sustainable fertilizers and biostimulants that can be tailored to culture and soil.



Figure 26. Fykia Biotech scientific areas.

3. Acceleration Programs

As a young start-up, Fykia Biotech has avidly demonstrated interest in the expansion of knowledge in business, commercialization, and entrepreneurial skills.

Acceleration programs consist of intense and fast-paced hands-on trainings, that offer powerful tools for early-stage companies that want to be market ready. These programs often provide access to mentorship, networking, and venture capital.

4. Social media in Biotech

Digital presence is, nowadays, crucial for companies in every sector. Social media can help businesses to engage and attract potential customers, get feedback, and build loyalty relationships, thus increasing market reach and awareness.

In the biotechnology sector, social media has been playing an important role in facilitating transparent knowledge to the general population and demystifying science with an educational background. This represents a huge opportunity for companies to easily share their milestones and scientific achievements and provide community building. LinkedIn is one of the platforms selected by most companies.

5. Objectives

In parallel with laboratory work for HVC microalgae screening, a set of business and entrepreneurial activities were also developed.

Fykia's nutraceutical team applied to several entrepreneurial programs – Business Ignition Program (BIP) Proof (by Spin up project, University of Porto and Santander), EIT Food Seedbed (by the European Institute of Innovation & Technology) and HiTech (by

HiSeedTech association). Program applications often required the assembly of: state of the art and framework of the proposed project/idea; a roadmap with work milestones; funding goals; and video pitches. The company was selected for the HiTech program. For several months, the nutraceutical team worked was enrolled in this program, as an outcome the team developed a business case for a nutraceutical product - targeted for obesity and overweight treatment.

As part of business work, the company's LinkedIn account was created and managed by three of the company interns. Part of the activities included content creation and network expansion.

II. HiTech acceleration program

1. HiTech program overview

With the project entitled “hpa, a compound to fight obesity”, the nutraceutical team of Fykia Biotech was actively enrolled in the HiTech program - from 15th march to 13th July of 2022. The team was composed by the researchers Pedro Leão and Ralph Urbatzka; and three interns, the master students Ana Rita Vieira, Filipe Henriques and Hugo Amaral.

HiTech, provided by the HiSeedTech non-profit association, is a hybrid hands-on training program that aims to support Portuguese research teams to create value from scientific discoveries. HiTech provides skills in knowledge commercialization that enable the translation of science to technology and ultimately to a product. The main goals of the course were the development of a business case and a pitch presentation for a product concept (or service).

The acceleration program was segmented into three parts: (A) from technology to product concept, (B) from product concept to value proposition, and (C) from value proposition to business case. Throughout the program course, advisors, mentors, and tutors (American partners) guided and supported the team's progress with individual meetings. In webinars and technological sessions, the topics covered were: technology and application fields; opportunity space; business model and strategic assessment; product selection; value propositions; market assessment and validation; intellectual property; pricing point; sales plan; roadmap; financials; and pitch. The described topics were worked on by each team with documents and materials provided by the HiSeedTech team.

2. hpa, a compound to fight obesity: project framework

The product concept developed in the HiTech program was the result of prior technology research conducted by a team led by Ralph Urbatzka in the Blue Biotechnology Group of CIIMAR. The team found that the compound 13²-hydroxy-pheophytin a (hpa), a chlorophyll derivative (**Figure 27**) showed anti-obesity activity by reducing neutral lipids [51]. Hpa was first discovered by bioassay-guided fractionation. This compound can be obtained in high quantities from the cyanobacteria *Spirulina*, approved by human consumption worldwide with GRAS status. Zebrafish animal model was used to find and determine hpa activity.

In **Figure 27** an example of the zebrafish Nile red fat metabolism assay is shown, where the red fluorescence marks the presence of neutral lipids, stored as fat. Comparing the untreated zebrafish with the exposed zebrafish to hpa, a neutral lipid-reducing activity of 73% was observed. Furthermore, during the 48h exposure, no further lipid accumulation occurred in the zebrafish. Hence, hpa as a single compound presented a double action -

decreasing lipid content and preventing future accumulation. No malformations were observed in the tested animals.

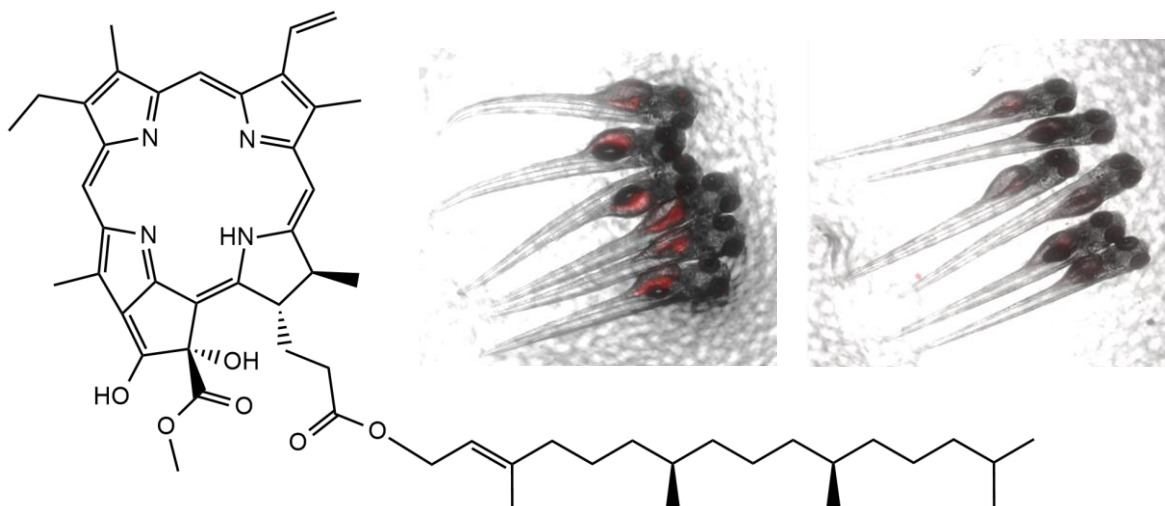


Figure 27. (A) Structure of 13²-hydroxy-pheophytin a (hpa). (B) Images from Zebrafish bioassay. Highlighted in red are neutral lipids, on the left for untreated fish and on the right for hpa-treated larvae.

Hpa represents a huge potential for being incorporated as an effective, non-toxic and carbon-negative nutraceutical for lipid reduction. A national patent was submitted on 2019-03-01 with the number 20191000013283 at INPI (Instituto Nacional da Propriedade Industrial) [145]. The patent relates to hydroxypheophorbide compounds with bioactivities towards obesity and obesity-related co-morbidities.

In order to compare the efficacy of hpa, the compound was tested against a weight-loss nutraceutical competitor (Sinetrol) that has clinical data. At the same concentration, the results showed that hpa was at least twice as effective in reducing lipids.

3. Product concept development

The product concept was developed to tackle obesity and overweight, a public health problem of utmost concern. According to World Health Organization (WHO), a whopping 13% of the worldwide population was considered obese and 39% as overweight in 2016. In some countries, these numbers are even more elevated, such is the case of the USA (**Figure 28**). The National Institute of Health (NIH) statistics reveal that 42.4% of American adults were obese in 2018, and this number is expected to keep increasing to around 50% of the US population will be by 2030, according to the Harvard School of Public Health.

The global anti-obesity nutraceutical market was estimated at USD 55 billion in 2021 (Grand View Research and National Institutes of Health (NIH) statistics). The anti-obesity nutraceutical market of the USA was then valued at around USD 20 billion.

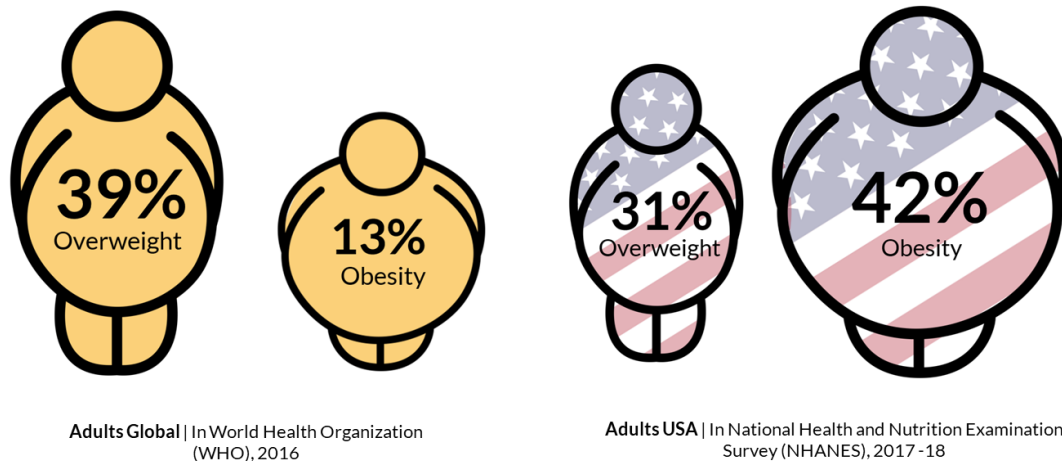


Figure 28. Overweight and obesity rates distribution among adults, globally on the left and USA on the right.

After opportunity space and market assessment, a product concept was developed to enter the USA market – a carbon-negative nutraceutical, from microalgae, for fat reduction and prevention of future accumulation. The nutraceutical would be shaped in a capsule format composed of a hpa enriched extracted. A possible package representation is shown in **Figure 29**. A business model was designed for the launch of the nutraceutical product into the USA market. The final project was pitched in a closing session - conducted at BBVA Theatre in Lisbon - to an audience comprised of HiSeedTech associates, capital investors and biotech companies.



Figure 29. Package representation of the nutraceutical product composed by hpa enriched extract capsules.

III. LinkedIn Management

In order to have an interactive digital space for Fykia Biotech company, a LinkedIn page was created and managed by an intern team composed by the master students Ana Freire, Ana Rita Vieira and Filipe Henriques.

The LinkedIn page was released in April 2022. Digital content was designed with the help of Adobe Illustrator 2020 software version 24.0.2. The first content to be launched was the presentation of the team members – initially the founder team members followed by the company collaborators. **Figure 30** shows an example of the type of content shared in the founder's presentation series.



Figure 30. Example of content shared on Fykia Biotech's LinkedIn page – founder team presentation.

Besides team presentation, content related to team activities, company networks and team projects were also shared – an example is demonstrated in **Figure 31**.



Figure 31. Example of content shared on Fykia Biotech's LinkedIn page network and projects.

Since Fykia Biotech's LinkedIn creation until the end of August, the page had a total of 2632 views with 736 unique visitors summing up to a total of 570 page followers. The engagement peak was experienced in April month. Regarding visitor demographics, Fykia Biotech was mainly visited by people with job functions in the Research and Education fields (49%) mainly from a biotechnology field background. Visitor analytics were obtained from LinkedIn's built-in function that displays aggregated traffic metrics.

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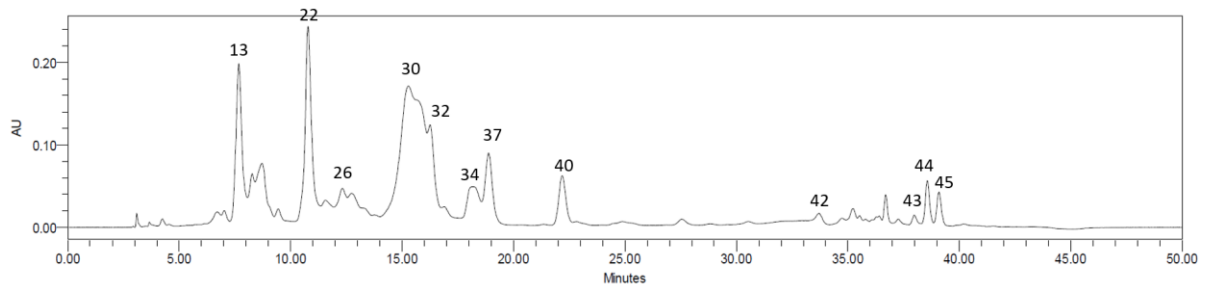
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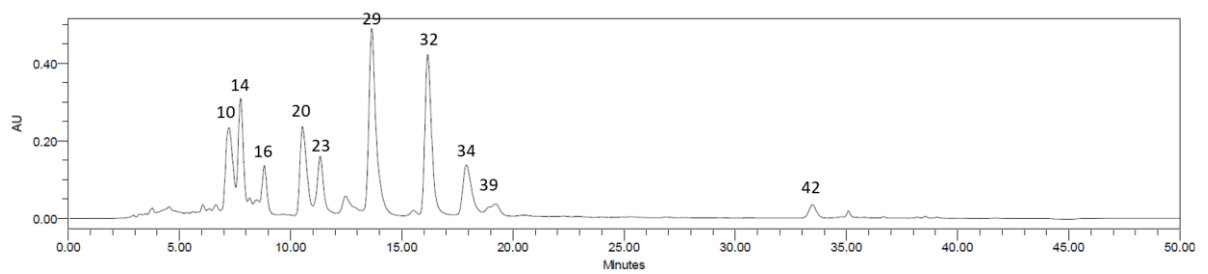
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Appendixes

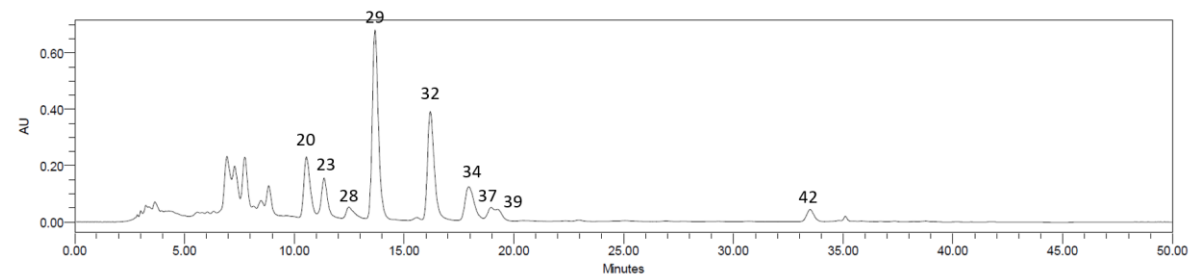
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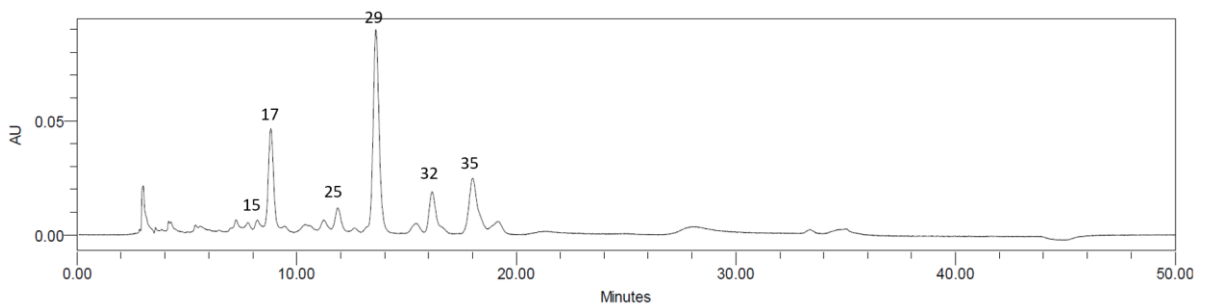
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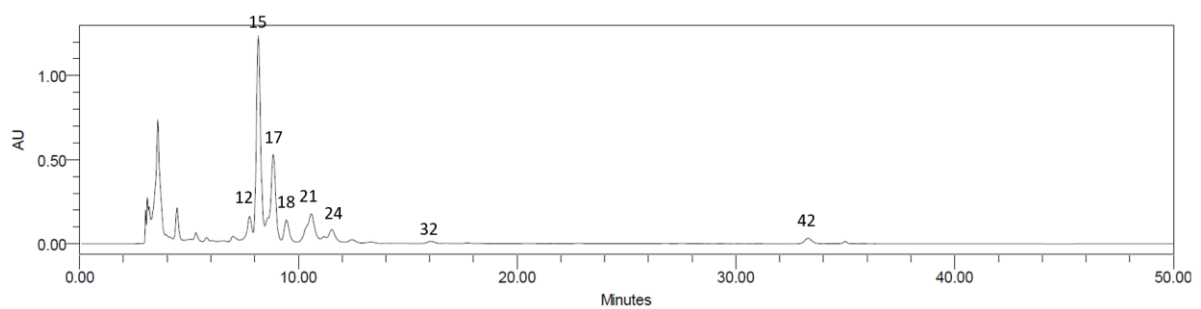
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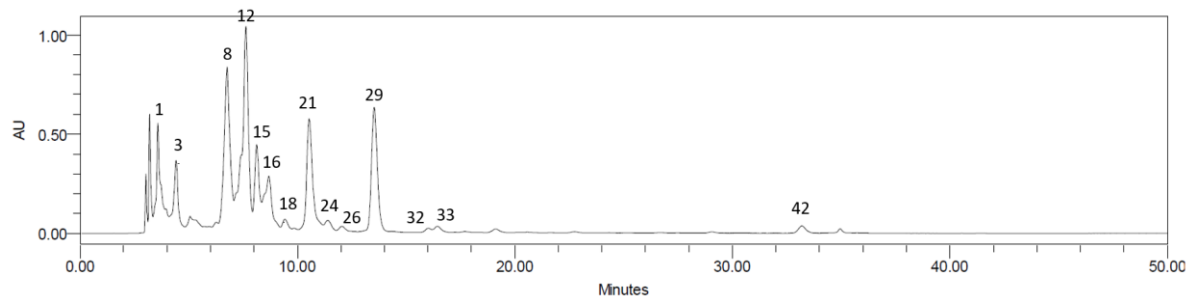
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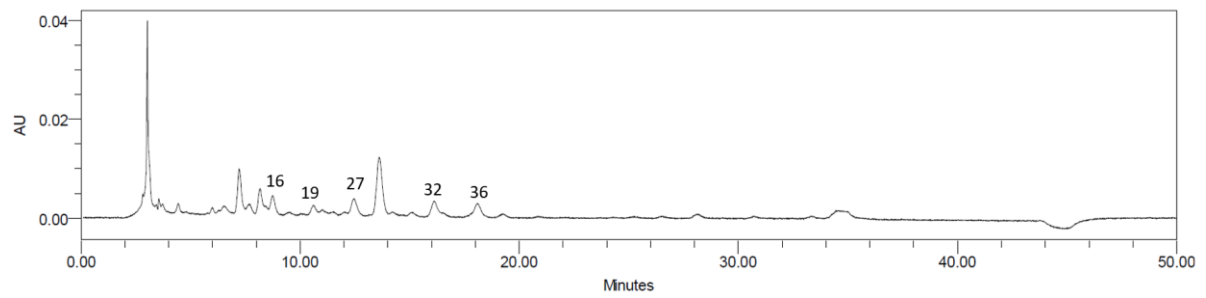
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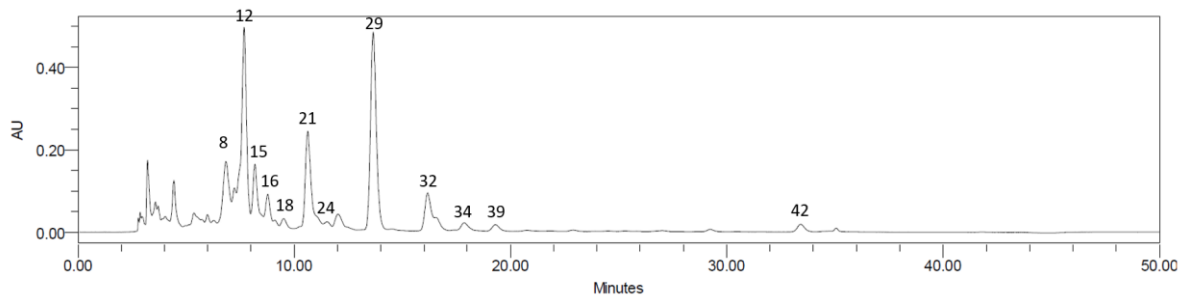
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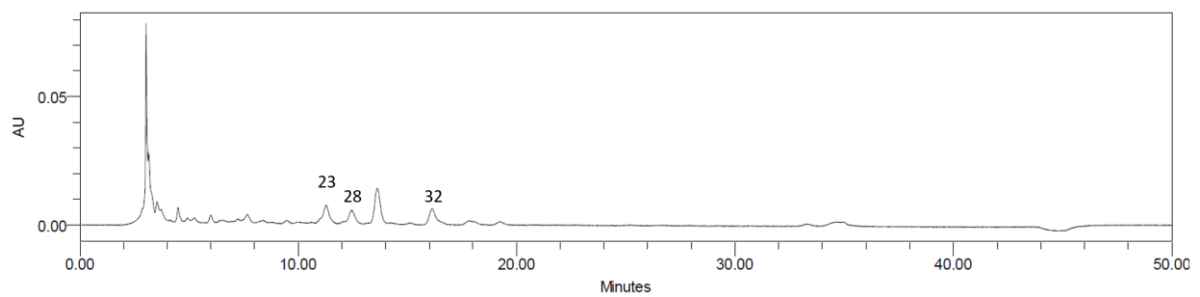
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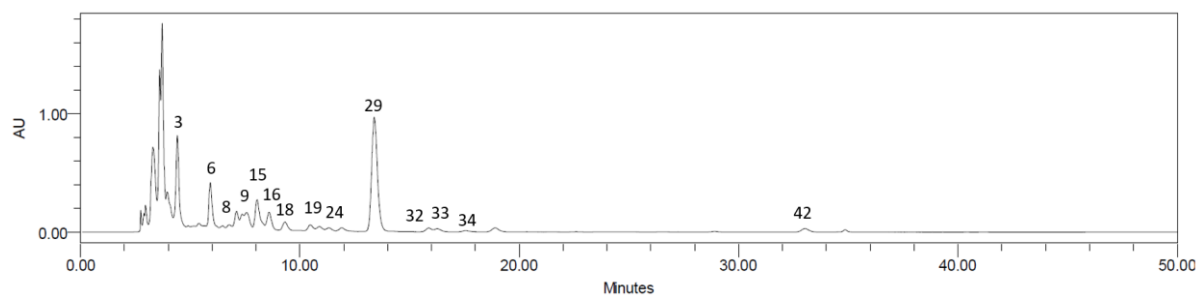
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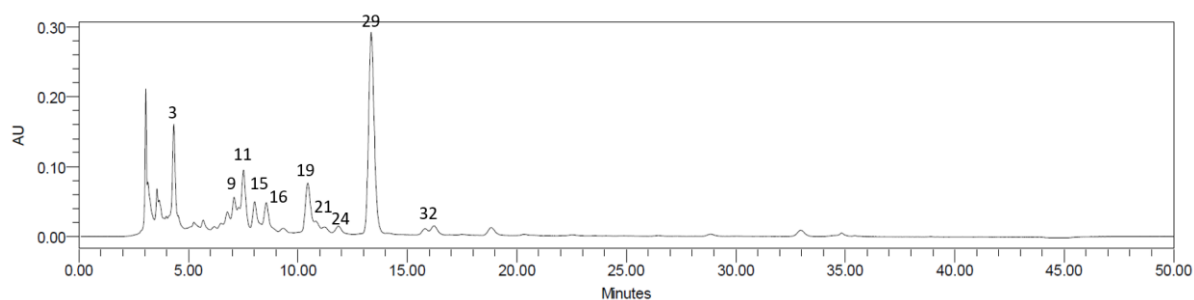
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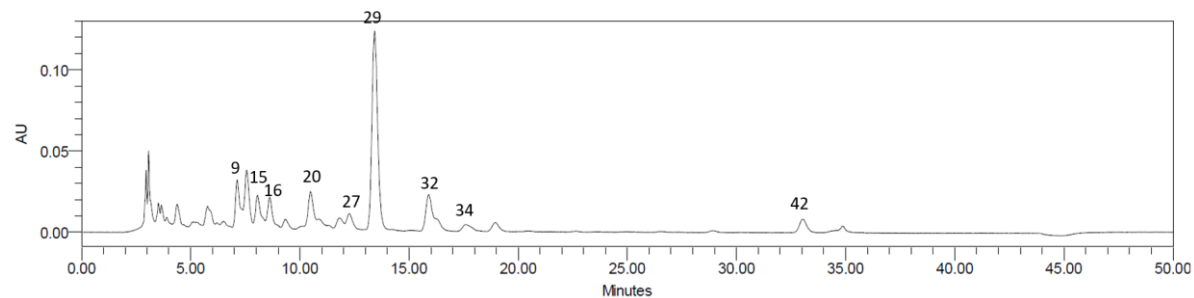
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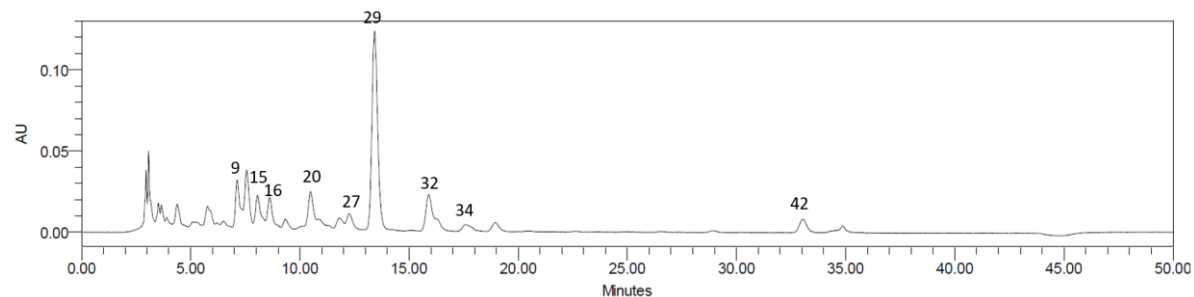
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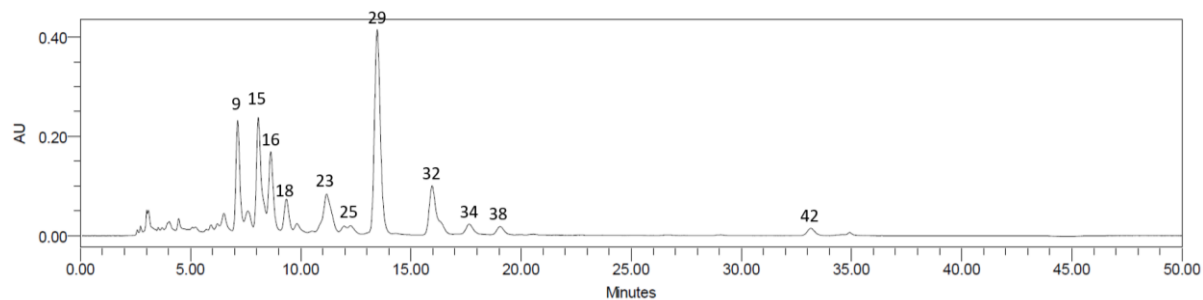
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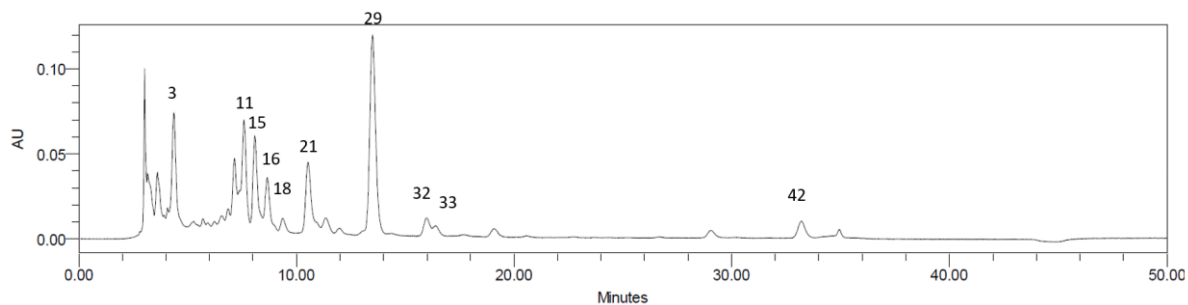
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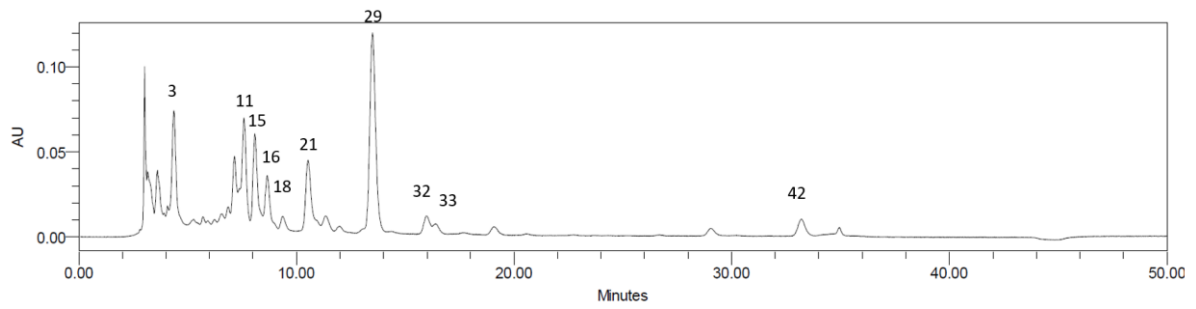
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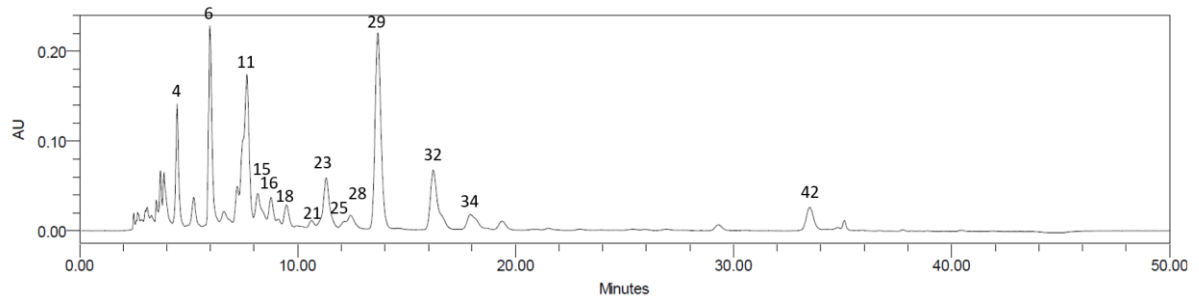
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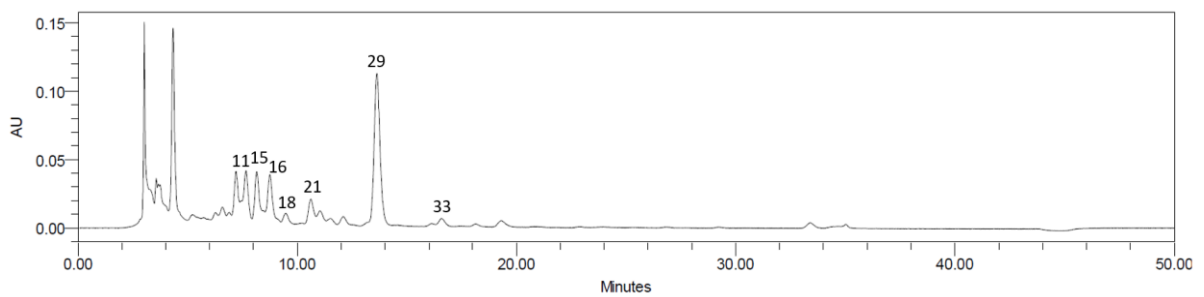
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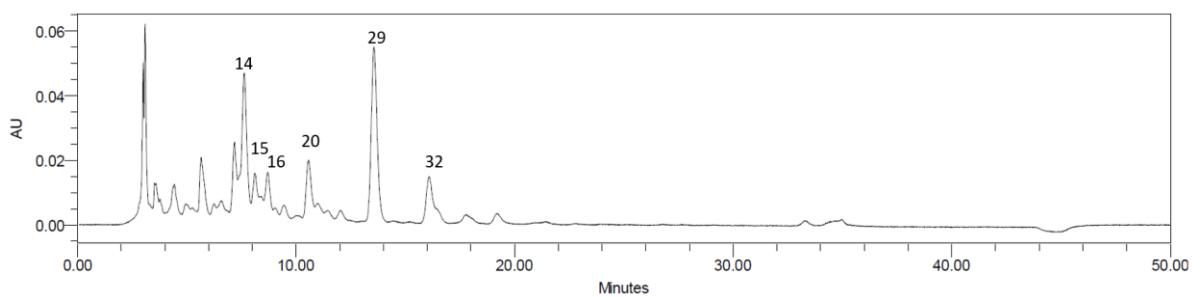
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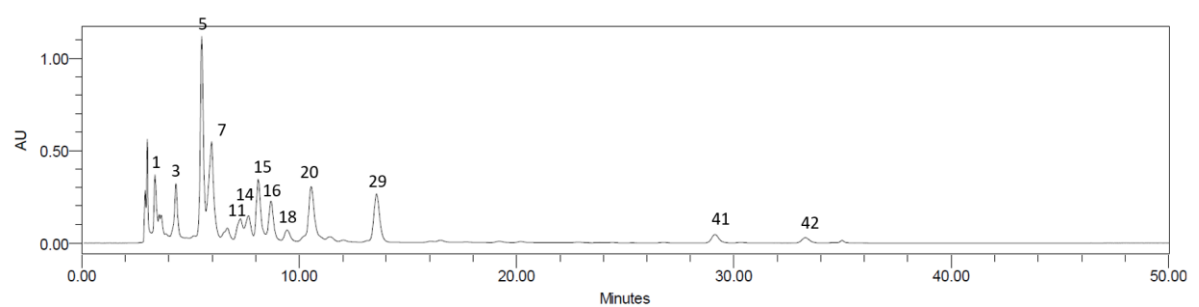
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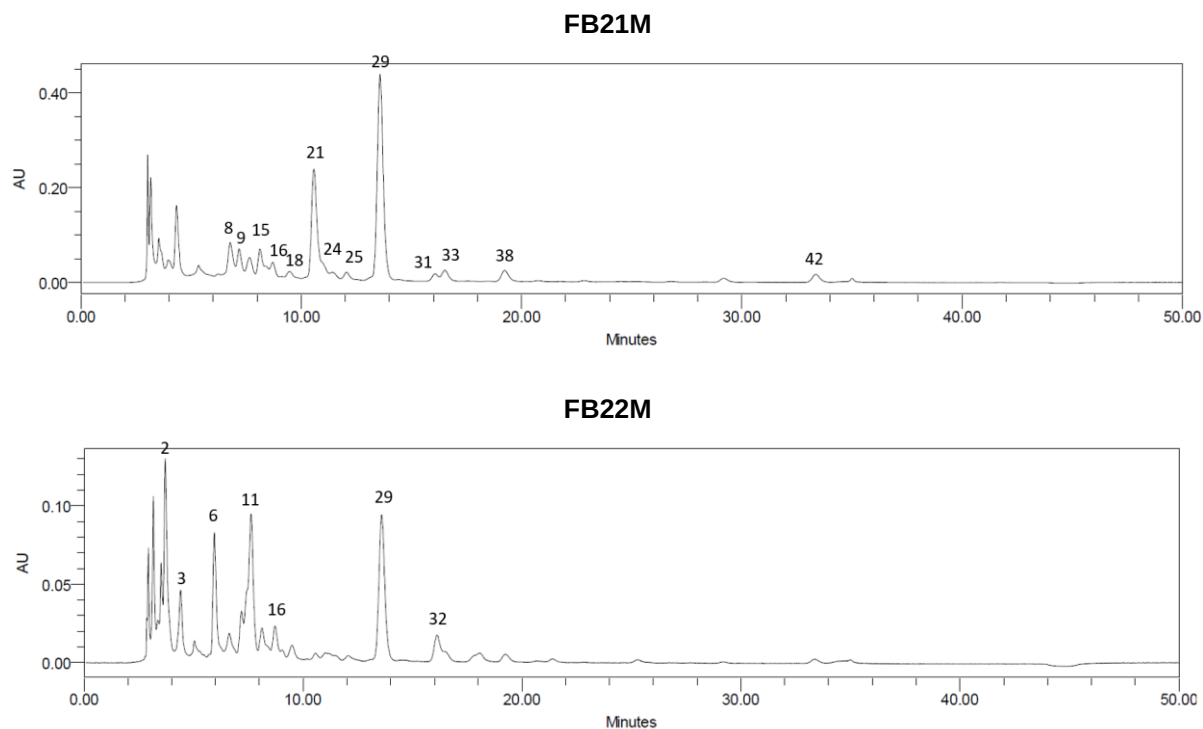


FB19M



FB20M





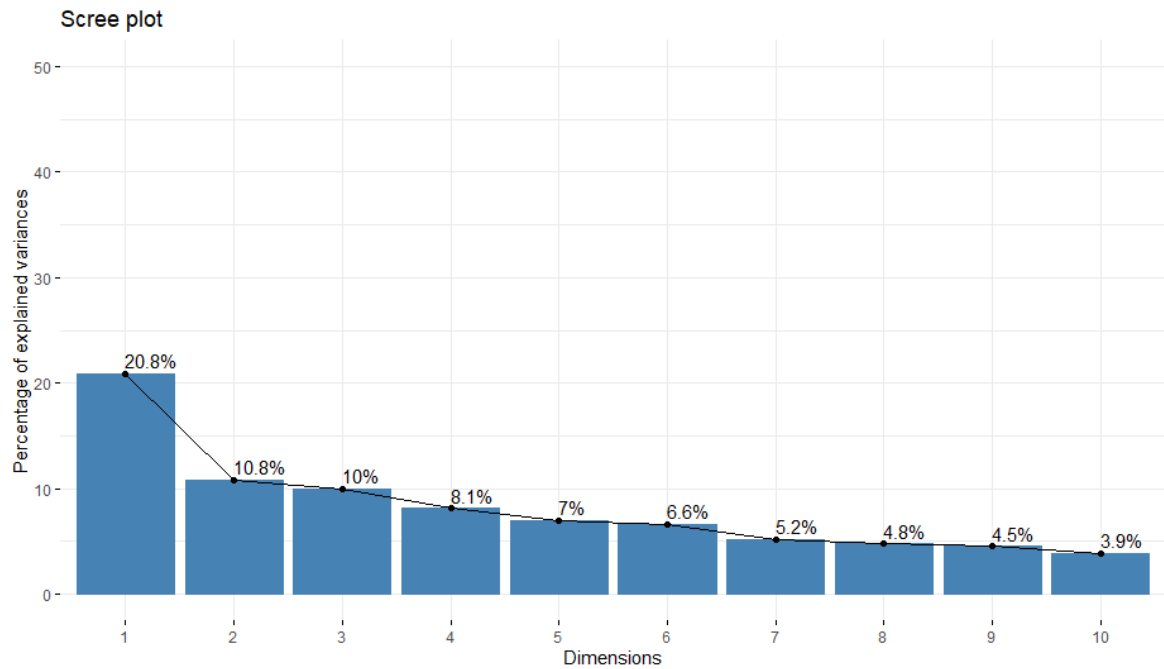
Appendix 1. HPLC-PDA carotenoid and chlorophyll chromatograms of microalgae ethanolic extracts at 450 nm. (1, 2, 3, 4, 7, 10, 13, 14, 19, 21, 23, 26 and 28) unidentified chlorophyll; (32) chlorophyll a; (6, 20, 22 and 34) chlorophyll a derivatives; (5, 8, 18, 30, 43 and 45) unidentified carotenoids; (9, 12, 25, 33, 35 and 38) violaxanthin derivative; (11, 7, 39 and 41) lutein derivative; (15) violaxanthin; (16) neoxanthin; (24) neoxanthin derivative; (27) astaxanthin; (29) lutein; (31) zeaxanthin; (36) canthaxanthin; (37 and 40) zeaxanthin derivative; (42) β -Carotene and; (44) lycopene.

Appendix 2. Carotenoids and chlorophyll content of all microalgae strains by HPLC-PDA. Pigments are expressed in mg per g of dry biomass $\pm$ standard deviation (SD).

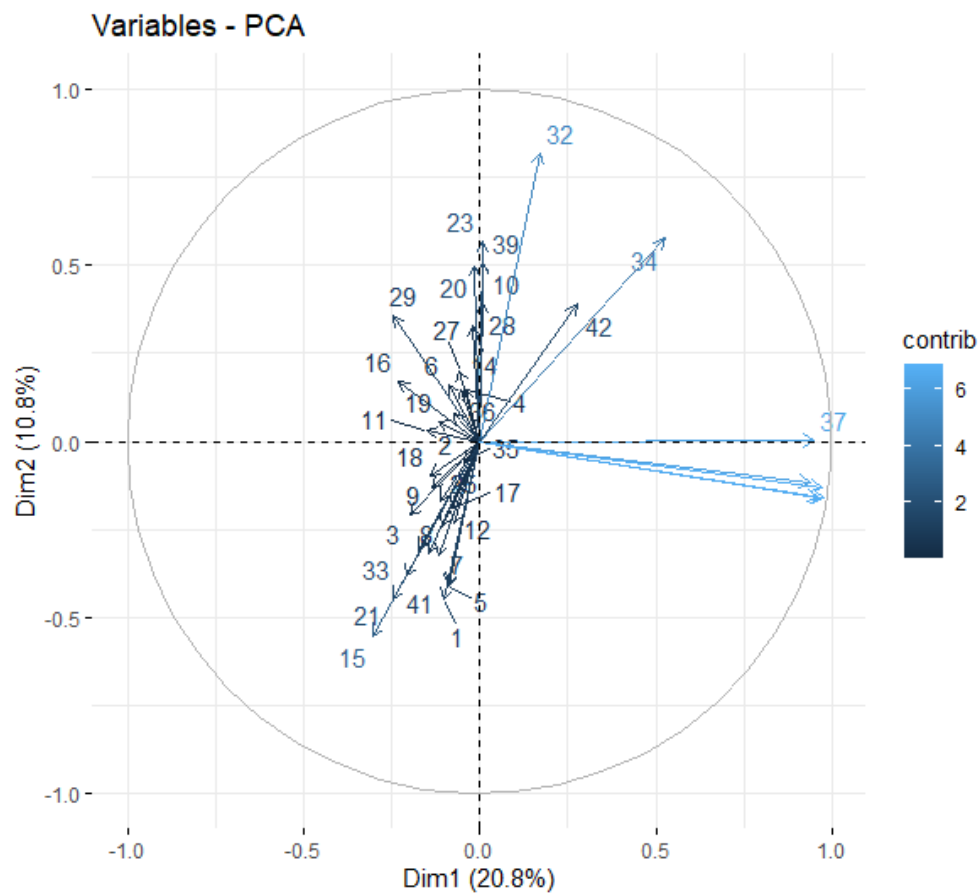
Peak	Pigment	RT/ min	FB01M	FB02M	FB03M	FB04M	FB05M	FB06M	FB07M
1	Unidentified chlorophyll	3.4						2.15 $\pm$ 0.11	
2	Unidentified chlorophyll	3.7							
3	Unidentified chlorophyll	4.4						1.53 $\pm$ 0.08	
4	Unidentified chlorophyll	4.5							
5	Unidentified carotenoid	5.5							
6	Chlorophyll a derivative	5.9							
7	Unidentified chlorophyll	6.0							
8	Unidentified carotenoid	6.7						0.77 $\pm$ 0.04	
9	Violaxanthin derivative	7.1							
10	Unidentified chlorophyll	7.2		2.67 $\pm$ 0.71					
11	Lutein derivative	7.5							
12	Violaxanthin derivative	7.6					1.84 $\pm$ 0.02	60.56 $\pm$ 1.99	
13	Chlorophyll a derivative	7.7	0.59 $\pm$ 0.02						
14	Unidentified chlorophyll	7.8		2.30 $\pm$ 0.37					
15	Violaxanthin	8.1				0.12 $\pm$ 0.01	21.71 $\pm$ 0.91	12.87 $\pm$ 1.11	
16	Neoxanthin	8.7		0.58 $\pm$ 0.01				0.64 $\pm$ 0.01	0.10 $\pm$ 0.00
17	Lutein derivative	8.8				0.03 $\pm$ 0.00	0.26 $\pm$ 0.01		
18	Unidentified carotenoid	9.4							0.10 $\pm$ 0.00
19	Unidentified chlorophyll	10.4							
20	Chlorophyll a derivative	10.5		3.24 $\pm$ 0.50	1.80 $\pm$ 0.03				
21	Unidentified chlorophyll	10.6					0.81 $\pm$ 0.01	4.64 $\pm$ 0.00	
22	Chlorophyll a derivative	10.8	0.92 $\pm$ 0.09						
23	Unidentified chlorophyll	11.3		1.71 $\pm$ 0.48	1.26 $\pm$ 0.00				
24	Neoxanthin derivative	11.5					0.22 $\pm$ 0.00		
25	Violaxanthin derivative	11.9				0.60 $\pm$ 0.04			
26	Unidentified chlorophyll	12.0	0.25 $\pm$ 0.05					0.48 $\pm$ 0.00	
27	Astaxanthin	12.3							0.09 $\pm$ 0.00
28	Unidentified chlorophyll	12.5			0.65 $\pm$ 0.01				
29	Lutein	13.6		1.61 $\pm$ 0.24	1.17 $\pm$ 0.02	0.11 $\pm$ 0.00		1.19 $\pm$ 0.00	
30	Unidentified carotenoid	15.3	0.29 $\pm$ 0.01						
31	Zeaxanthin	16.0							
32	Chlorophyll a	16.2	0.48 $\pm$ 0.03	4.94 $\pm$ 0.10	3.26 $\pm$ 0.02	0.34 $\pm$ 0.00	0.16 $\pm$ 0.00		0.10 $\pm$ 0.00
33	Violaxanthin derivative	16.4						2.64 $\pm$ 0.00	
34	Chlorophyll a derivative	17.9	0.39 $\pm$ 0.02	2.34 $\pm$ 0.04	1.52 $\pm$ 0.01				
35	Violaxanthin derivative	18.0				1.97 $\pm$ 0.04			
36	Canthaxanthin	18.1							0.08 $\pm$ 0.00
37	Zeaxanthin derivative	18.9	0.08 $\pm$ 0.01		0.09 $\pm$ 0.00				
38	Violaxanthin derivative	19.0							
39	Lutein derivative	19.2		0.13 $\pm$ 0.02					
40	Zeaxanthin derivative	22.2	0.05 $\pm$ 0.00						
41	Lutein derivative	28.9							
42	β -Carotene	33.3	0.04 $\pm$ 0.02	0.16 $\pm$ 0.01	0.37 $\pm$ 0.35				
43	Unidentified carotenoid	37.9	0.08 $\pm$ 0.01						
44	Lycopene	38.6	0.12 $\pm$ 0.00						
45	Unidentified carotenoid	39.1	0.03 $\pm$ 0.01						
Total carotenoids			0.69 $\pm$ 0.03	2.49 $\pm$ 0.24	1.63 $\pm$ 0.35	2.83 $\pm$ 0.06	24.03 $\pm$ 0.91	78.67 $\pm$ 2.28	0.28 $\pm$ 0.00
Total chlorophylls			2.64 $\pm$ 0.11	17.20 $\pm$ 1.06	8.49 $\pm$ 0.04	0.34 $\pm$ 0.00	0.97 $\pm$ 0.01	8.79 $\pm$ 0.13	0.20 $\pm$ 0.00

Peak	Pigment	RT/ min	FB08M	FB09M	FB10M	FB11M	FB12M	FB13M	FB14M
1	Unidentified chlorophyll	3.4							
2	Unidentified chlorophyll	3.7							
3	Unidentified chlorophyll	4.4			2.09 ± 0.09	0.43 ± 0.01			
4	Unidentified chlorophyll	4.5							
5	Unidentified carotenoid	5.5							
6	Chlorophyll a derivative	5.9			1.34 ± 0.01				
7	Unidentified chlorophyll	6.0							
8	Unidentified carotenoid	6.7	0.64 ± 0.72					0.47 ± 0.02	
9	Violaxanthin derivative	7.1			2.46 ± 0.03	0.43 ± 0.06	0.68 ± 0.04		12.28 ± 0.14
10	Unidentified chlorophyll	7.2							
11	Lutein derivative	7.5				0.02 ± 0.00		1.84 ± 0.03	
12	Violaxanthin derivative	7.6	22.99 ± 3.48						
13	Chlorophyll a derivative	7.7							
14	Unidentified chlorophyll	7.8							
15	Violaxanthin	8.1	4.92 ± 0.09		8.45 ± 0.05	0.82 ± 0.02	0.55 ± 0.06	12.68 ± 1.43	14.08 ± 0.12
16	Neoxanthin	8.7	0.32 ± 0.01		0.35 ± 0.00	0.17 ± 0.00	0.22 ± 0.00	0.49 ± 0.02	0.63 ± 0.02
17	Lutein derivative	8.8							
18	Unidentified carotenoid	9.4							
19	Unidentified chlorophyll	10.4			0.45 ± 0.00	0.40 ± 0.00		1.36 ± 0.00	
20	Chlorophyll a derivative	10.5					0.28 ± 0.02		
21	Unidentified chlorophyll	10.6	1.86 ± 0.01			0.20 ± 0.00			
22	Chlorophyll a derivative	10.8							
23	Unidentified chlorophyll	11.3		0.20 ± 0.01				1.39 ± 0.01	1.45 ± 0.00
24	Neoxanthin derivative	11.5	0.28 ± 0.00		0.26 ± 0.00	0.16 ± 0.00			1.28 ± 0.07
25	Violaxanthin derivative	11.9							
26	Unidentified chlorophyll	12.0							
27	Astaxanthin	12.3					0.39 ± 0.15		
28	Unidentified chlorophyll	12.5		0.19 ± 0.00					
29	Lutein	13.6	0.76 ± 0.00		1.33 ± 0.01	0.27 ± 0.00	0.14 ± 0.00	0.98 ± 0.01	0.98 ± 0.00
30	Unidentified carotenoid	15.3							
31	Zeaxanthin	16.0							
32	Chlorophyll a	16.2	1.08 ± 0.00	0.19 ± 0.00	0.38 ± 0.00	0.24 ± 0.00	0.35 ± 0.01	4.17 ± 0.03	1.62 ± 0.00
33	Violaxanthin derivative	16.4			1.53 ± 0.03				
34	Chlorophyll a derivative	17.9	0.42 ± 0.00		0.26 ± 0.00		0.23 ± 0.00	1.34 ± 0.01	0.64 ± 0.00
35	Violaxanthin derivative	18.0							
36	Canthaxanthin	18.1							
37	Zeaxanthin derivative	18.9							
38	Violaxanthin derivative	19.0						2.34 ± 0.01	1.92 ± 0.00
39	Lutein derivative	19.2							
40	Zeaxanthin derivative	22.2							
41	Lutein derivative	28.9							
42	β-Carotene	33.3					0.04 ± 0.00		
43	Unidentified carotenoid	37.9							
44	Lycopene	38.6							
45	Unidentified carotenoid	39.1							
Total carotenoids			29.91 ± 3.55	0.00 ± 0.00	14.39 ± 0.07	1.88 ± 0.06	2.01 ± 0.17	18.79 ± 1.43	31.16 ± 0.20
Total chlorophylls			3.36 ± 0.01	0.58 ± 0.01	4.52 ± 0.09	1.27 ± 0.01	0.86 ± 0.02	8.26 ± 0.03	3.70 ± 0.01

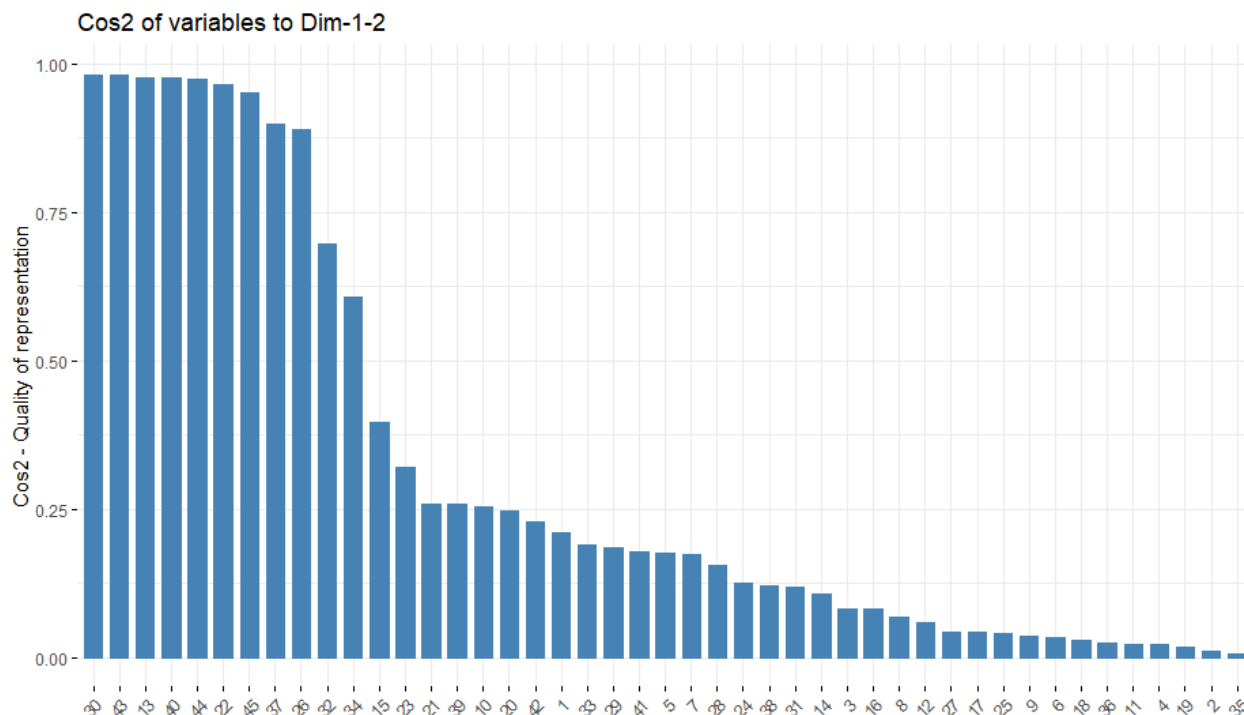
Peak	Pigment	RT/ min	FB15M	FB16M	FB17M	FB18M	FB19M	FB20M	FB21M	FB22M
1	Unidentified chlorophyll	3.4						2.07 ± 0.28		
2	Unidentified chlorophyll	3.7								0.30 ± 0.00
3	Unidentified chlorophyll	4.4	0.48 ± 0.01					1.77 ± 0.04		0.17 ± 0.00
4	Unidentified chlorophyll	4.5			1.20 ± 0.00					
5	Unidentified carotenoid	5.5						0.88 ± 0.13		
6	Chlorophyll a derivative	5.9		0.98 ± 0.03	1.86 ± 0.05					0.22 ± 0.01
7	Unidentified chlorophyll	6						4.26 ± 1.29		
8	Unidentified carotenoid	6.7							0.11 ± 0.01	
9	Violaxanthin derivative	7.1							2.04 ± 0.30	
10	Unidentified chlorophyll	7.2								
11	Lutein derivative	7.5	0.09 ± 0.01		0.48 ± 0.01	0.03 ± 0.01				0.06 ± 0.00
12	Violaxanthin derivative	7.6								
13	Chlorophyll a derivative	7.7								
14	Unidentified chlorophyll	7.8		1.93 ± 0.00			1.41 ± 0.00	0.91 ± 0.05		
15	Violaxanthin	8.1	2.07 ± 0.00	2.54 ± 0.92	2.17 ± 0.41	0.70 ± 0.02	0.74 ± 0.17	19.56 ± 0.44	2.18 ± 0.24	
16	Neoxanthin	8.7	0.28 ± 0.01	0.30 ± 0.01	0.49 ± 0.00	0.19 ± 0.00	0.67 ± 0.00	0.80 ± 0.03	0.37 ± 0.00	0.12 ± 0.00
17	Lutein derivative	8.8								
18	Unidentified carotenoid	9.4	0.04 ± 0.00		0.10 ± 0.01	0.03 ± 0.00				
19	Unidentified chlorophyll	10.4								
20	Chlorophyll a derivative	10.5					0.91 ± 0.01	3.57 ± 0.27		
21	Unidentified chlorophyll	10.6	0.54 ± 0.00		0.52 ± 0.00	0.24 ± 0.01			2.51 ± 0.02	
22	Chlorophyll a derivative	10.8		0.74 ± 0.03						
23	Unidentified chlorophyll	11.3		0.74 ± 0.01	1.17 ± 0.00					
24	Neoxanthin derivative	11.5							0.38 ± 0.00	
25	Violaxanthin derivative	11.9			0.69 ± 0.06				1.43 ± 0.05	
26	Unidentified chlorophyll	12		0.34 ± 0.00						
27	Astaxanthin	12.3								
28	Unidentified chlorophyll	12.5		0.38 ± 0.02	0.65 ± 0.00					
29	Lutein	13.6	0.17 ± 0.00	0.70 ± 0.00	0.62 ± 0.00	0.11 ± 0.00	0.16 ± 0.00	0.65 ± 0.02	0.96 ± 0.00	0.06 ± 0.00
30	Unidentified carotenoid	15.3								
31	Zeaxanthin	16							0.07 ± 0.00	
32	Chlorophyll a	16.2	0.32 ± 0.00	2.01 ± 0.00	1.48 ± 0.00		0.96 ± 0.01			0.17 ± 0.01
33	Violaxanthin derivative	16.4	0.43 ± 0.01			0.27 ± 0.03			2.29 ± 0.02	
34	Chlorophyll a derivative	17.9		0.75 ± 0.00	0.82 ± 0.01					
35	Violaxanthin derivative	18								
36	Canthaxanthin	18.1								
37	Zeaxanthin derivative	18.9								
38	Violaxanthin derivative	19							2.56 ± 0.01	
39	Lutein derivative	19.2								
40	Zeaxanthin derivative	22.2								
41	Lutein derivative	28.9						0.096 ± 0.01		
42	β-Carotene	33.3	0.06 ± 0.00	0.10 ± 0.00	0.13 ± 0.05				0.10 ± 0.00	
43	Unidentified carotenoid	37.9								
44	Lycopene	38.6								
45	Unidentified carotenoid	39.1								
Total carotenoids			3.14 ± 0.01	3.65 ± 0.92	4.68 ± 0.42	1.34 ± 0.03	1.56 ± 0.17	21.89 ± 0.46	12.48 ± 0.39	0.23 ± 0.00
Total chlorophylls			1.34 ± 0.01	7.86 ± 0.05	7.68 ± 0.06	0.24 ± 0.01	3.27 ± 0.01	12.58 ± 1.35	2.51 ± 0.02	0.86 ± 0.01



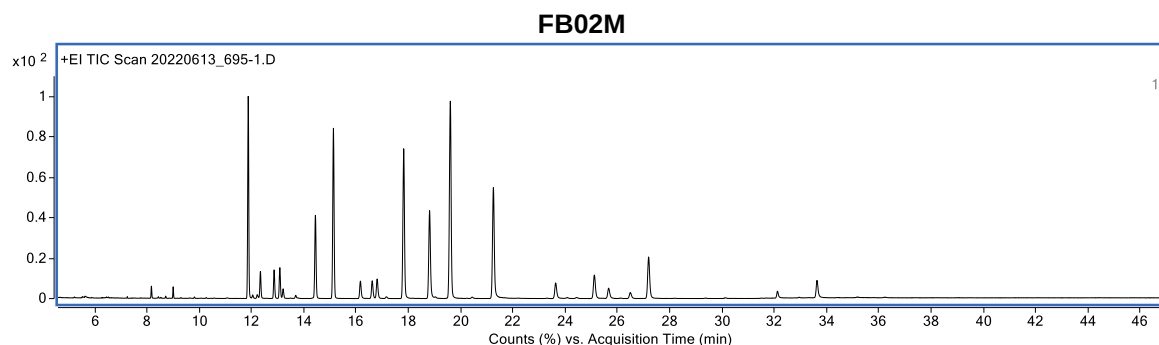
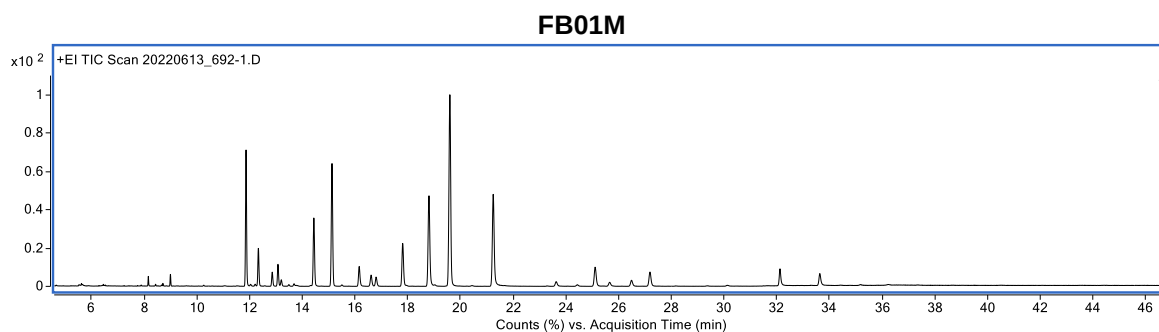
Appendix 3. Pigment PCA analysis scree plot. Eigenvalues are ordered from the largest to the smallest percentage of explained variance.



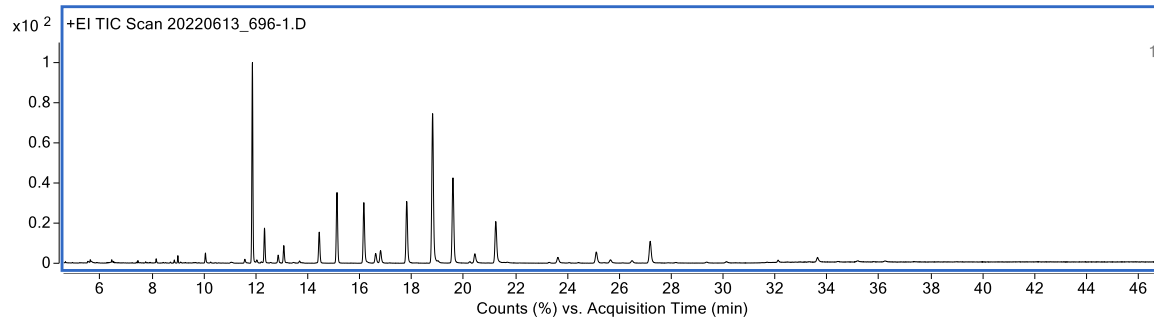
Appendix 4. Pigments PCA graph of variables, with the highlighted contribution.



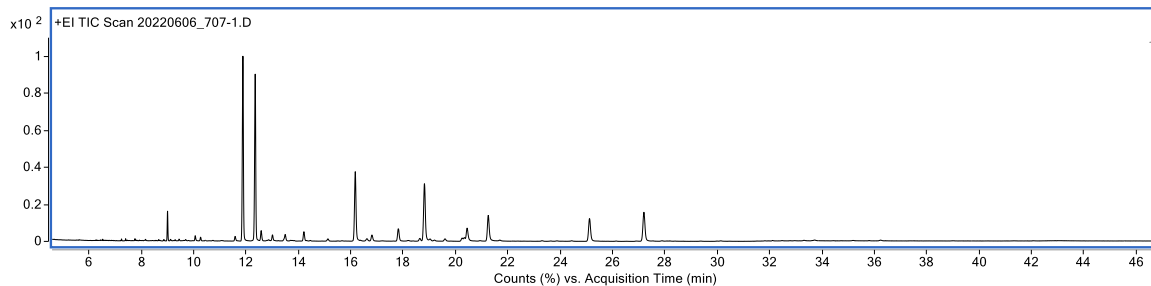
Appendix 5. Contributors (pigments) for PCA (Dim1:2) of pigment PCA analysis. (1, 2, 3, 4, 7, 10, 13, 14, 19, 21, 23, 26 and 28) unidentified chlorophyll; (32) chlorophyll a; (6, 20, 22 and 34) chlorophyll a derivatives; (5, 8, 18, 30, 43 and 45) unidentified carotenoids; (9, 12, 25, 33, 35 and 38) violaxanthin derivative; (11, 7, 39 and 41) lutein derivative; (15) violaxanthin; (16) neoxanthin; (24) neoxanthin derivative; (27) astaxanthin; (29) lutein; (31) zeaxanthin; (36) canthaxanthin; (37 and 40) zeaxanthin derivative; (42) β -Carotene and; (44) lycopene.



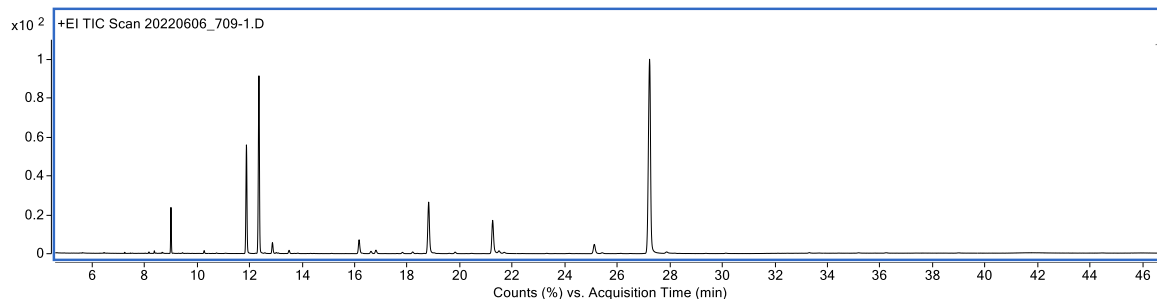
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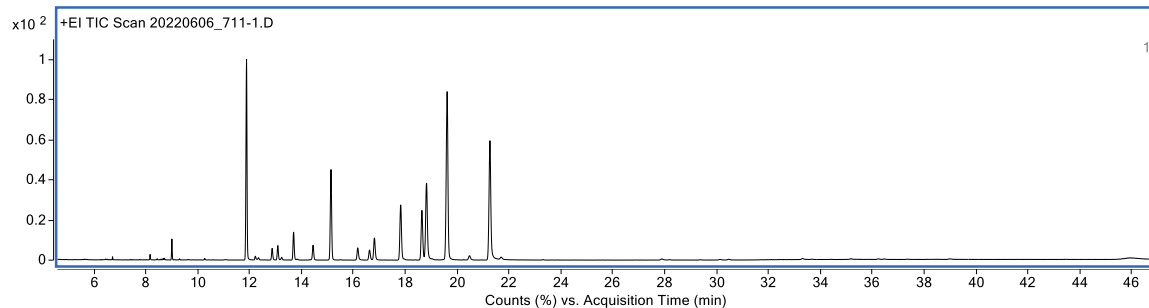
FB04M



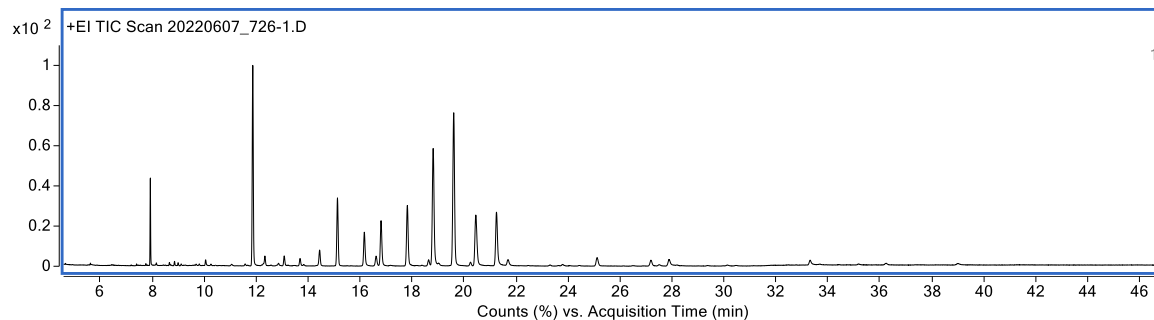
FB05M



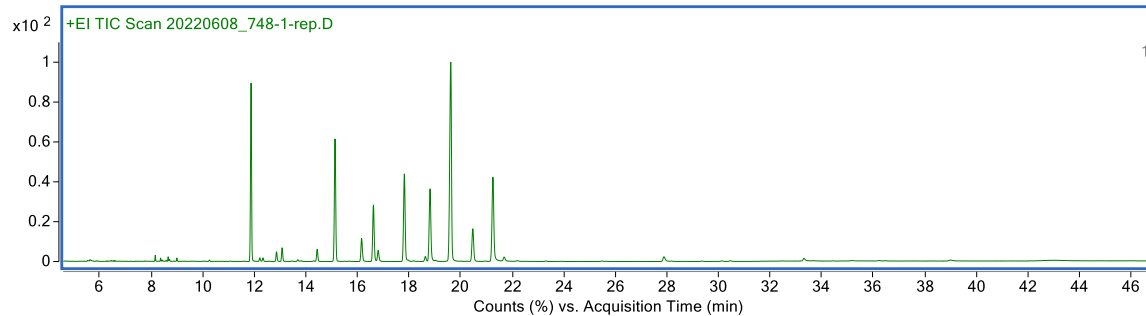
FB06M



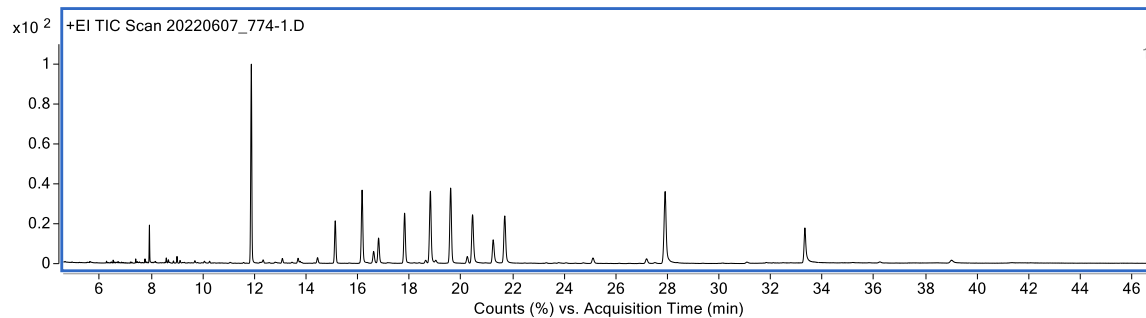
FB07M



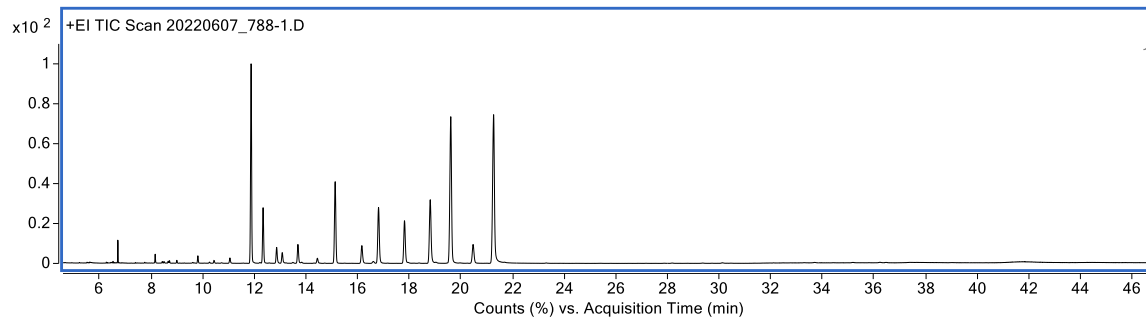
FB08M



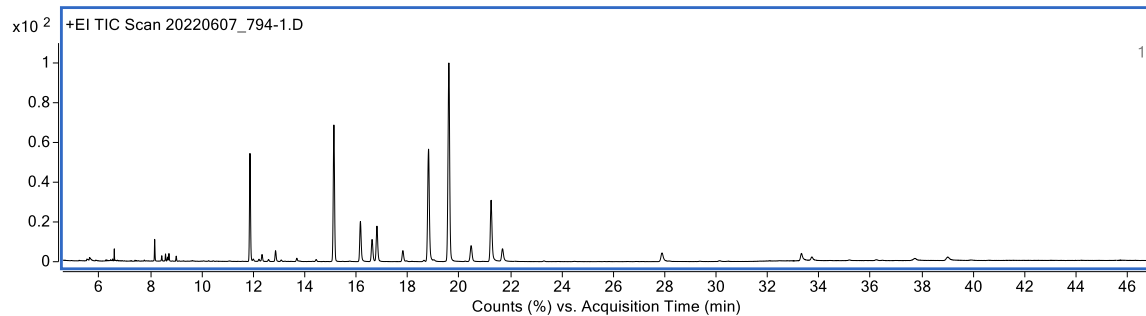
FB09M



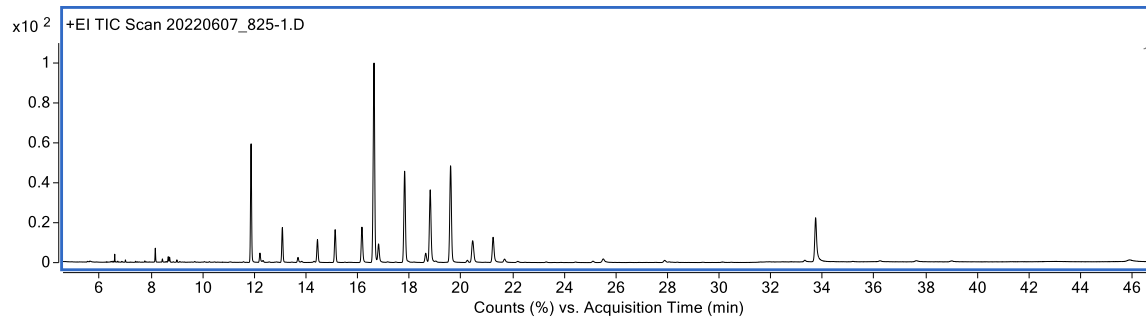
FB10M



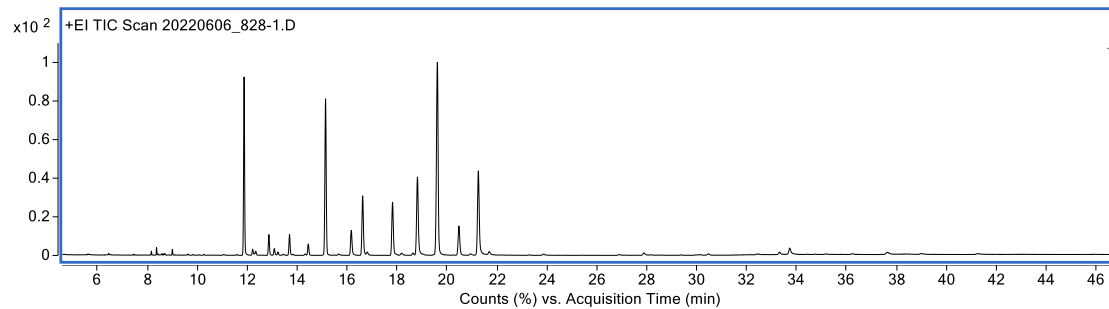
FB11M



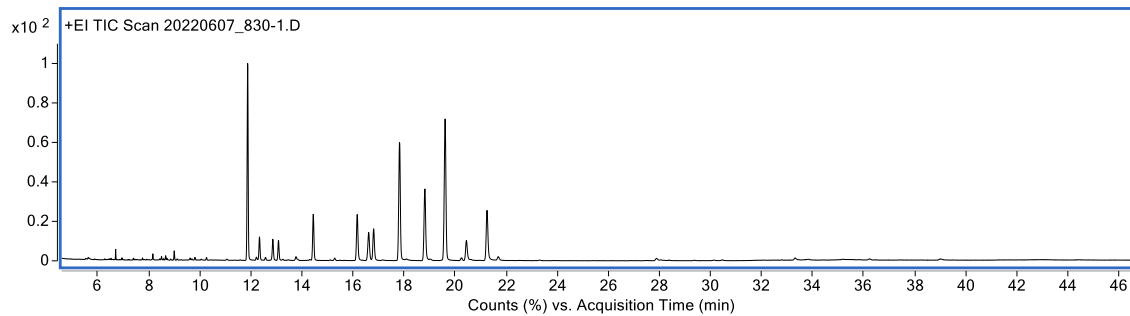
FB12M



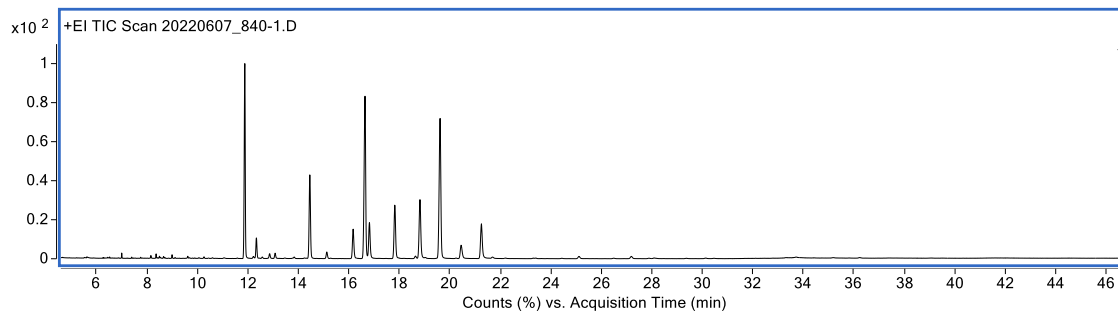
FB13M



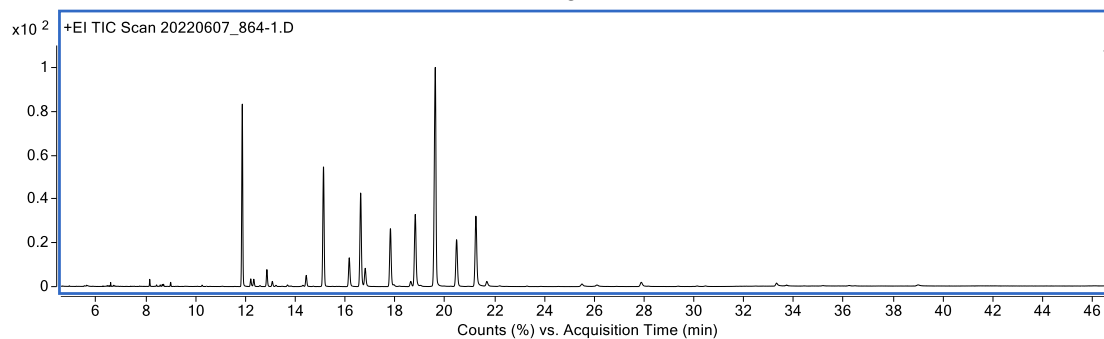
FB14M



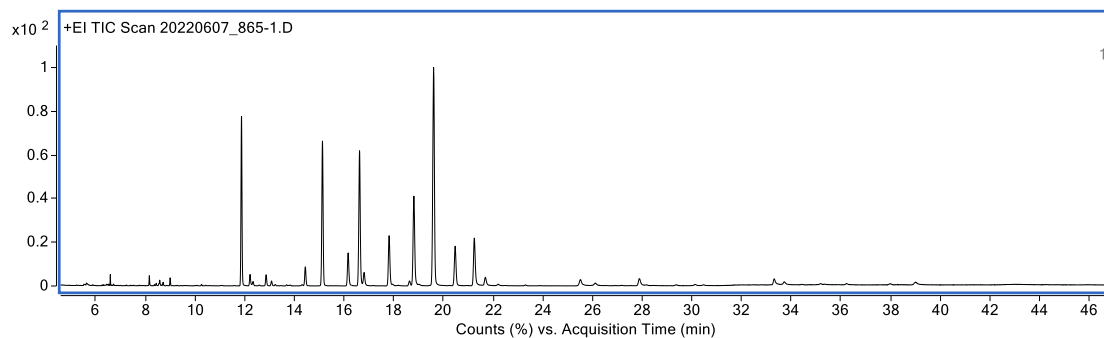
FB15M



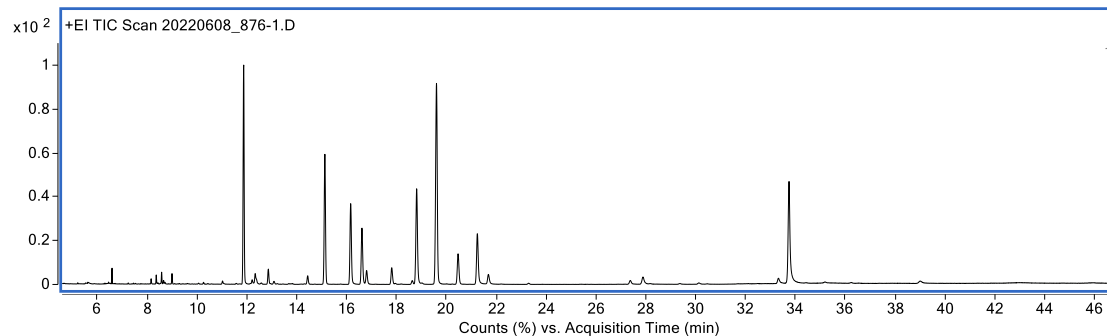
FB16M



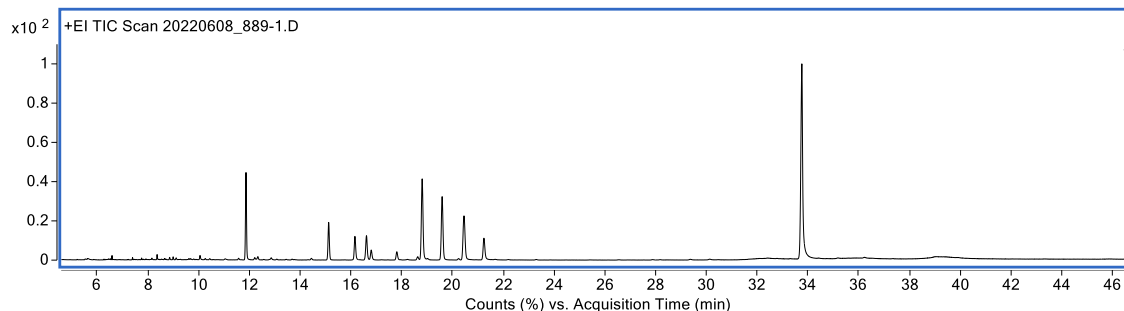
FB17M



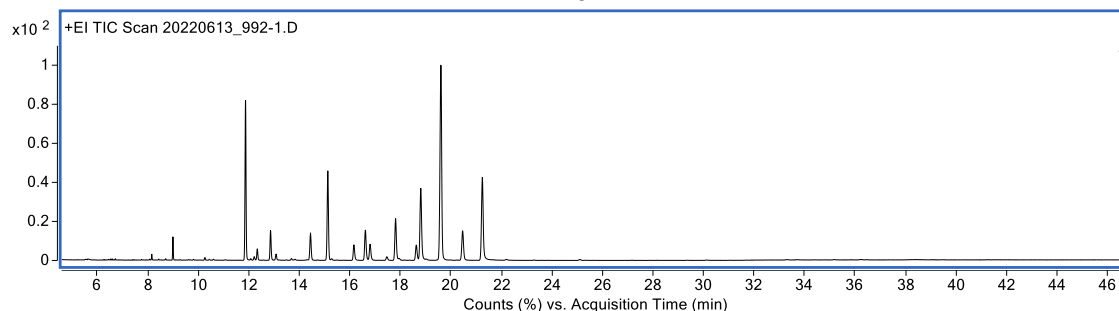
FB18M



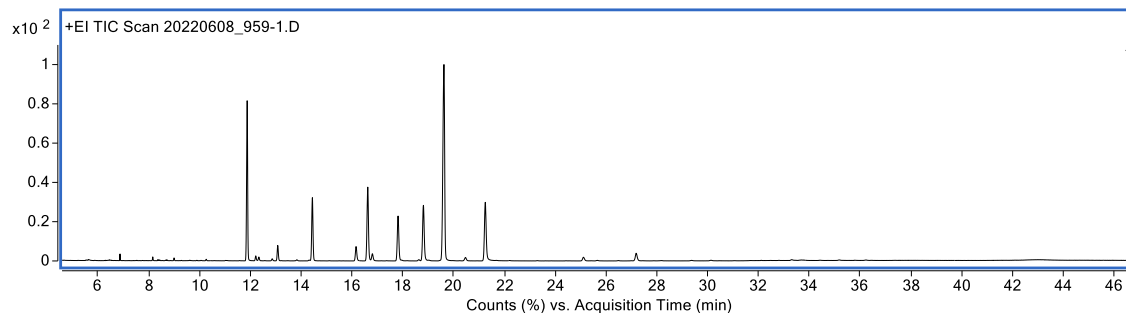
FB19M



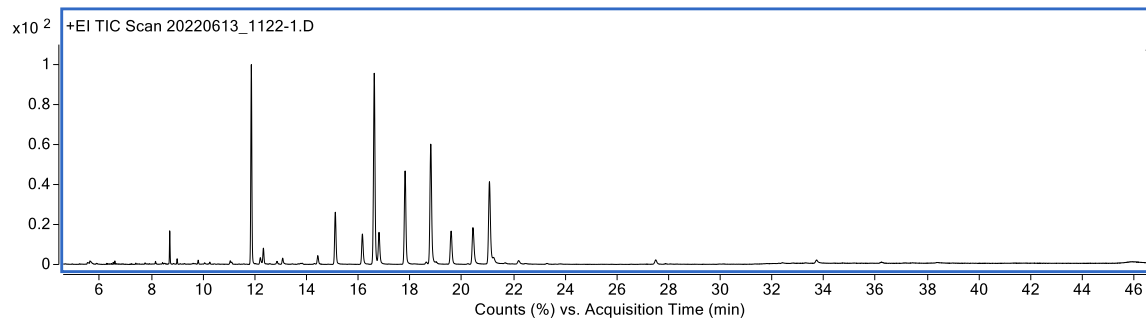
FB20M



FB21M



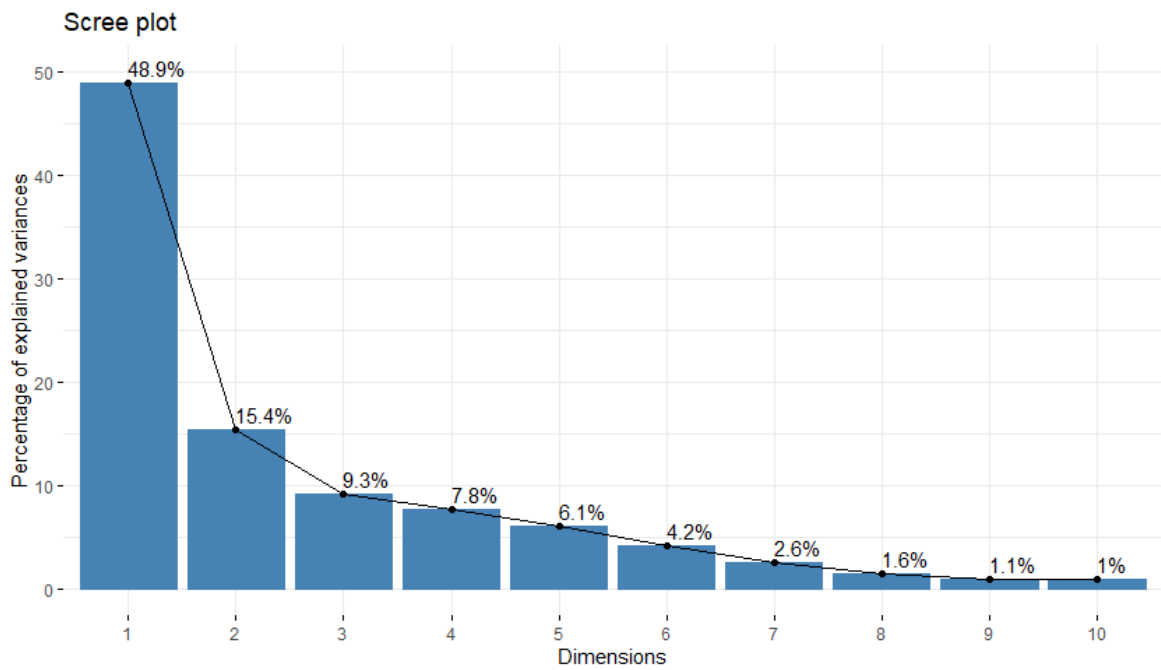
FB22M



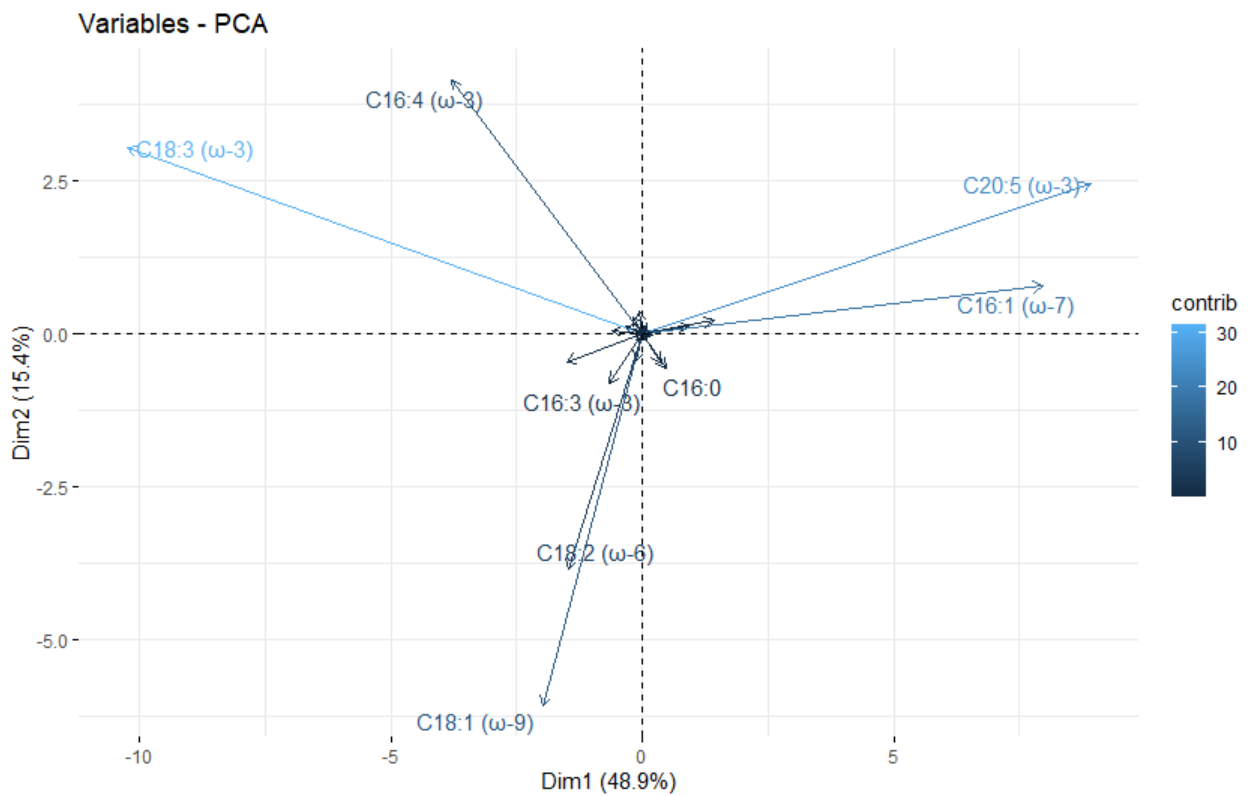
Appendix 7. Fatty acid of microalgae strains by GC-MS. Fatty acids are expressed in mg g⁻¹ dry biomass (%), values are the means of three analytical samples (n = 3) ± standard deviation (SD).

Peak	Fatty Acid	FB01M	FB02M	FB03M	FB04M	FB05M	FB06M	FB07M	FB08M	FB09M	FB010M	FB11M
1	C14:0				0.09 ± 0.03	2.75 ± 0.43						
2	C16:0	1.89 ± 2.28	7.07 ± 0.63	5.95 ± 4.19	0.79 ± 0.30	11.10 ± 2.16	21.06 ± 1.52	0.99 ± 0.14	9.57 ± 1.27	0.89 ± 0.39	14.84 ± 6.20	1.14 ± 0.41
3	C16:1 Δ ¹¹ (ω-5)				0.05 ± 0.01							
4	C16:1 Δ ⁹ (ω-7)	0.54 ± 0.81	0.95 ± 0.06	0.67 ± 0.34	1.00 ± 0.15	20.92 ± 3.91					2.69 ± 1.89	0.08 ± 0.03
5	C16:1 Δ ⁷ (ω-9)		1.20 ± 0.07	0.37 ± 0.28		1.26 ± 0.18	1.27 ± 0.08				1.06 ± 0.07	0.10 ± 0.06
6	C16:2 Δ ^{9,12} (ω-4)				0.04 ± 0.01							
7	C16:2 Δ ^{7,10} (ω-6)	0.42 ± 0.37	1.38 ± 0.13	1.64 ± 1.32			1.77 ± 0.28	0.06 ± 0.01	0.73 ± 0.18		1.22 ± 0.92	
8	C16:3 Δ ^{7,10,13} (ω-3)	1.35 ± 1.66	4.09 ± 0.06	1.28 ± 0.88			1.89 ± 0.18	0.11 ± 0.02	0.67 ± 0.18			
9	C16:3 Δ ^{6,9,12} (ω-4)				0.05 ± 0.01							
10	C16:4 Δ ^{6,9,12,15} (ω-1)						3.48 ± 0.37				1.93 ± 1.22	
11	C16:4 Δ ^{4,7,10,13} (ω-3)	2.48 ± 3.15	8.32 ± 0.99	4.18 ± 3.06			13.84 ± 1.62	0.49 ± 0.08	8.51 ± 1.70	0.23 ± 0.04	8.96 ± 4.61	1.75 ± 0.94
12	C18:0	0.40 ± 0.44	0.82 ± 0.10	1.66 ± 1.12	0.30 ± 0.20	1.67 ± 0.49	1.47 ± 0.15	0.21 ± 0.06	1.74 ± 0.28	0.35 ± 0.21	1.45 ± 0.33	0.63 ± 0.12
13	C18:1 Δ ¹³ (ω-5)						2.68 ± 0.22	0.26 ± 0.03		0.14 ± 0.07	5.24 ± 1.45	0.57 ± 0.06
14	C18:1 Δ ⁹ (ω-9)	0.15 ± 0.13	0.59 ± 0.07	0.52 ± 0.45					3.41 ± 0.08	0.05 ± 0.03		0.28 ± 0.14
15	C18:2 Δ ^{9,12} (ω-6)	1.18 ± 1.09	8.76 ± 0.28	6.00 ± 4.64	0.10 ± 0.02		9.84 ± 1.90	0.49 ± 0.05	6.59 ± 1.36	0.30 ± 0.13	6.76 ± 5.41	0.22 ± 0.09
16	C18:3 Δ ^{9,12,15} (ω-3)	5.00 ± 6.56	12.68 ± 1.67	5.44 ± 3.83			34.66 ± 4.20	1.38 ± 0.12	18.83 ± 3.57	0.55 ± 0.11	20.33 ± 9.96	3.37 ± 1.60
17	C18:3 Δ ^{6,9,12} (ω-6)						8.15 ± 0.61	0.06 ± 0.01				
18	C18:4 Δ ^{6,9,12,15} (ω-3)						1.17 ± 0.05		3.11 ± 0.67	0.31 ± 0.15	2.43 ± 0.49	0.47 ± 0.14
19	C20:0							0.06 ± 0.01		0.35 ± 0.19		
20	C20:2 Δ ^{11,14} (ω-6)	0.34 ± 0.19	1.29 ± 0.05	0.71 ± 0.50								
21	C20:3 Δ ^{11,14,17} (ω-3)	0.27 ± 0.16	0.99 ± 0.10	0.31 ± 0.18								
22	C20:4 Δ ^{5,11,14,17} (ω-3)	0.28 ± 0.25	0.59 ± 0.04									
23	C20:4 Δ ^{5,8,11,14} (ω-6)	0.81 ± 0.59	1.79 ± 0.13	1.47 ± 1.12	0.24 ± 0.04	2.08 ± 0.39		0.11 ± 0.01		0.05 ± 0.02		
24	C20:5 Δ ^{5,8,11,14,17} (ω-3)	0.75 ± 0.41	3.00 ± 0.26	1.80 ± 1.26	0.27 ± 0.06	38.99 ± 7.59		0.10 ± 0.01		0.06 ± 0.01		
25	C22:0							0.08 ± 0.00	0.55 ± 0.14	0.73 ± 0.37		0.15 ± 0.13
26	C22:5 Δ ^{4,7,10,13,16} (ω-6)	0.56 ± 0.58	0.65 ± 0.12									
27	C22:6 Δ ^{4,7,10,13,16,19} (ω-3)	0.61 ± 0.37	1.37 ± 0.13									
28	C24:0							0.10 ± 0.01	0.86 ± 0.13	0.31 ± 0.16		
Σ SFA		2.29 ± 2.32	7.89 ± 0.64	7.61 ± 4.34	1.18 ± 0.36	15.52 ± 2.26	22.53 ± 1.53	1.44 ± 0.15	12.72 ± 1.31	2.63 ± 0.63	16.29 ± 6.21	1.92 ± 0.45
Σ MUFA		0.69 ± 0.82	2.74 ± 0.12	1.56 ± 0.63	1.05 ± 0.15	22.18 ± 3.91	3.95 ± 0.23	0.26 ± 0.03	3.41 ± 0.08	0.19 ± 0.08	8.99 ± 2.38	1.03 ± 0.17
Σ PUFA		14.05 ± 7.63	44.91 ± 2.00	22.83 ± 7.16	0.70 ± 0.08	41.07 ± 7.60	74.80 ± 4.95	2.80 ± 0.16	38.44 ± 4.24	1.50 ± 0.23	41.63 ± 12.34	5.81 ± 1.86
Σ PUFAω-6		3.31 ± 1.43	13.87 ± 0.36	9.82 ± 4.98	0.34 ± 0.04	2.08 ± 0.39	19.76 ± 2.02	0.72 ± 0.05	7.32 ± 1.37	0.35 ± 0.13	7.98 ± 5.49	0.22 ± 0.09
Σ PUFAω-3		10.74 ± 7.49	31.04 ± 1.97	13.01 ± 5.14	0.27 ± 0.06	38.99 ± 7.59	51.56 ± 4.51	2.08 ± 0.15	31.12 ± 4.01	1.15 ± 0.19	31.72 ± 10.99	5.59 ± 1.86

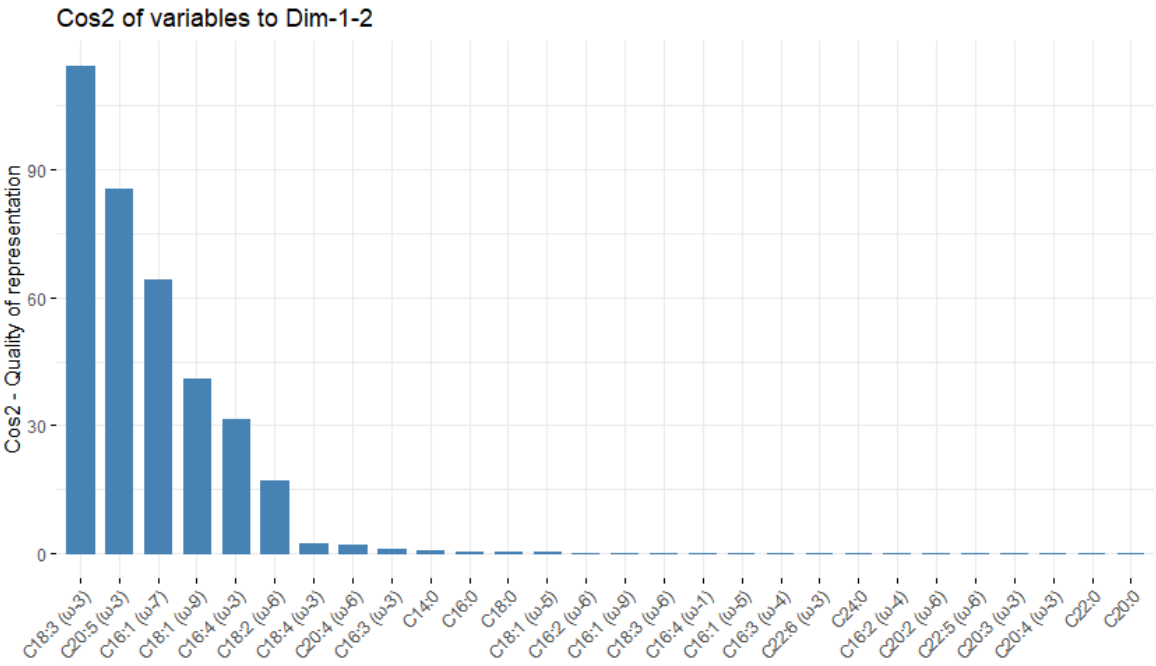
Peak	Fatty Acid	FB12M	FB13M	FB14M	FB15M	FB16M	FB17M	FB18M	FB19M	FB20M	FB21M	FB22M
1	C14:0											
2	C16:0	1.31 ± 0.22	12.92 ± 1.75	1.99 ± 0.48	4.33 ± 0.56	7.23 ± 2.79	2.53 ± 0.55	2.26 ± 0.79	1.04 ± 0.30	15.54 ± 1.76	11.22 ± 3.80	1.06 ± 0.45
3	C16:1 Δ^{11} (ω -5)											
4	C16:1 Δ^9 (ω -7)			0.20 ± 0.09	0.42 ± 0.05							0.13 ± 0.13
5	C16:1 Δ^7 (ω -9)		1.79 ± 0.28	0.28 ± 0.04		0.84 ± 0.23		0.15 ± 0.09			1.66 ± 0.91	
6	C16:2 $\Delta^{9,12}$ (ω -6)											
7	C16:2 $\Delta^{7,10}$ (ω -6)	0.46 ± 0.08		0.25 ± 0.03	0.39 ± 0.24					1.54 ± 0.36		
8	C16:3 $\Delta^{7,10,13}$ (ω -3)	0.32 ± 0.06	1.08 ± 0.13	0.66 ± 0.07	2.43 ± 0.17		0.34 ± 0.04	0.11 ± 0.02		7.29 ± 1.92	2.02 ± 0.90	0.04 ± 0.01
9	C16:3 $\Delta^{6,9,12}$ (ω -4)											
10	C16:4 $\Delta^{6,9,12,15}$ (ω -1)		1.62 ± 0.55									
11	C16:4 $\Delta^{4,7,10,13}$ (ω -3)	0.50 ± 7.00	17.37 ± 1.82		0.25 ± 0.09	7.51 ± 2.46	2.90 ± 0.30	2.02 ± 0.34	0.56 ± 0.06		6.56 ± 2.82	
12	C18:0	0.58 ± 0.06	1.82 ± 0.68	0.59 ± 0.24	0.84 ± 0.16	1.34 ± 0.38	0.62 ± 0.04	1.01 ± 0.51	0.44 ± 0.12	2.10 ± 1.90	2.07 ± 0.78	0.19 ± 0.04
13	C18:1 Δ^{13} (ω -5)	0.20 ± 0.06		0.35 ± 0.09	0.84 ± 0.05	0.81 ± 0.11		0.20 ± 0.16	0.13 ± 0.07		1.00 ± 0.39	0.22 ± 0.19
14	C18:1 Δ^9 (ω -9)	3.21 ± 0.61	6.30 ± 0.79	0.42 ± 0.08	3.76 ± 1.46	4.55 ± 3.40	2.71 ± 0.49	0.80 ± 0.12	0.31 ± 0.06	7.68 ± 2.78	2.21 ± 1.14	1.09 ± 0.32
15	C18:2 $\Delta^{9,12}$ (ω -6)	1.67 ± 0.27	6.44 ± 1.56	2.10 ± 0.22	2.49 ± 0.55	3.40 ± 2.11	1.14 ± 0.26	0.32 ± 0.05	0.18 ± 0.05	6.25 ± 1.13	3.94 ± 1.89	0.79 ± 0.30
16	C18:3 $\Delta^{9,12,15}$ (ω -3)	2.02 ± 0.35	31.16 ± 4.38	2.95 ± 0.35	7.07 ± 1.51	17.36 ± 7.25	5.30 ± 0.98	3.74 ± 1.27	1.31 ± 0.26	35.23 ± 4.69	19.03 ± 9.27	0.56 ± 0.48
17	C18:3 $\Delta^{6,9,12}$ (ω -6)	0.19 ± 0.04				0.46 ± 0.15			0.09 ± 0.01		1.46 ± 0.68	0.04 ± 0.03
18	C18:4 $\Delta^{6,9,12,15}$ (ω -3)	0.60 ± 0.07	4.80 ± 0.61	0.30 ± 0.15	0.45 ± 0.21	3.75 ± 1.68	1.15 ± 0.11	0.82 ± 0.19	0.94 ± 0.08	0.82 ± 0.19	3.24 ± 1.49	0.29 ± 0.14
19	C20:0					0.63 ± 0.12						
20	C20:2 $\Delta^{11,14}$ (ω -6)											
21	C20:3 $\Delta^{11,14,17}$ (ω -3)											
22	C20:4 $\Delta^{5,11,14,17}$ (ω -3)											
23	C20:4 $\Delta^{5,8,11,14}$ (ω -6)									0.88 ± 0.13		
24	C20:5 $\Delta^{5,8,11,14,17}$ (ω -3)				0.23 ± 0.03					1.72 ± 0.20		
25	C22:0					0.55 ± 0.13						
26	C22:5 $\Delta^{4,7,10,13,16}$ (ω -6)						0.36 ± 0.11	0.23 ± 0.13				
27	C22:6 $\Delta^{4,7,10,13,16,19}$ (ω -3)											
28	C24:0	0.13 ± 0.00				0.67 ± 0.13	0.37 ± 0.10			0.82 ± 0.09		
	Σ SFA	2.02 ± 0.23	14.74 ± 1.88	2.58 ± 0.54	5.17 ± 0.58	10.42 ± 2.82	4.21 ± 0.58	3.71 ± 0.96	1.48 ± 0.32	18.46 ± 2.59	13.29 ± 3.88	1.25 ± 0.45
	Σ MUFA	3.41 ± 0.61	8.09 ± 0.84	1.25 ± 0.16	5.02 ± 1.46	6.20 ± 3.41	2.71 ± 0.49	1.15 ± 0.22	0.44 ± 0.09	7.68 ± 2.78	4.87 ± 1.51	1.44 ± 0.39
	Σ PUFA	5.76 ± 7.02	62.47 ± 5.06	6.26 ± 0.45	13.31 ± 1.65	32.48 ± 8.12	10.83 ± 1.06	7.01 ± 1.33	3.08 ± 0.28	53.73 ± 5.21	36.25 ± 10.05	1.72 ± 0.58
	Σ PUFA ω -6	2.32 ± 0.28	6.44 ± 1.56	2.35 ± 0.22	2.88 ± 0.60	3.86 ± 2.12	1.14 ± 0.26	0.32 ± 0.05	0.27 ± 0.05	8.67 ± 1.19	5.40 ± 2.01	0.83 ± 0.30
	Σ PUFA ω -3	3.44 ± 7.01	54.41 ± 4.78	3.91 ± 0.39	10.43 ± 1.54	28.62 ± 7.84	9.69 ± 1.03	6.69 ± 1.33	2.81 ± 0.28	45.06 ± 5.08	30.85 ± 9.84	0.89 ± 0.50



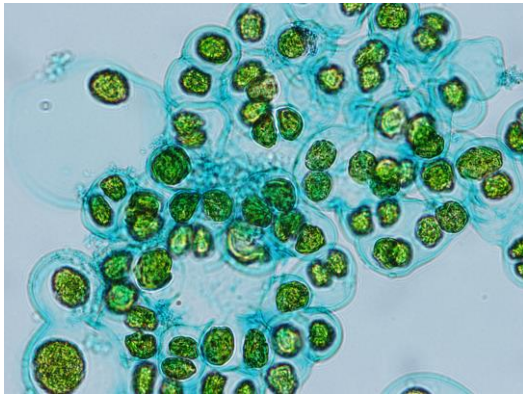
Appendix 8. Fatty acid PCA analysis scree plot. Eigenvalues are ordered from the largest to the smallest percentage of explained variance.



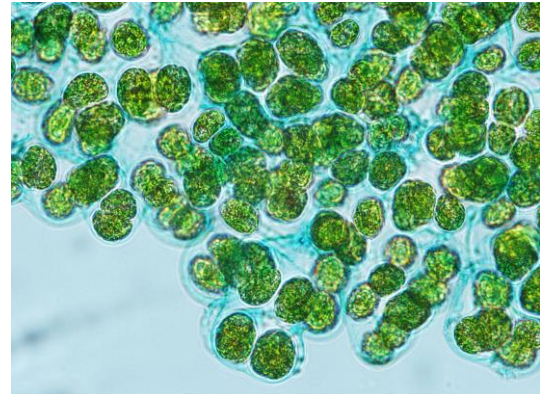
Appendix 9. Fatty acid PCA graph of variables, with the highlighted contribution.



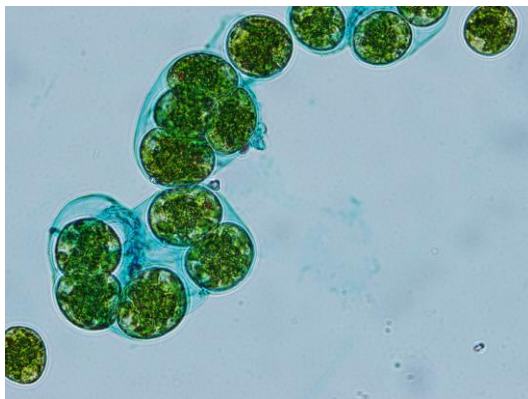
Appendix 10. Contributors (fatty acids) for PCA (Dim1:2) of fatty acid PCA analysis.



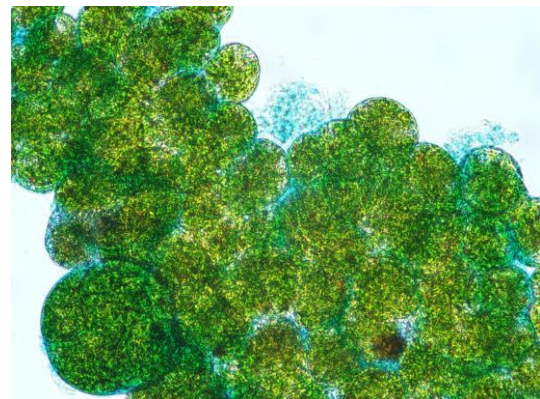
FB01M with AB 0.5% in Ethanol (50%)



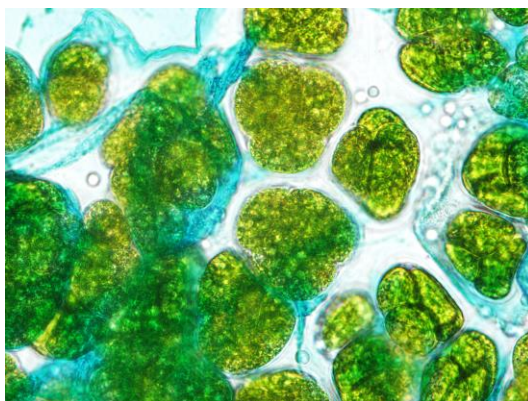
FB01M with AB 0.5% in Acetic acid



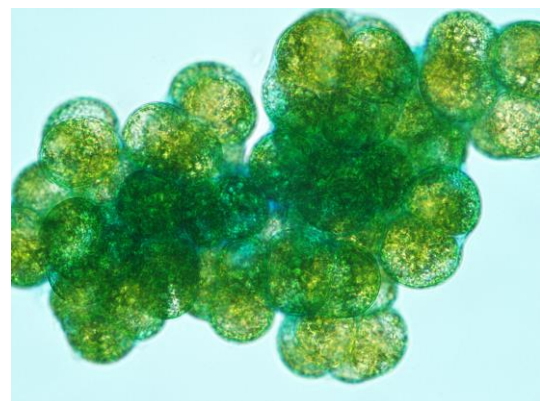
FB02M with AB 0.5% in Ethanol (50%)



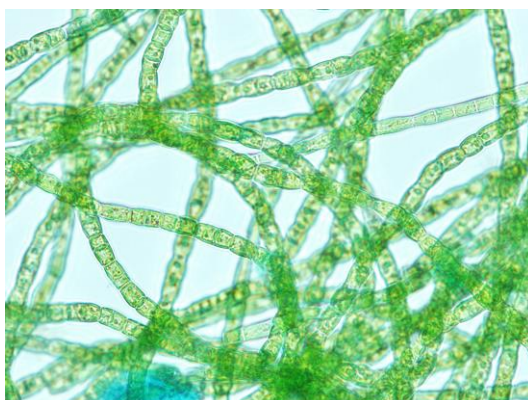
FB02M with AB 0.5% in Acetic acid



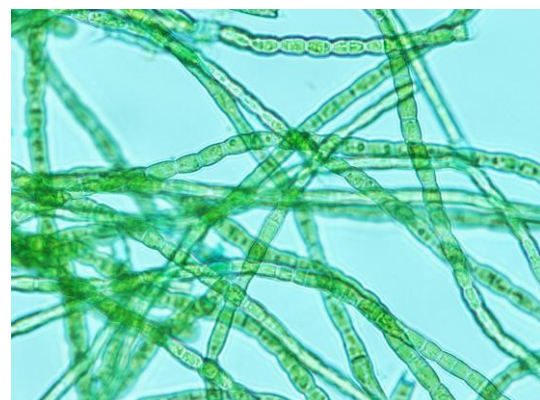
FB03M with AB 0.5% in Ethanol (50%)



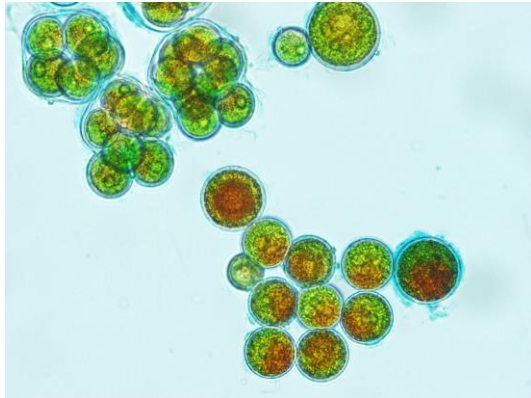
FB03M with AB 0.5% in Acetic acid



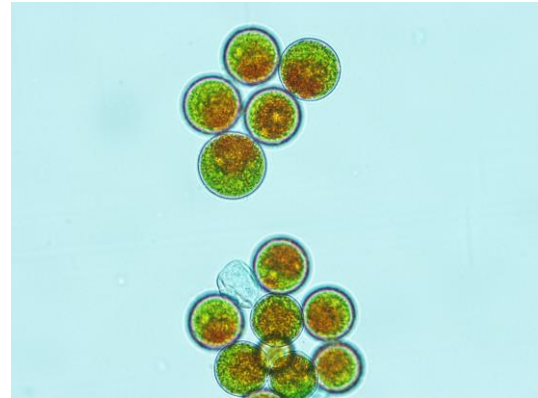
FB04M with AB 0.5% in Ethanol (50%)



FB04M with AB 0.5% in Acetic acid



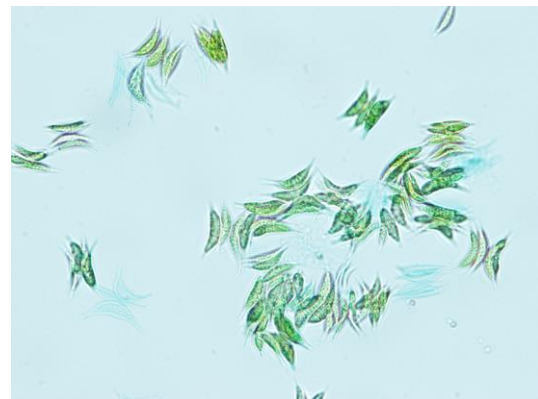
FB07M with AB 0.5% in Ethanol (50%)



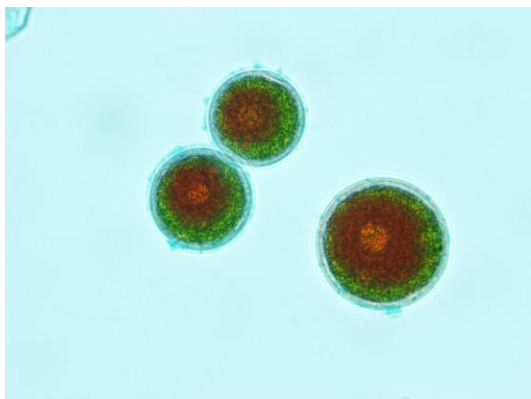
FB07M with AB 0.5% in Acetic acid



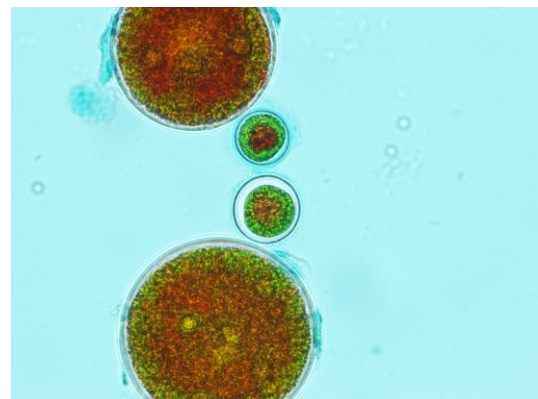
FB08M with AB 0.5% in Ethanol (50%)



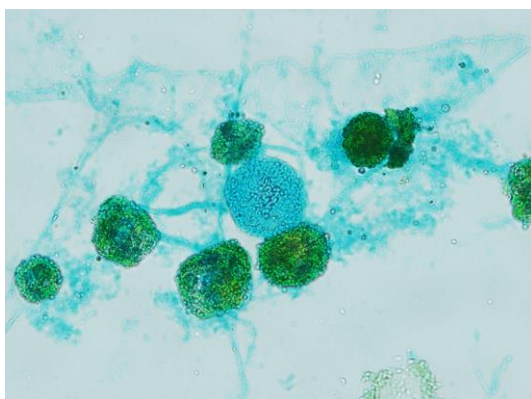
FB08M with AB 0.5% in Acetic acid



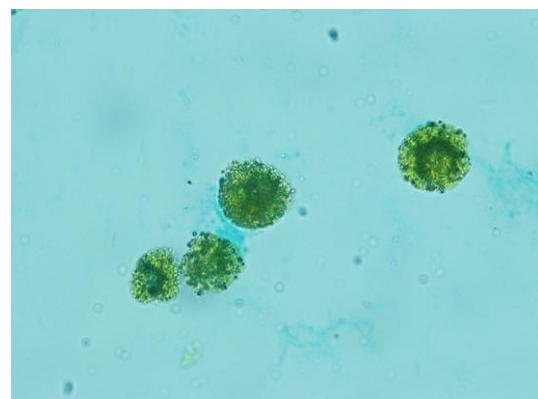
FB09M with AB 0.5% in Ethanol (50%)



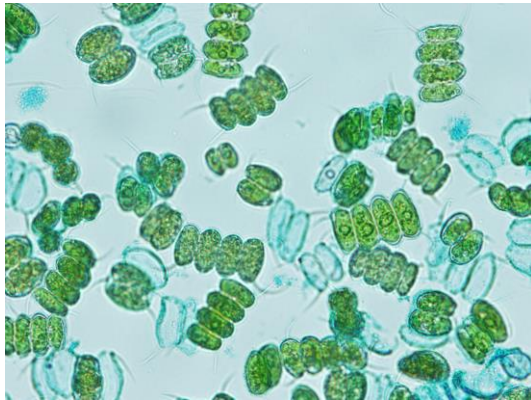
FB09M with AB 0.5% in Acetic acid



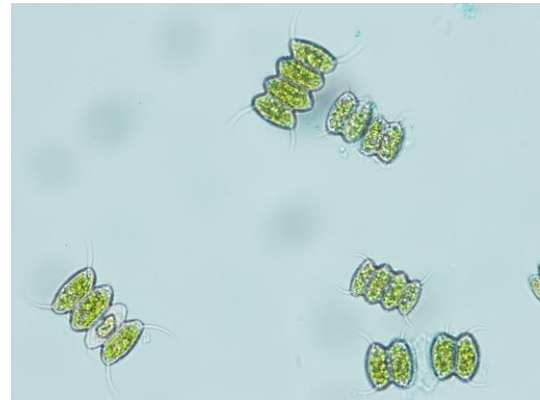
FB10M with AB 0.5% in Ethanol (50%)



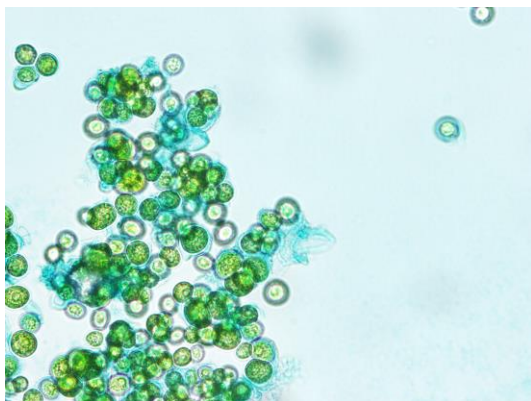
FB10M with AB 0.5% in Acetic acid



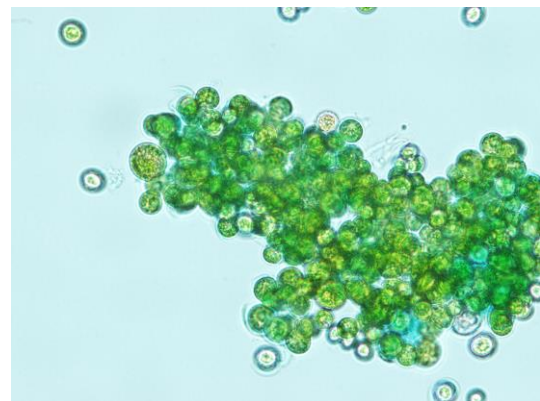
FB11M with AB 0.5% in Ethanol (50%)



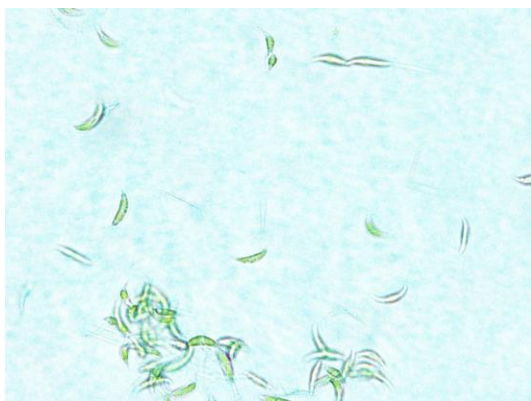
FB11M with AB 0.5% in Acetic acid



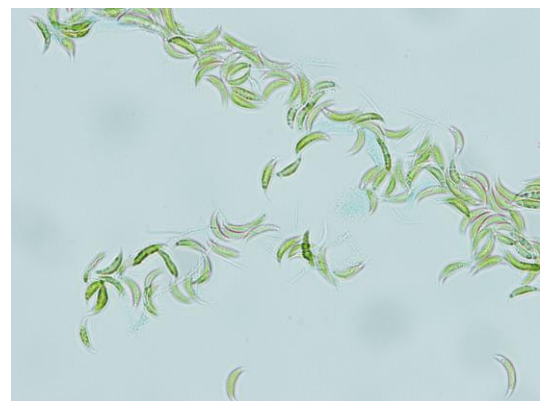
FB12M with AB 0.5% in Ethanol (50%)



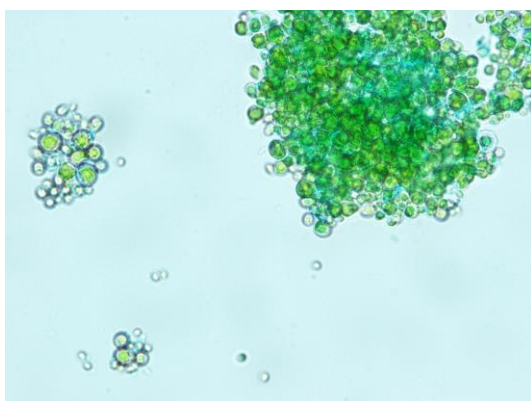
FB12M with AB 0.5% in Acetic acid



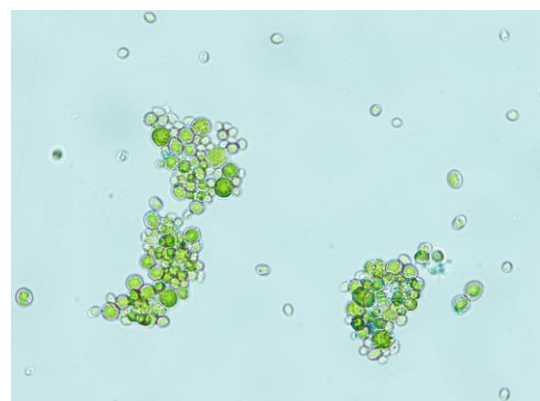
FB13M with AB 0.5% in Ethanol (50%)



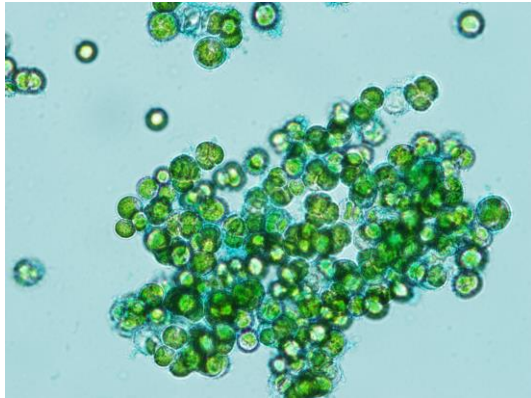
FB13M with AB 0.5% in Acetic acid



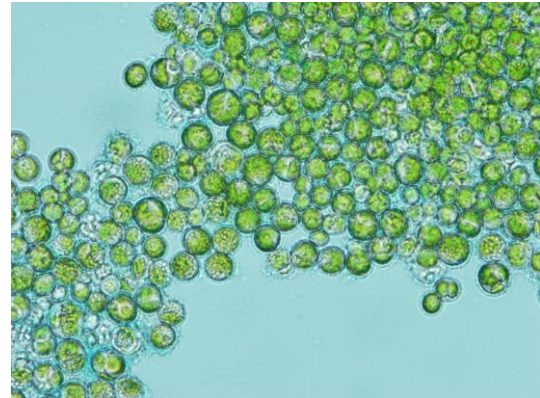
FB14M with AB 0.5% in Ethanol (50%)



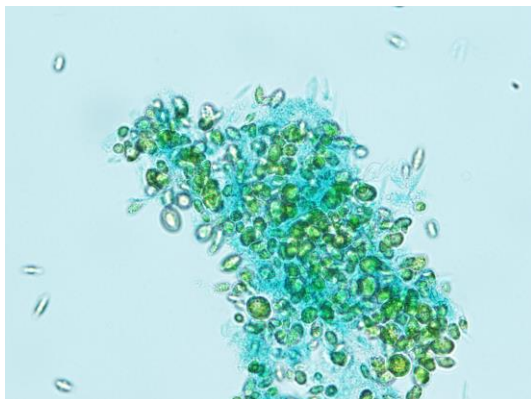
FB14M with AB 0.5% in Acetic acid



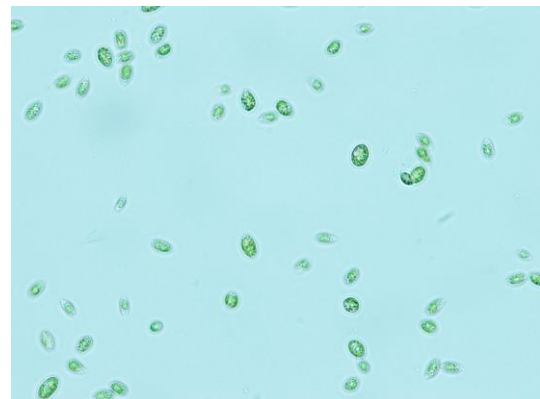
FB15M with AB 0.5% in Ethanol (50%)



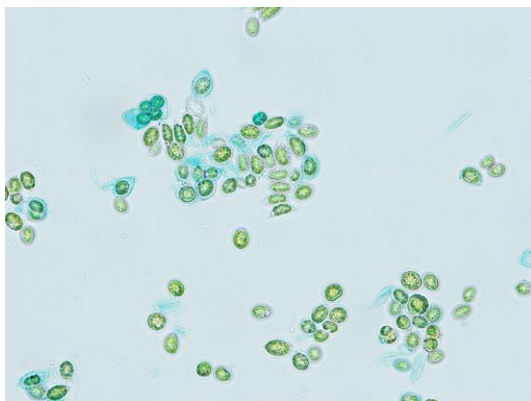
FB15M with AB 0.5% in Acetic acid



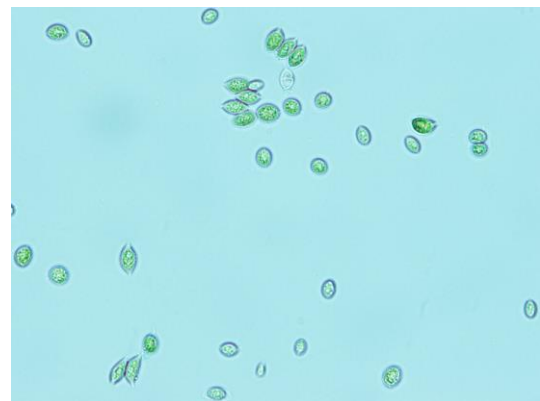
FB16M with AB 0.5% in Ethanol (50%)



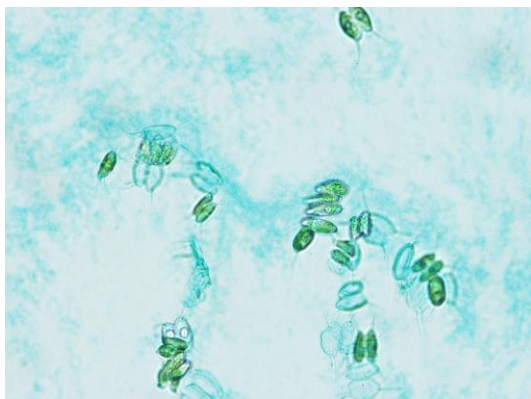
FB16M with AB 0.5% in Acetic acid



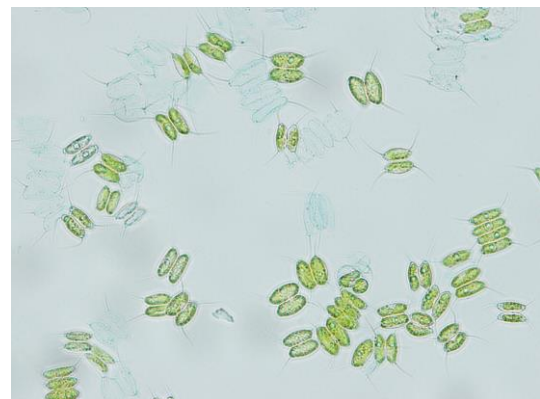
FB17M with AB 0.5% in Ethanol (50%)



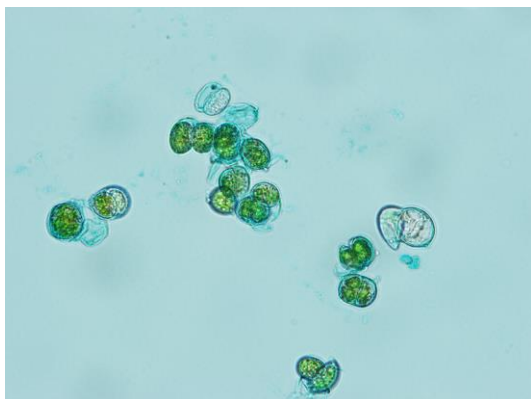
FB17M with AB 0.5% in Acetic acid



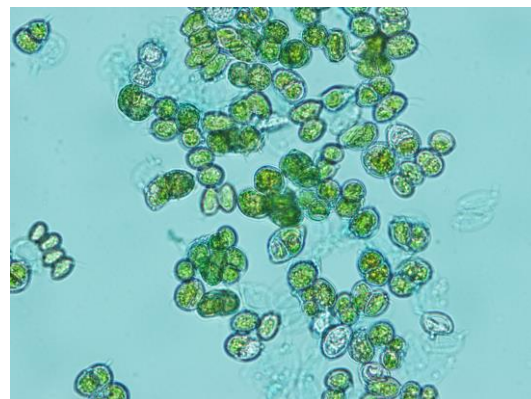
FB18M with AB 0.5% in Ethanol (50%)



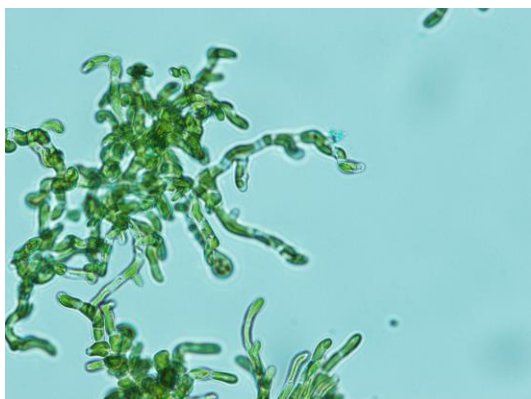
FB18M with AB 0.5% in Acetic acid



FB19M with AB 0.5% in Ethanol (50%)



FB19M with AB 0.5% in Acetic acid



FB20M with AB 0.5% in Ethanol (50%)



FB20M with AB 0.5% in Acetic acid

Appendix 11. Microscopic visualization of microalgae strains FB01M, FB02M, FB04M, FB07M, FB08M, FB09M, FB10M, FB11M, FB12M, FB13M, FB14M, FB15M, FB16M, FB17M, FB18M, FB19M and FB20M with Alcian Blue dye 0.5% in (A) ethanol (50%) and (B) acetic acid.