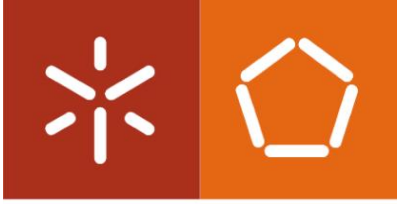

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Mestrado em Biotecnologia

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

RESUMO

A grande capacidade das microalgas para subsistir à exposição a condições severas ou limitantes é amplamente conhecida, constituindo uma fonte singular de biocompostos específicos e de valor acrescentado. O potencial dos seus extratos, no que concerne às propriedades funcionais e bioativas, encontra-se bem estabelecido, com características interessantes para o cuidado da pele, enquanto agentes de consistência, hidratação e antioxidantes. O interesse crescente por metodologias mais sustentáveis culmina numa maior procura por formulações naturais. Todavia, o potencial e a utilidade das microalgas encontram-se dependentes das técnicas de extração aplicadas. A seleção do método de extração preferencial constitui um fator determinante para a qualidade do produto final, o qual deverá exceder os requisitos do consumidor, enquanto oferece um processo economicamente apelativo.

Desta forma, esta tese objetivou evidenciar o potencial da microalga *Phaeodactylum tricornutum*, ao fornecer úteis informações para a formulação de um produto cosmecêutico natural. Procurou-se obter duas frações, responsáveis por funções bioativas e tecnológicas, explorando o impacto das condições de crescimento em culturas de *P. tricornutum* e estabelecendo uma metodologia de extração, que inclui o aquecimento óhmico (OH) como potencial alternativa aos processos de extração convencionais.

Inicialmente, implementou-se uma evolução laboratorial adaptativa (ALE) acoplada à mutagénese por UV-C nas culturas de *P. tricornutum*. Apesar dos resultados não evidenciarem um claro impacto positivo na atividade antioxidante dos extratos, esta estratégia apresentou-se potencialmente útil no aumento da extração lipídica. Utilizou-se a água e diferentes misturas de etanol/água como solventes para explorar os efeitos da extração baseada em OH e convencional nas propriedades dos extratos, com objetivando-se um espetro de polaridade. A funcionalidade de *P. tricornutum* foi avaliada relacionando a composição dos extratos (estabelecida por fluorescência intrínseca, eletroforese SDS-PAGE, FTIR e cromatografia de permeação em gel) com o seu comportamento térmico, reológico e emulsificante. A triagem do seu potencial bioativo baseou-se em três ensaios de determinação de atividade antioxidante (FRAP, ABTS e DPPH) e num ensaio de viabilidade em linhas celulares normais e de cancro.

Os extratos demonstraram potencial tecnológico e bioactivo, com interessantes capacidades de gelificação, emulsificação e antioxidante. O seu perfil bioquímico e de atuação foi dependente do solvente utilizado. Na generalidade, não se observaram efeitos significativos aquando da aplicação de OH. Em suma, os objetivos delineados foram cumpridos, reconhecendo-se o potencial dos extratos de *P. tricornutum* para a indústria cosmecêutica, bem como para o desenvolvimento de alimentos funcionais.

PALAVRAS–CHAVE: Aquecimento óhmico; Bioatividade; Extração; Propriedades funcionais; *Phaeodactylum tricornutum*

ABSTRACT

Microalgae superior ability to sustain harsh, stressing or limiting conditions is widely known, while being also a unique source of specific and valuable biocompounds. Microalgae extracts' potential has been well recognized for their interesting functional and bioactive properties, showing specially interesting features for skin care products' manufacturing mainly as thickening, water-binding and antioxidant agents. The expanding interest in addressing a more sustainable and eco-friendly product development results in a growing community searching for natural-based formulations. However, microalgae potential and usefulness are dependent on the extraction techniques applied. The selection of the preferential method presents a major impact on the final product quality, which should exceed costumer's requests, while simultaneously offering an economically appealing process.

Thus, this thesis aimed at acknowledging *Phaeodactylum tricornutum* potential, by ultimately providing important information for the formulation of an algae-based cosmeceutical product. The present work focused on achieving two different main fractions accountable for bioactive and technological functions, underlying *P. tricornutum* growth conditions impact and designing the extraction methodology, including ohmic heating (OH) as a potential alternative to conventional extraction processes.

Firstly, an adaptive laboratory evolution (ALE) taking advantage of UV-C mutagenesis was performed in *P. tricornutum* cultures. Although the results did not allow a clear positive impact in global extracts' antioxidant activity, it presented as a potentially useful tool to enhance lipidic extraction. In order to explore the OH-based and conventional extraction implications on the microalgae extracts properties, water and different ethanol/water mixtures were used as solvents, addressing a polarity spectrum. *P. tricornutum* functionality was evaluated by relating the extracts composition (assessed by intrinsic fluorescence analysis, SDS-PAGE electrophoresis, FTIR and gel permeation chromatography) with their thermal, rheological and emulsifying behavior. Bioactive potential was analyzed by three different antioxidant activity measuring tests (FRAP, ABTS and DPPH) and a viability assay in normal and cancer cell lines.

This diatom extracts showed promising technological and bioactive potential, with interesting gelling, emulsification and antioxidant capacities. Extracts showed different biochemical and performance profiles, depending on the solvent used. Generally, no significant effects were observed for OH application, having this process lower energy consumptions. In conclusion, the major goals of the present work were successfully accomplished, underlining *P. tricornutum* extracts potentiality not only for cosmeceutical industry, but also for functional foods development.

KEY WORDS: Bioactivity, Extraction, Functional properties, Ohmic heating, *Phaeodactylum tricornutum*

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

λ_{max}	Maximum of the fluorescence emission spectra
1-UVR	One UV stimulus
3-UVR	Three UV stimuli
ABTS	2,2-azino-bis(3-ethylbenzothiazoline)-6 sulphonic acid
ALE	Adaptive Laboratory Evolution
APX	Ascorbate peroxidase
ARA	Arachidonic acid
ATR	Attenuated Total Reflectance
BSA	Bovine Serum Albumin
CE	Catechin Equivalents
DE	Dry Extract
DHA	Docosahexaenoic acid
DichEt	Dichloromethane:ethanol 50%
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSC	Differential Scanning Calorimetry
DSP	Downstream processing
DW	Dry weight
ECM	Extracellular matrix
EF	Electric Field
EI	Emulsification Index
em	Emission
EMS	Ethyl methanesulfonate
EPA	Eicosapentaenoic acid
EPS	Exopolysaccharide
eq	Equivalent
EtOH	Ethanol
EU	European Union
ex	Excitement
FBS	Fetal Bovine Serum

FRAP	Ferric Reducing Antioxidant Power
FTIR	Fourier Transform Infrared
G'	Storage modulus
G''	Viscous/Loss modulus
GAE	Gallic Acid Equivalents
GMO	Genetically Modified Organism
GPC	Gel Permeation Chromatography
GR	Glutathione Reductase
GRAS	Generally recognized as safe
h	Hour
HA	Hyaluronic acid
HPLC	High-performance liquid chromatography
HVED	High Voltage Electric Discharges
IC_{50}	Half-maximal inhibitory concentration
LAP	Laboratory Analytical Procedures
LED	Light-emitting diode
MAA	Mycosporine-like Amino Acid
MEF	Moderate Electric Fields
min	Minute
MW	Molecular Weight
NREL	National Renewable Energy Laboratory
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
OD	Optical density
OH	Ohmic heating
p	p-value
PCA	Principal Component Analysis
PEF	Pulsed Electric Fields
POH	Pulsed Ohmic Heating
PSA	Phenol-sulfuric acid
PUFA	Polyunsaturated fatty acid
RI	Refractive Index
RNA	Ribonucleic acid

ROS	Reactive oxygen species
rpm	Rotation per minute
s	Second
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
SLE	Solid-liquid extraction
SOD	Superoxide dismutase
TAG	Triacylglycerol
TEM	Transmission Electron Microscopy
TFC	Total Flavonoid Content
T_g	Temperature of gelling
T_m	Temperature of melting
TPC	Total Phenolic Content
TPTZ	2,4,6-tripyridyl-s-triazine
Trolox	(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
Trp	Tryptophan
Tyr	Tyrosine
USP	Upstream processing
UV	Ultraviolet

LIST OF FIGURES

LIST OF TABLES

1. STATE OF THE ART

The aquatic environment is a crucial and growing source of bioactive compounds, comprising approximately a half of the total global biodiversity (Aneiros and Garateix 2004; Ruocco *et al*. 2016). It has been classified as the largest remaining reservoir of natural molecules to be evaluated for drug activity, providing an unique source of microorganisms, animals and plants with particular characteristics, due to the very demanding, competitive and aggressive surrounding environment. This singular way of life leads to the production of specific and promising active natural molecules. With oceans accounting for about 70 % of the Earth's surface and corresponding to about 90 % of the biosphere, it becomes understandable its increasing exploration interest, having into account the revealing great potential of its source in several fields (Ruocco *et al*. 2016; Aneiros and Garateix 2004; J. Kim *et al*. 2018; Ariede *et al*. 2017). In fact, there are plenty of known studies reporting that ocean's biological activity overcomes to those isolated from terrestrial sources (J. Kim *et al*. 2018).

Algae species, due to their harsh environment and phototropic life, are submitted to high oxidative and free-radical stresses in their habitat. This leads its evolution towards the development of natural protective systems and different adaptation strategies, which enable them to survive in these hostile conditions (Bleakley and Hayes 2017; H. D. Wang *et al*. 2017; Ruocco *et al*. 2016).

The increasing environmental pollution that has been noticed in the last few decades has emerged as one of the major problems around the globe, owing to rapid industrial expansion and the growing global population. With their health hazard origin and eminent danger to the living ecosystems as simultaneously socioeconomic awareness and ecological apprehensions, scientific research aims to address a more sustainable and ecofriendly product development (Ahmed, M. N. Iqbal, and Dhama 2017; Bilal *et al*. 2018). Therefore, the expanding interest on searching for bioactive formulations achieved by natural sources, instead of synthetic made products, can present as a considerable solution to this core humanity concerning (J. Kim *et al*. 2018). Since 2012, the European Union (EU) has established a sustainable strategy, which proposes a comprehensive approach in order to increase green products and limit the adverse ambient impact, revealing a gap opportunity for algae as a natural source (Vigani *et al*. 2015).

The algae extended application for food ingredient conception as a source of functional health ingredients has been extensively reviewed in literature, as well as its enhanced potential of providing a wide range of biologically important compounds useful for cosmeceutical benefit in humans and

pharmacological industry (Kadam, Tiwari, and O'Donnell 2013; Thomas and Kim 2013; Ruocco *et al.* 2016). These relates to the possibility that offers of maximizing resources, since they do not require freshwater or arable land to grow, and thereby have the ability to grow at higher rates than terrestrial plants, increasing the positive socioeconomic impact of its use. Furthermore, its strong ability to respond to external factors as the consequent physiology change due to environment alteration appeals their extensive variety of high-value molecules for diverse purposes. A practical application is the production of secondary metabolites like pigments (e.g., carotenes, chlorophylls, and phycobiliproteins) and polyphenols (e.g., catechins, flavonols, and phlorotannins), which can impart health benefits to the consumer when eaten (J. Kim *et al.* 2018; Vigani *et al.* 2015; H. D. Wang *et al.* 2017; Ruocco *et al.* 2016; Bleakley and Hayes 2017). In the other hand, it has been reported that, when compared to the terrestrial plants and animal-based foods, algae offers a richer source of some health-promoting molecules, such as dietary fiber, ω-3 fatty acids, essential amino acids and vitamins A, B, C, and E, important aspect for cosmeceutical product development (Thomas and Kim 2013).

Hence, algae species are considered important biomass producers for many different applications, being capable of producing bioactive compounds with potential interest in the cosmetic industry. Important features for cosmeceutical application relate to the presence of thickening, water-binding and antioxidants agents (Ariede *et al.* 2017; Ruocco *et al.* 2016).

Having the ecological preservation in mind as well as the current interest by the consumers in natural made products, there is an important need to account to these demands. Marine organisms have been proved as a valuable source of structurally diverse biologically active compounds for cosmeceutical industry, offering a considerable contribution to prevent aging, inflammation and skin degradation linked to free radicals studies (J. Kim *et al.* 2018; Bilal *et al.* 2018; Ruocco *et al.* 2016). Moreover, since Portugal has one of the largest Exclusive Economic Zones around the world, accounting for 18.7 times its size, the marine exploration becomes of greater potential (Tomé 2014; "Ordenamento Do Espaço Marítimo Nacional, Plano de Situação," n.d.). Furthermore, taking into consideration the well-recognized algae potential and its remarkable but confined used as a powerful healthy nutrient based diet, the assessment of this ecosystem becomes of further importance. In fact, despite the greater bioavailability content in Portugal, the current confined and non-traditional alimentation based ingredient, allows a non-competitive handling to other human benefits, referring to land or nutritional use (To *et al.* 2018; Vigani *et al.* 2015; L. Pereira 2008; Sathasivam *et al.* 2019).

There has been a growing national effort to adopt a more integrate and flexible management of the coastal zones, which is reflected by the major importance recognized to algae production (Lillebo and

Gooch 2015). In fact, the algae bloom phenomena characterized by an excessive growth of this species has been considered an ecological concern. It leads to drastic negative consequences as a result of the water clarity and oxygen levels decreasing. Thereby, its exploration could result in the repression or even prevention of this incident (Ribeiro 2008). Portugal provides the largest european microalgae production center, which applies a circular economy advantage system (M. Fernandes 2018). As a result, cosmeceutical algae-based products have a promising future in Portugal, following the current demand of more sustainable and ecological approaches to fulfill costumer's needs.

1.1.Algae definition

Algae assemble a multiplicity of species with a polyphyletic origin, which had suffered a heterogeneous evolution process and can be broadly described as photosynthetic oxygen-producers, unicellular or multicellular organisms, excluding embryophyte terrestrial plants and lichens (Ariede *et al.* 2017; Sasso *et al.* 2012; Bleakley and Hayes 2017). They are known for being capable of growing by themselves or in symbiosis with other organisms in a variety of aquatic habitats – such as lakes, ponds, rivers, oceans and wastewater – and indulge in a wide range of temperatures, salinities and pH values, as well as different light exposure and conditions (Khan, Shin, and Kim 2018). It is recognized that these environmental factors, the physiological status, and the aerobic or anaerobic conditions can influence its chemical composition. Thereby, they must be taken into consideration when processes development and optimization are aimed (H. D. Wang *et al.* 2017). Algae cells´ ability to perform the conversion of solar energy into chemical energy through photosynthesis process allows the storage of different bioactive compounds (Bilal *et al.* 2018). This has enhanced its application as a natural resource (Ariede *et al.* 2017).

Algae classification based on its size results into macroalgae (seaweeds) and microalgae. Macroalgae are defined as multicellular and large-size marine plants, usually found in coastal areas, with simpler structures than terrestrial plants (H.-M. D. Wang *et al.* 2015; J. Kim *et al.* 2018). It is possible to classify macroalgae into three main taxonomic groups based on their pigmentation: *Phaeophyta* (brown macroalgae), *Chlorophyta* (green macroalgae), and *Rhodophyta* (red macroalgae) (Kadam, Tiwari, and O'Donnell 2013; Bleakley and Hayes 2017; J. Kim *et al.* 2018). Microalgae comprise an enormous biodiversity, including prokaryotic and eukaryotic microorganisms with high growth rates due to their unicellular or simple multicellular structure (Mata, Martins, and Caetano 2010; Suganya *et al.* 2016). In fact, data from the literature report that only 40-50.000 species are described or analyzed from a total of

20.000-800.000 species estimated (Suganya *et al*. 2016). Microalgae species may be found in all ecosystems and in oceanic water area as phytoplankton (Ariede *et al*. 2017; J. Kim *et al*. 2018). The evolutionary and phylogenetic diversity of this group provides a wide variety in chemical composition, enhancing its interest for bioprospecting and exploitation as commercial sources (Borowitzka 2013) since commonly display exceptionally high market values (Koller, Muhr, and Braunegg 2014). Microalgae´s essential role to sustain earth's ecosystems is highlighted by the production of approximately half of the atmospheric oxygen (O_2), while allowing the greenhouse gas carbon dioxide (CO_2) levels to decrease thought out an exceptional CO_2 fixation capacity (Suganya *et al*. 2016; Koller, Muhr, and Braunegg 2014).

Algae have been traditionally classified based on their color, characteristic that still remains of certain importance. Additional criteria may include cytological and morphological features, such as flagellate cells presence and flagella structure, nuclear and cell division type, the existence of an endoplasmic reticulum envelope around the chloroplast and endoplasmic reticulum connection with the nuclear membrane. It is known that microalgae may have different cell organization types, namely unicellular, filamentous and colonial, with possible diverse shapes of colonial organization, such as flat, spherical, cubic, palmelloid, dendroid, flagellate and non-flagellate (Richmond 2004). The current systems take mainly into consideration the kind of pigments present, chemical nature of storage products and cell wall components. Thus, there are two groups of prokaryotic microalgae (Cyanophyta and Prochlorophyta) (Mourelle, Gómez, and Legido 2017) and ten phyla of eukaryotic cells (Glaucophyta, Rhodophyta, Chlorophyta, Euglenophyta, Dinophyta, Apicompexa, Cryptophyta, Heterokontophyta and Prymnesiophyta) (R. E. Lee 2008). The most abundant microalgae divisions are Bacillariophyta (diatoms), Chlorophyta (green algae), Chrysophyta (golden algae) and Cyanophyta (blue-green algae) (Bleakley and Hayes 2017).

Microalgae superior ability to sustain harsh or limiting conditions, as well as environmental stressors such as photo-oxidation, osmotic pressure, and ultraviolet (UV) radiation exposure, due to its microscopic size, is widely known. Their ability to provide a broad range of bioproducts and incorporate many valuable bioactive components such as polysaccharides, proteins, pigments, polyunsaturated fatty acids (PUFAs), tocopherols, sterols, vitamins, antioxidants, and minerals has been explored by different areas. In addition, when compared to plants, they present several advantages such as increased productivity, confined season variation, easier extraction processes and a possibility of obtaining a great number of raw materials (Christaki *et al*. 2013). Several of these species have been applied to a multiplicity of biotechnological purposes, namely cosmeceuticals, animal feed, health supplements, pharmaceuticals, wastewater treatment, atmospheric CO_2 mitigation and biofuel production, either as

biomass or as high-value products source (Acién *et al.*, 2017; Bleakley & Hayes, 2017; Christaki *et al.*, 2013; Khan *et al.*, 2018). However, there are biological and economic challenges regarding large-scale microalgae cultivation, as well as their biorefinery process, which should be addressed to ensure a sustainable production achievement (Barkia, Saari, and Manning 2019).

1.2.Cosmeceutical market

The cosmeceutical industry is a worldwide business, being used on a daily basis by millions of people by taking advantage of the aging effects and of the beauty patterns established by the society (Ariede *et al.* 2017; Kligman 2000). Statistical data from 2014 show that skin care consumption, when compared to make-up and fragrances, accounts to the highest sales value, with 305.3 € million versus 79.2 and 195.5 € million, respectively. Portugal was recognized, in 2018, as the 12th EU country with the highest consumption value of cosmetics and personal care in Europe (statista 2019). In a social opportunity point of view, a previous study from 2016 reported that Portuguese consumers reveal a preference towards international brands, due to its availability and quality (Lopes 2016). Therefore, there is a useful gap for Portuguese-made products distribution that should be taken into consideration and possibly explored.

Nowadays, cosmeceuticals have been showing as an important and increasing demand by consumers, due to its known potential beneficial effects on human health. Cosmeceuticals can be defined based on its etymology, fusion result of the words "cosmetic" and "pharmaceutical" (Ruocco *et al.* 2016; S.-K. Kim *et al.* 2008). Cosmetics products are any substance or preparation that is applied to the external parts of human body for its reinforcement, beautification and appearance improvement that must not affect its structure and functions, establishing its inert and pharmacological inactive role (Ruocco *et al.* 2016; Kligman 2000; H.-M. D. Wang *et al.* 2015). Taking into account this definition, it is understandable the relevance of cosmeceuticals in society, as a cosmetic product with drug-like benefits that enhance or protect the appearance of human body, which includes anti-aging creams and moisturizers (S.-K. Kim *et al.* 2008). Its formula contains active ingredients, such as vitamins, minerals, phytochemicals, enzymes, antioxidants and essential oils, useful to promote a healthier skin, hair and nails at a cellular level (S.-K. Kim *et al.* 2008; Ruocco *et al.* 2016). Some of the costumer's requirements include safety, effectiveness, protection, natural formulation and good sensatory quality (Ruocco *et al.* 2016).

The skin, as the largest organ in the human body, is constantly exposed to environment conditions and has a crucial role as first defense barrier and in many physical functions (Yasin *et al.* 2018; H.-M. D. Wang *et al.* 2015; Review 2011). Skin structure complexity and aging responsible factors

acknowledgment will enhance its prevention, by anti-aging products development or improvement (Yasin *et al*. 2018).

This organ's ability to offer protection against exogenous factors, such as toxic or immune inductive response substances penetration and UV radiation, while prevents excessive water loss, is directly dependent on the structural epidermal integrity (Lorencini *et al*. 2014; Review 2011). Skin is composed of the epidermis, dermis and hypodermis (H.-M. D. Wang *et al*. 2015; Lorencini *et al*. 2014). In the epidermis, the predominant keratinocyte cells repair skin damage, with water content being vital for the skin cells functions, the sequential differentiation process and the skin's final appearance. The dermis is mainly composed of connective tissues, including blood vessels, sweat glands, nerves, fibroblasts, hyaluronic acid (HA), collagen and elastin, in which these two last polymers are cross-linked and provide support to the skin. HA is known for its important role in tissue repair and water retention, with its size assuming a critical parameter for its various functions (Review 2011; H.-M. D. Wang *et al*. 2015). An expecting consequent of aging is the breakdown of collagen and HA occurrence, leading to the depletion of skin firmness and to wrinkles appearance. The hypodermis is characterized by a fat and loose connective tissue layer (H.-M. D. Wang *et al*. 2015).

It is widely recognized that moisturization is the basic and first anti-aging gesture, helping to maintain the skin appearance and elasticity, while simultaneously strength its barrier function (H.-M. D. Wang *et al*. 2015). Hyaluronic acid proprieties have been of great interest for cosmetics industry, performing as an important functional ingredient due to its excellent moisture-retention ability. However, its limited amount and highly price enhances the search for novel abundant, natural and cheaper alternatives. Algae derived polysaccharides presents an excellent additive solution, with a previously study reporting a better performance of polysaccharides extracted from *Saccharina japonica* than HA (J. Wang *et al*. 2013; H.-M. D. Wang *et al*. 2015).

Skin aging is a slow and complex biological process, comprising intrinsic and extrinsic factors, that has been reported as the primal cause of thinning, dryness, laxity, fragility, enlarged pores, fine lines and wrinkles (H.-M. D. Wang *et al*. 2015). In fact, aging involves major variable cutaneous alterations with consequent important skin functions deterioration. Intrinsic aging is related to genetic predisposition, cellular metabolic pathways and qualitative and/or quantitative hormonal alterations, while extrinsic aging results from UV light exposure and less frequently due to chemical, toxins and pollution exposure. (Nikolakis, Makrantonaki, and Zouboulis 2013).

At a molecular and cellular level, the skin aging process involves several modifications, including disappearance of epidermal HA ant its size reduction, increase extracellular matrix (ECM) degradation,

cellular senescence and collagen and elastin degradation by ascorbic acid – hydroxylase cofactor required in reactive species oxygen (ROS) elimination process, ultimately leading to a skin aging acceleration (Nikolakis, Makrantonaki, and Zouboulis 2013; H.-M. D. Wang *et al*. 2015; Kammeyer and Luiten 2015; Masaki 2010).

Therefore, anti-aging strategies are based in hindering or even reducing the evidence of aging, being the use of formulations with active substances a commonly and successful approach (Ramos-e-Silva *et al*. 2013). Antioxidant properties display an important role for anti-aging effect, since it prevents skin cellular level damage by decreasing free radicals negative influence (Ramos-e-Silva *et al*. 2013).

Taking these into consideration, it becomes understandable the vitamins and antioxidants application acceptance as primary cosmetics ingredients. However, to assure the compound bioactivity advantageously effect, several factors must be accomplished: molecule stability during production, storage and manipulation phases, nontoxic impact for the consumer and activity evidence at the target site. Thus, stability analysis is an indispensable tool to assess formulation usefulness, since it has a major impact on product quality, efficacy and safey (Baby *et al*. 2007).

1.3.Algae potential in cosmeceutical

Algae extracts are currently being used for a variety of cosmeceutical products, since its composition offers a multiplicity of bioactive ingredients suitable for skin care manufacturing, mainly as thickening, water-binding and antioxidants agents. Brown and red algae add to the highest explored group due to the presence of vitamins, minerals, amino acids, sugars, lipids and other biological active molecules involved in moisture loss reduction and cell regeneration, thickening and skin health promotion processes (S.-K. Kim *et al*. 2008; Ariede *et al*. 2017). It is important to enhance the potential use of polysaccharides in algae cell walls as texture-improving and stabilizing agents: fucoidans in brown algae, carrageenan's in red algae and ulvan's in green algae (J. Kim *et al*. 2018; Ruocco *et al*. 2016).

Microalgae extracts potential for its interesting bioactive properties has been widely recognized, with antioxidant activity accounting for a major application in cosmetics, pharmaceutics and nutrition fields, due to its health-promoting effects (M. Raposo, De Morais, and Bernardo de Morais 2013). In fact, microalgae phototrophic growth regime and consequent exposure to high oxygen and radical stress result in the development of numerous efficient protective systems. This enables them to prevent free radicals and ROS accumulation and thus to counteract cell-damaging activities (Pulz and Gross 2004). Oxidative stress has been associated with various chronic diseases including atherosclerosis, diabetes mellitus, neurodegenerative disorders, and certain types of cancer (Banskota *et al*. 2019), as well as with skin

aging acceleration (J. Kim *et al*. 2018). Microalgae extracts are mainly used in cosmeceutical industry as antiaging creams, refreshing or regenerating skin care products, antioxidant and emollient products formulation. Moreover, it has been reported that some of microalgae secondary metabolites can repair damaged skin, maintain skin moisture and inhibit inflammation and accelerate healing processes (Ariede *et al*. 2017; S.-K. Kim *et al*. 2008).

Therefore, a variety of compounds with antioxidant properties and antiaging effects acquired from algae exploration have been widely applied in skin care and protection (H.-M. D. Wang *et al*. 2015). Some include the use of carotenoids, vitamins, polyphenols, sulfated polysaccharides and PUFAs, with macro and microalgae extracts already being used as sources of cosmeceuticals (Mourelle, Gómez, and Legido 2017; Masaki 2010; H.-M. D. Wang *et al*. 2015; Borowitzka 2013), as evidenced in Table 1. In another perspective, antioxidant activity presents of greater relevance for cosmeceutical development due to its ability to preserve the product organoleptic properties (H.-M. D. Wang *et al*. 2015).

Table 1 – Microalgae extract or bioactive compound whose reported activity is potential interesting for cosmeceutical industry and respectively commercially available products.

Bioactive compound	*Algae organism*	*Potential activities and cosmetic application*	*Examples of commercially available products*	*Ref*
Polysaccharides				
Fucoidans	*Laminaria japonica* *Fucus vesculosus* *Undaria pinnatifida* *Cladosiphon okamuranus* *Hizikia fusiforme*	Skin protection and elasticity maintenance by moisture increasing, antioxidant and anti-aging effects	Tiande® Fucoidan line	(Tiande® n.d.; S.-K. Kim *et al*. 2008; Wijesinghe and Jeon 2011)
	Saccharina japonica	Higher moisture absorption and retention ability than hyaluronic acid		
	Costaria costata *Mekabu* *Ascophyllum nodosum*	Skin anti-aging		(Tiande® n.d.; J. Kim *et al*. 2018)
	Sargassum tenerrimum *Turbinaria conoides*	Antioxidant		
Carrageenans	*Chondrus* sp.	Viscosity-altering and thickening agents	Satiagum™ and Satiagel™	(Necas and Bartosikova 2013; "Cargill: Skin Care/Face" n.d.; S.-K. Kim *et al*. 2008)
Alginate/ Alginic acid	Brown algae	Thicken and stabilize emulsions at low pH; quickly water retention;	ALGOBOTOrelax, Teana laboratories	(S.-K. Kim *et al*. 2008; H.-M. D. Wang *et al*.

			provide cellular support to keep the cells from collapsing		2015; "Teana Laboratories: ALGOBOTORELAX" n.d.)
	Agar	*Pterocladia* *Pterocladiella* *Gelidium* *Gracilaria gracilis* *Gracilaria chilensis*	Viscosity-altering and thickening agent	Hot Chocolate Aromatherapy Powder Facial Mask, CALM SkinCare Inc®	(Inc n.d.; S.-K. Kim *et al.* 2008; H.-M. D. Wang *et al.* 2015)
	Ulvans	*Ulva* sp	Antioxidant and skin anti-aging	–	(J. Kim *et al.* 2018)
Sulfated polysaccharides		*Phaeodactylum tricornutum*	Anti-inflammatory activity and immunomodulatory agent	–	(M. Raposo, De Morais, and Bernardo de Morais 2013; Talakoub, Neuhaus, and Yu 2016)
		Chlorella stigmatophora			(M. Raposo, De Morais, and Bernardo de Morais 2013)
		Porphyridium	Anti-inflammatory activity; immunomodulatory agent and biolubricant properties	Astaderm™	(M. Raposo, De Morais, and Bernardo de Morais 2013; Coast Southwest™ n.d.)
Proteins		*Chlorella sp*	Skin moisture; excellent emulsifying properties; anti-photoaging.	–	Ariede *et al.*, 2017; Sathasivam, Radhakrishnan,

					Hashem, & Abd_Allah, 2019; Ursu *et al.*, 2014
Polyunsaturated fatty acids (PUFA)					
	Eicosapentaenoic acid (EPA)	*Nannochloropsis oceanica. Phaeodactylum tricornutum Chlorella vulgaris*	Anti-photoaging effect; cell regeneration and skin health assistance;	GENIUS Liquid Collagen™, Algenist® Skincare (San Francisco)	(Huang *et al.* 2018; Jeong *et al.* 2017; Sathasivam *et al.* 2019; Yang *et al.* 2017; Algenist ® n.d.)
	Docosahexaenoic acid (DHA)	*Nannochloropsis oceanica*			(H. Kim *et al.* 2019; Algenist ® n.d.)
	Arachidonic acid (ARA)/ omega-6	*Porphyridium cruentum*	Cell regeneration and skin health assistance.		(Barkia, Saari, and Manning 2019; Huang *et al.* 2018; Algenist ® n.d.)
Carotenoids					
	Violaxanthin	*Nannochloropsis oceanica*	Anti-photoaging agent	–	H. Kim *et al.*, 2019
	Fucoxanthin	*Phaeodactylum tricornutum*	Effective radical scavenger with higher antioxidant properties; skin photoage and wrinkle formation prevention	–	J. Peng, Yuan, Wu, & Wang, 2011 (S. M. Kim *et al.* 2012; Borowitzka 2013)
	Astaxanthin	*Haematococcus pluvialis, Chlorella* sp. *Chlorococcum* sp. and *Scenedesmus* sp	Ultraviolet radiation protection; strong antioxidant	Astaxanthin Collagen All-in-One Gel, DHC	(Bhalamurugan, Valerie, and Mark 2018; Panis and Carreon 2016; M. F. de J. Raposo, Morais,

					and Morais 2013; "DHC: Astaxanthin Collagen All-in-One Gel" n.d.)
	Lycopene	*Dunaliella salina, Haematococcus pluvialis*	Antioxidant and anti-aging agent by skin roughness decreasing	–	Masaki, 2010; Takaichi, 2011
	β-Carotene (pro-vitamin A)	*Dunaliella salina*	Antioxidant; epithelial cell growth and differentiation regulation; roughness and facial wrinkling decreasing; keratinization normalization	AHAVA Skincare	(Lupo 2001; Varela *et al.* 2015; AHAVA n.d.)
Vitamins					
	Vitamin E (α-Tocopherol)	*Dunaliella tertiolecta, Tetraselmis suecica, Nannochloropsis oculata, Chaetoceros calcitrans* and *Porphyridium cruentum*	Antioxidant and wrinkle reduction effect.	Algenist® Skincare (San Francisco)	(Galasso *et al.* 2019; Lupo 2001; Algenist ® n.d.)
	Vitamin C (ascorbic acid)	*Skeletonema marinoi*	Antioxidant; collagen biosynthesis and deposition stimulation, vitamin E regeneration.	GENIUS Ultimate Anti-Aging Vitamin C+ Serum, Algenist® Skincare (San Francisco)	(Galasso *et al.* 2019; Lupo 2001; Algenist ® n.d.)
Microalgal extracts					
Chlorella vulgaris extract			Collagen synthesis stimulation (anti-aging); supporting tissue	Dermochlorella, Codif, St. Malo, France ;	(de Melo *et al.* 2019; ELEMIS n.d.; Mourelle, Gómez, and Legido

	regeneration; wrinkle reduction and wound healing effects.	Pro-Collagen Marine Cream SPF 30, ELEMIS.	2017; Spolaore *et al.* 2006)
Arthrospira extract	Early skin aging signs repair, tightening effect and stria formation prevention	Protulines, Exsymol S.A.M., Monaco	Spolaore *et al.*, 2006
Nannochloropsis oculate extract	Skin-tightening properties by protecting the cells from oxidative stress and stimulating collagen-I synthesis	Pepha®-Tight, Pentapharm (Basel, Switzerland)	(Pentapharm, n.d.; Spolaore *et al.* 2006)
Dunaliella salina extract	Cell proliferation and turnover stimulation and skin metabolism positively influence	AHAVA Skincare Pepha®Ctive, Pentapharm (Basel, Switzerland)	(Spolaore *et al.* 2006; AHAVA n.d.)
Phaeodactylum tricornutum lipidic extract	Anti-aging effect by limiting the accumulation of harmful proteins; skin protection from UV exposure adverse effects; skin elasticity and firmness improvement; skin-depigmenting properties	Givaudan, Switzerland	(Ariede *et al.* 2017; Mourelle, Gómez, and Legido 2017)

Despite the extensive research and development that has been made related with cosmeceutical algae-based products to enhance biological effects on the skin, the complete acknowledgement of its potential is still not well recognized. Most of these studies are present in patents, which do not report details concerning the cause-effect relation that would enable the full cosmetic performance comprehension, by not establishing the biocompounds and mechanisms accountable for the respective biological activity. Furthermore, the singular or combined action of each biological element, as well as their part in the overall algae outcome for the cosmetic products, is not clarified. Therefore, the bioactive compounds and extracts characterization regarding their effect on the skin must be further explored in order to promote an improved development approach for new cosmeceutical products with commercial purposes. Moreover, parameters related to stability, physical-chemical and toxicological research should be considered to evaluate the product potential in a commercial perspective.(Ariede *et al*. 2017)

Beside these bioactive components, microalgae generally contain large amounts of structural biopolymers, including proteins and carbohydrates. These structural biopolymers might possibly display interesting technological functionalities in cosmeceutical products, by showing a potential role as texturizer, stabilizer, or emulsifier (Bernaerts *et al*. 2019). Rheological properties are one of the most important quality aspects to consider in these products (Gilbert *et al*. 2013), not only for their physical appearance (e.g. creating a desired texture or preventing phase separation phenomena during storage), but also regarding consumer sensory perception. There is a lack of knowledge on the functionality role of microalgae cell wall related polysaccharides, despite cell wall polysaccharides of many taxonomically related macroalgae are among the common thickening and gelling agents used, such as carrageenans, agars, and alginates. This might be due to the diversity and complexity of molecular structures of the cell wall related polysaccharides, but enhances the possibly use of microalgae as a sustainable source of functional agents (Bernaerts *et al*. 2019). Moreover, microalgae exopolysaccharides have been recognized for being shelf-life extenders (Levasseur, Perré, and Pozzobon 2020). A natural sulfated polysaccharide compound isolated from *Porphyridium* sp., that acts as a physical barrier, creating a thick protective layer around the cell, also evidenced an active protection against skin photo damaging and aging (Martins *et al*. 2014). Therefore, a synergetic effect between bioactive and technological properties may be attained (Lupo 2001).

On the other hand, microalgae have been granted with the generally recognized as safe (GRAS) status, which emphasis their use as a cell factory. This GRAS classification is critical since allows to simplify downstream processes, such as purification steps, leading to a more economical appealing production by costs decreasing and higher global efficiency (Fu *et al*. 2016).

1.4. *Phaeodactylum tricornutum*

The class *Bacillariophyceae* or diatoms are golden brown unicellular eukaryotes, which color results from the combination of green due to chlorophylls a, c1 and c2 content with brown and yellow pigmentation of the fucoxanthin and β-carotene (Richmond 2004). Diatoms are characterized by a protoplast embedded in a frustule composed of two overlapping thecae: the larger, upper epitheca and the smaller, lower hypotheca. The frustule is composed of three successive layers: (1) diatotepum, which is the inner-most organic layer; (2) a mineral and silicified shell that contains organic matter and (3) an external organic coat designated as cell wall-bound exopolysaccharides (EPSs) (Gügi *et al*. 2015). Two major diatom groups can be establish according to frustule symmetry: pennate diatoms characterized by a bilateral symmetry; central diatoms with radial symmetry (Richmond 2004; De Tommasi *et al*. 2018). This class is responsible for about 20-25 % of the global CO_2 fixation (Villanova *et al*. 2017), while provides approximately half of the marine primary food sources (Yi *et al*. 2019). Their essential role in marine ecosystems is enhanced, accounting for the production of a large portion of the oceans total energy, as well as for global silicon (Yi *et al*. 2015) and carbon cycle (Armbrust 2009). Diatoms complex evolutionary history based on the union between heterotrophic host and photosynthetic red alga may explain the distinctive range of attributes found (Armbrust 2009). In fact, resulting from a secondary endosymbiotic event, diatoms display a complex combination of genes and metabolic pathways acquired from endosymbiotic events and horizontal transfer with bacteria and viruses. This chimeric metabolism is believed to be an essential component of the great evolutionary success and the high biotechnological potential of these microalgae species. Their potential industrial applications rely on the use of silica for nanostructures, food and production of triacylglycerols (TAGs) for biofuel under low-input conditions. Given their metabolic flexibility, different growth systems can be induced in microalgae and diatoms in particular (Villanova *et al*. 2017).

Phaeodactylum tricornutum is a marine pennate raphid diatom, on which interest has been increasing over the years. This is due to its high growth rates and known ability to thrive in large-scale production facilities, enabling the obtention of bioactive compounds such as carotenoids, phytosterols, vitamins, and antioxidants, as well as different biotechnological applications (Quelhas *et al*. 2019; Zhao *et al*. 2015). In general, this diatom shows good tolerance to growth at 9-30 % of salinity and temperatures between 15 and 25 °C (Sigaud and Aidar 1993; García *et al*. 2005), with temperatures above 28°C leading to a drop in proteins and PUFAs concentration, due to metabolic stress (Bitaubé Pérez, Caro Pina, and Pérez Rodríguez 2008). Literature report that *Phaeodactylum tricornutum* under photoautotrophic regime contains around 34.8-39.6 % of crude protein, 16.8-26.1 % available carbohydrate and 16.1-

18.2 % lipid, on a dry weight (DW) basis (Barkia, Saari, and Manning 2019; S. M. Kim *et al.* 2012; Tibbetts, Milley, and Lall 2015).

Furthermore, *P. tricornutum* is the first pennate diatom for which its genome has been sequenced being currently used as a model organism (Le Costaouëc *et al.* 2017; Quelhas *et al.* 2019), despite the unique and atypical pleiomorphy of this species that highlights its great adaptation ability (Ovide *et al.* 2018; Martin-Jézéquel and Tesson 2012). In fact, it has four convertible morphotypes: oval, fusiform, triradiate and cruciform, with the last one only being more recently discovered. All have similar cellular ultrastructure except for cell wall and vacuolar organization (He, Han, and Yu 2014; Tesson, Gaillard, and Martin-Jézéquel 2009; Ovide *et al.* 2018; Zhao *et al.* 2015), such that all three morphotypes (oval, fusiform and triradiate) possess silicified structures on epitheca. However, unlike typical diatoms, the cell wall is essentially composed of organic compounds and poorly silicified, despite also presenting a three-layered based structure (Martin-Jézéquel and Tesson 2012; Song, Lye, and Parker 2020). This may be advantageous for biotechnological purposes when compared to other diatoms rigid cell walls regarding intracellular product recovery (Song, Lye, and Parker 2020). Moreover, a sulfated glucuronomannan was described as the prominent polysaccharide in *P. tricornutum* cell wall. It comprises a backbone of α-1,3-linked mannose residues with side chains of mannose and glucuronic acid (Bernaerts *et al.* 2019), which may enhance its potential to attain value-added products for different application areas, such as nutraceuticals, therapeutic agents and cosmetics (M. Raposo, De Morais, and Bernardo de Morais 2013; Le Costaouëc *et al.* 2017). The oval cells are the only form capable to synthesize a silicified valve (frustule) with pores and displaying an identical morphology as raphid pennate diatoms with central raphes (Martin-Jézéquel and Tesson 2012; Bernaerts *et al.* 2019; Tesson, Gaillard, and Martin-Jézéquel 2009). Ultrastructural organization of the different diatom cells is represented in Figure 1.

Numerous researchers have tried to understand the cause-effect relation behind this species polymorphism (Tesson, Gaillard, and Martin-Jézéquel 2009). A correlation between the environmental factors change and the morphogenesis phenomenon has been suggested. Data report that different shapes of *P. tricornutum* are more prevalent under different culture conditions. Oval cells are enhanced under stressed conditions (e.g. nutrient limitation, hyposaline conditions, low temperature, low light, unshaken liquid culture and solid substrate), converting to fusiform or triradiate cells under favorable conditions, while the fusiform morphotype accounts for the most widespread phenotype (Tesson, Gaillard, and Martin-Jézéquel 2009; Zhao *et al.* 2015; Ovide *et al.* 2018). In fact, different morphotypes provide more favorable acclimation processes under different environments, with oval cells allowing a better sedimentation and surface adhesion (Yi *et al.* 2019; Song, Lye, and Parker 2020). Despite these

observations, limited information is available about conditions manipulation to attain each cell morphotype of *P. tricornutum* (Song, Lye, and Parker 2020).

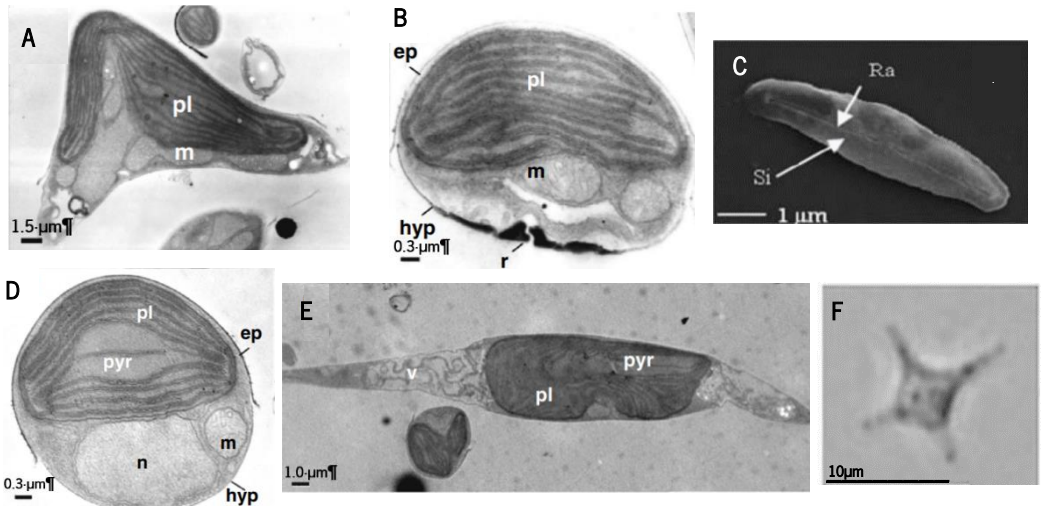

Figure 1 – *Phaeodactylum tricornutum's* morphotypes ultrastructural organization. (A) Triradiate cell by transmission electron microscopy (TEM); (B) oval cell in transversal section in the raphe area (r) by TEM; (C) oval cell with silicified valve by scanning electron micrograph; (D) and (E): fusiform cell in longitudinal and transversal sections, respectively; (F) Cruciform under light microscopy. c: chloroplast; ep: epivalve; hyp: hypovalve; m: mitochondria; n: nucleus; pl: plastid; pyr: pyrenoid; ra: raphe; v: vacuole. Adapted from Ovide *et al.* (2018); De Martino *et al.* (2011); Martin-Jézéquel and Tesson (2012).

1.4.1. Main applications: cosmeceutical potential

Phaeodactylum tricornutum is widely known for its interesting lipid profile with significant amounts of PUFA, in particular eicosapentaenoic acid (EPA), while assembles a rich source of fucoxanthin up to at least ten times more abundant than in macroalgae (S. M. Kim *et al.* 2012; Song, Lye, and Parker 2020).

Fucoxanthin has been found to have several health-conducive effects, including antioxidant, anti-inflammatory, anticancer and antiobesity activities (McClure *et al.* 2018; S. M. Kim *et al.* 2012). Since most of these studies rely on fucoxanthin derived from macroalgae, little is known about beneficial effects of fucoxanthin obtained from *P. tricornutum*, although this microalga was shown to comprise 16.5 to 26.1 mg fucoxanthin per gram dry matter. A study evidenced the antioxidant and antiproliferative *in vitro* ability of the carotenoid fucoxanthin extracted from this diatom, enhancing its role against oxidative stress, which could be potentially interesting for the cosmeceutical industry. This may be related to its location in the thylakoids of chloroplasts, as well as its natural occurrence as a light harvesting complex with

chlorophyll a/c (Neumann *et al*. 2019). In fact, carotenoids protect chlorophyll from the effects of excess light exposure, by scavenging ROS such as singlet oxygen molecules and free radicals (Sathasivam and Ki 2018).

It has been recognized that over exposure to UV radiation from sunlight leading to the generation of ROS, inflammatory reaction, and angiogenesis of the skin may be the primary causative agent in the damage of cellular constituents and some cutaneous disease such as pigmentation, laxity, wrinkling, and erythema. Several results show that topical treatment with fucoxanthin prevented skin photoaging and wrinkle formation in UV-B irradiated mice, possibly through its antioxidant and antiangiogenic effects. These studies suggest the possible effective use of fucoxanthin in cosmetics formulations as UV protect ingredient (Peng *et al*. 2011). Therefore, rational biotechnological approaches should be developed in order to make fucoxanthin production in diatoms feasible, while further encourage its full potential exploration.

Moreover, as it has been previously underline, microalgae structural biopolymers such as proteins, storage polysaccharides, and cell wall related polysaccharides might possibly alter the rheological properties of the enriched cosmetic product (Bernaerts *et al*. 2019). Environmental conditions have shown to influence the monosaccharide composition of *P. tricornutum* cell wall polysaccharides, thus affecting their respective structure (Gügi *et al*. 2015). Therefore, the effects of culture conditions on microalgae composition must be considered, since different techno-functional properties may be observed (Gügi *et al*. 2015; Bernaerts *et al*. 2019).

1.5. Microalgae cultivation strategy and requirements

The major elements of microalgae biomass are carbon (30 %–50 % DW), oxygen (30 %–50 % DW), hydrogen (3 %–7 % DW), nitrogen (4 %–9 % DW), phosphorus (1 %–3 % DW) and minor amounts of other elements such as sulfur, potassium, magnesium and calcium. These components should be present in culture medium to allow culture's performance maximization (Acién *et al*. 2017). In a broad way, microalgae biomass growth mainly depends on a carbon source and light suitable supply, in order to carry out photosynthesis. However, their ability to respond to environmental stimuli by internal adjustments like biochemical and physiological acclimation has been largely explored. Moreover, they are externally capable of excrete a diversity of compounds to amongst others, render nutrients available or limit competitor's growth (Mata, Martins, and Caetano 2010; Richmond 2004). Therefore, when considering microalgae cultivation there are several conditions that must not be overlooked, since they may have a great impact on its growth kinetics. This include abiotic factors such as temperature, pH,

salinity, light, nutrient concentration, O_2, CO_2 and toxic components, as well as biotic factors such as pathogens (bacteria, fungi, viruses) and competition by other algae. Moreover, operational factors such as shear produced by mixing, dilution rate, depth, harvest frequency, self-shading due to high cell density and addition of bicarbonate must also be consider (Mata, Martins, and Caetano 2010; Barkia, Saari, and Manning 2019). In this manner, establishing microalgae biochemical composition presents of greater importance in order to address the valorization of the high-value compounds produced and enhance the economic feasibility of the cultivation process, while promoting the main goal fulfillment (Branco-Vieira *et al*. 2018; Richmond 2004). To satisfy these requirements at a laboratory-scale is relatively easy although costly, but to carry it out at large-scale is more challenging, especially because it must consider the global cost of the process (Acién *et al*. 2017).

Microalgae species are mainly cultured under photoautotrophic conditions, although some species are capable to indulge different growth regimes and metabolic shift as an outcome of the environmental conditions' modification (Mata, Martins, and Caetano 2010; Acién *et al*. 2017). Such that can use organic carbon and oxygen to growth heterotrophically (Cerón-García *et al*. 2013). Mixotrophic can be broadly defined as a cultivation system in which CO_2 and organic carbon are simultaneously assimilated, combining energy sources and resulting in both respiratory and photosynthetic metabolism operating concurrently (Richmond 2004; Cerón-García *et al*. 2013). These potential allows to attain higher biomass yields, while maintaining a high content of valuable products (Cerón-García *et al*. 2013). Advantages and disadvantages of each cultivation system can be found in Table 2.

Table 2 – Main advantages and disadvantages of each cultivation system used for microalgae growth.

	Advantages	*Disadvantages*	*References*
Photoautotrophic growth	▪ Carbon emissions to atmosphere reduction; ▪ Potable water and arable land not required; ▪ Suitable for almost all microalgae species; ▪ Valuable compounds production.	▪ Cellular self-shading; ▪ Low maximum biomass concentration attainable; ▪ Decreased harvesting efficiency; ▪ Limited productivity ▪ Cost-ineffective system.	(Bart, Palmeri, and Cavallaro 2010; García *et al*. 2005; Cerón García *et al*. 2006; Richmond 2004)
Heterotrophic growth	▪ Higher cell densities; ▪ Light not required; ▪ Easier to control;	▪ Limited number of available	(Perez-Garcia and Bashan 2015; García *et al*. 2000)

	▪ Easier scaling-up process; ▪ Biomass harvesting cost effective.	heterotrophic algal species; ▪ Increased potential of bacterial contamination; ▪ Growth inhibition by low concentrations of organic substrates; ▪ Photosynthesis-related compounds production reduction or inability.	
Mixotrophic growth	▪ Low light intensities requirement, resulting in energy costs reduction; ▪ Simultaneously synthesis of compounds characteristic from heterotrophic and photosynthetic metabolisms; ▪ Higher productivities; ▪ Higher growth rates than either heterotrophic or photoautotrophic regimes; ▪ Prolonged exponential growth phase; ▪ Reduction or stopping of photo-inhibitory effect; ▪ Protection from photo-oxidative damage stimulated by accumulating oxygen in enclosed photo-bioreactors; ▪ Reduction of lost biomass from respiration during dark hours; ▪ Land surface and refrigeration reduction.	▪ Dual limiting process; ▪ Increasing costs by adding an organic substrate; ▪ Increased potential of bacterial contamination; ▪ Limited number of available mixotrophically algal species.	(Cerón García *et al.* 2006; García *et al.* 2000; García *et al.* 2005; Perez-Garcia and Bashan 2015)

Although mixotrophy presents as a promising alternative for enhancing the productivity of many interesting compounds, its potential must be analyzed regarding the product of interest. Optimal balance between heterotrophic and photoautotrophic stimuli should be addressed considering the specific culture

application (Cerón-García *et al*. 2013). For instance, at high cell density, light becomes limiting leading to photoautotrophic growth rate decreasing. Under this condition, both protein and chlorophyll contents are much lower than those of the autotrophic cultures (García *et al*. 2000). Several studies have reported the ability of *P. tricornutum* to growth mixotrophically in the presence of different organic substrates, namely, glycerol, glucose, fructose and mannose (Cerón-García *et al*. 2013; Villanova *et al*. 2017). Organic nutrient selection for potential use in commercial scale should rely on an inexpensive and easy to sterilize carbon source, while allows to enhance microalgae growth and desired bioproducts synthesis (Cerón García *et al*. 2006). Glycerol has been recognized as the preferable choice for promoting mixotrophic growth by this microalgae (Cerón García *et al*. 2006; Cerón-García *et al*. 2013), with significant impact on *P. tricornutum* biochemical profile, namely at fatty acid profile and content (Cerón-García *et al*. 2013). It acts like an osmoticum i.e. a substance capable of raising the solution osmotic strength and, consequently, keeping cells osmotic equilibrium. This may be related to its use as preferable substrate for the majority of species that naturally occur in habitats with elevated osmolarity, such as seawater and saline pounds. Glycerol is considered an economical carbon source for energy and carbon supply and requirements, with almost no toxic effects even at high concentrations (Perez-Garcia *et al*. 2011). Furthermore, since glycerol is a major by-product of biodiesel industry, its use as substrate could provide a more integrating by-product valorization and sustainable microalgae production (Choi and Yu 2015; Poddar, Sen, and Martin 2018).

The most suitable proportion of phototrophy/heterotrophy to be used must consider its influence on growth kinetics and biochemical profile. Since exclusively heterotrophic growth is not possible for *P. tricornutum*, this parameter selection becomes of greater importance to establish the minimum amount of light needed to maintain growth, while fulfilling the correct amount of organic nutrients. It is widely known that high substrate initial concentrations may negatively affect cell growth. A commonly strategy applied to overcome this limitation is fed-batch feeding, which relies on gradually adding the organic nutrient and only when the biomass concentration is sufficiently high to indulge culture photolimitation. This must be carefully planned as part of the process design, defining the addition frequency and dose in order to guarantee that the organic nutrient is consumed and does not accumulate (Cerón-García *et al*. 2013). However, mixotrophic growth consequences under *P. tricornutum* biochemical profile is not well recognized, with a limited number of studies focusing on pigments and lipid fractions variation (García *et al*. 2005; Cheirsilp and Torpee 2012; Cerón-García *et al*. 2013; Cerón García *et al*. 2006).

It has been established that culture conditions have a direct impact on microalgae cell composition, which can be optimized using a multistage cultivation strategy, providing the achievement of specific biotechnological goals. This concept allows ensuring maximum biomass production, as well as maximum induction and accumulation of the desired products, by their occurrence in separate phases through a two-stage process. In fact, the culture exposure to stressful environmental conditions may result in overall biomass productivity reduction and introduce potential instability of the culture, which often accounts for contamination and culture collapse (Richmond 2004). Commonly stress strategies include nutrient depletion – in particular nitrogen and phosphate –, unfavorable temperature, high light intensity and/or high salinity (X.-M. Sun *et al*. 2018; Poddar, Sen, and Martin 2018). Therefore, biomass concentration is increased prior to the stress stimuli (Yap *et al*. 2016).

Culture media formulation aims to supply nutrients in excess to ensure that they do not become the rate-limiting factor. However, for specific applications, nutrients are purposely supplied in limiting concentrations. Nitrogen, after carbon, is the most important nutrient responsible for biomass production. Typical responses to nitrogen limitation include cells discoloration through the decrease in chlorophylls and an increase in carotenoid content, as well as accumulation of organic carbon compounds such as polysaccharides and PUFAs (Richmond 2004). Moreover, several studies have reported the possibility to control cell metabolism to yield a high content of energy-rich compounds (B. Fernandes *et al*. 2013). Diatoms allocate organic carbon into two primary energy storage compounds: carbohydrates, specifically β–1,3 glucans also known as chrysolaminarin; and lipids, as neutral lipid TAGs (Caballero *et al*. 2016; Jensen *et al*. 2019). Chrysolaminarin is a glucose-based water soluble polysaccharide which accumulates in the vacuole (Chauton *et al*. 2013; Obata, Fernie, and Nunes-Nesi 2013) and may present potential biotechnological applications, since it has been demonstrated to have antioxidant activity, as well as antitumor effects (Gao *et al*. 2017). This polymer accumulation in *P. tricornutum* cells can be induced by nutrient replete conditions (Chauton *et al*. 2013; Obata, Fernie, and Nunes-Nesi 2013), as well as by high CO_2 concentration (Jensen *et al*. 2019). On the other hand, lipid accumulation in diatoms mainly occurs as oil bodies in the cytoplasm and, less usual, in the chloroplasts (Jensen *et al*. 2019). Lipid content can be enhanced by nitrogen or phosphate limitation, high salt or iron concentrations or growth under heterotrophic/mixotrophic culture conditions (B. Fernandes *et al*. 2013).

Despite the knowledge that glycerol addition enhances overall lipid production (Cerón-García *et al*. 2013), as well as increases carbohydrates storage (Villanova *et al*. 2017), no study was found regarding the combination of mixotrophic growth and nutrient stress for this species. Moreover, glycerol

presence results in higher mannitol and trehalose storage, which upon glycerol addition may also represent a response to an increased osmotic pressure (Villanova *et al*. 2017).

1.6.Adaptive laboratory evolution using UV mutagenesis

Adaptive laboratory evolution (ALE) is an increasingly popular biotechnological tool for developing and improving desirable traits of industrial microorganisms regarding their robustness to unfavorable conditions, such as unusual carbon source, hostile environment, and chemical inhibitors, as well as understanding biological phenomena (Sandberg *et al*. 2019; Su *et al*. 2018). ALE process comprises prolonged iterative cultivation under artificially specified stress, which application has been recognized by its simplicity and effectiveness (Su *et al*. 2018; Winkler and Kao 2014). It takes advantage of the microorganisms great ability to rapidly adapt to changing environments, which occur by random genomic mutations and subsequent positive selection towards best performance (X.-M. Sun *et al*. 2018). Therefore, this approach is based on two main assumptions: intrinsic spontaneous and random mutations in cells occurrence; defined selective pressure preserves mutants with improved adaptability to the given environment. This outcomes in microbial population direction evolution to desirable phenotypes over time and consequently relies on the evolutionary engineering field (Sandberg *et al*. 2019; Su *et al*. 2018).

Physical or chemical mutagenesis can be used to induce a higher frequency of mutation than the natural rate of a particular organism (Fu *et al*. 2016). Mutagens stimuli have been successfully applied for improving microalgae strain performance or enhancing carotenoids production. These include physical mutagens such as UV light and gamma and X-rays radiations, as well as chemical mutagens such as EMS (ethyl methanesulfonate) and NTG (N-methyl-N′-nitro-N-nitrosoguanidine) (Fu *et al*. 2016; Yi *et al*. 2015).

ALE has been extensively employed with *Escherichia coli* and *Saccharomyces cerevisiae* for strain improvement over the last 25 years, whereas its application with non-conventional hosts like microalgae is recently emerging. Considering that environmental stimuli are decisive factors for microalgae biochemical composition, ALE suits the purpose for developing algal strains resistant to growth stresses and with improved production performance. In addition, when compared to other strain improvement strategies, that is, genetic engineering, ALE does not require a priori genetic knowledge, allowing to better understand molecular mechanisms by identifying the mutations underlying the improved phenotype (Su *et al*. 2018).

Three alternative methods of ALE have been identified, namely, batch cultivation in parallel serial cultures, colony transfer and chemostat cultures, as evidenced in Figure 2. In batch cultivation,

microorganisms undergo successive propagation in shake flasks, with an aliquot of culture being periodically transferred to fresh medium at regular intervals (X.-M. Sun *et al*. 2018; Su *et al*. 2018). An identical strategy is applied for colony transfer, where an aliquot of the culture is transferred to a new culture dish (X.-M. Sun *et al*. 2018). Both these methods are advantageous for their cheap equipment and easy setup. However, batch variations in nutrient supply, environmental conditions, population density, and growth rate are the main shortcomings. This is overcome by the stable equilibrium achieved through the continuous addition of medium and simultaneous removal of culture broth, where a tight control of nutrient supply and environmental conditions is possible. Furthermore, growth rates and population densities changes can be experimentally controlled by culture rate dilution modulation. Although chemostat culture requires complex procedures leading to an enhanced operation cost, which is much higher than the serial batch cultures, it provides more genetic diversity. Moreover, continuous selection may result in shorter ALE periods, reducing the overall project cost (Su *et al*. 2018; X.-M. Sun *et al*. 2018).

When considering ALE experiments, several key elements must be taken into consideration: selection pressure driving the evolution (X.-M. Sun *et al*. 2018; Su *et al*. 2018), passage size (X.-M. Sun *et al*. 2018), cultivation period and achievable performance improvement (Dragosits and Mattanovich 2013). In practice, the stress stimuli depend on the experiment purpose or the desired microorganism feature, while their levels need to suit the selection required, that is, growth inhibition of undesired variants and avoiding whole population death. When microalgal population approaches to adaptation to the designed stress, represented by population majority in mutants with high fitness, growth rate further improvement becomes lower. Therefore, ALE should proceed by changing or enhancing the stress (Su *et al*. 2018). Passage size determines how much of the population is used to propagate to each subsequent batch culture and may have a significant impact on ALE success. It has been previously recognized that small population passage can result in the beneficial mutation loss, leading to a ceased or slowed evaluation rate. Alternatively, higher volumes could be used in order to decrease the possibility of this loss, but an increase in the resources required to sustain would be observed (X.-M. Sun *et al*. 2018). Therefore, an adjustment should be fulfilled to address an economical and time feasible strategy. In addition, in serial batch cultures, the transfer usually occurs before the stationary phase is reached, to avoid stationary phase adaptation, while comprises the higher growth rate phase. In continuous cultures, since growth rate is kept constant (or under certain experimental setups stepwise) by a major growth nutrient limitation, cells selected under growth limiting conditions can show growth trade-offs in non-limiting conditions and *vice versa*. Regarding ALE experiment duration it has been established a period

between a few weeks up to a few months, comprising 100 to 2000 generations. During ALE, several phenotypes will firstly occur and compete for dominance, with factors such as clonal interference, bet hedging, genetic hitchhiking and growth environment variation being responsible for a significant population heterogeneity. In this manner, it cannot be assumed that a homogenous population is present during any point of the experiment. It must be taken in consideration that the best phenotype is not necessarily the one with the highest fitness for a specific condition, but the one with increased performance and lowest trade-offs in other environmental conditions (Dragosits and Mattanovich 2013).

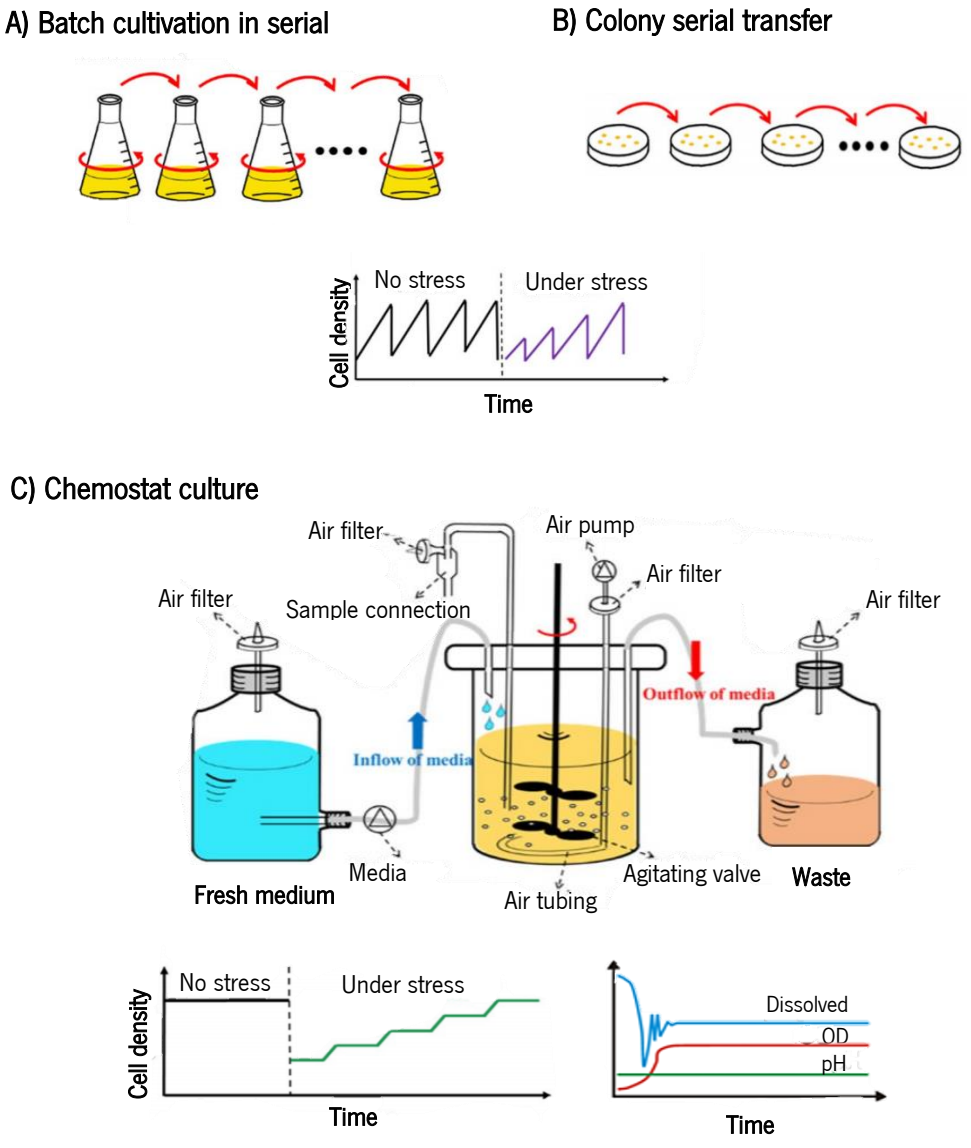

Figure 2 – Adaptive laboratory evolution (ALE) can be performed in the laboratory using three broad approaches. (A) Sequential serial passages at regular intervals in shake flasks with liquid medium, where nutrients will not be limited, and certain growth parameters can heavily vary. (B) Colony transfer is similar to serial transfer but is performed on plates with solid medium. (C) A chemostat comprises a culture vessel in which the population grows under continuous agitation and aeration, where cell density and

environmental conditions can be kept constant. Fresh medium is added into the vessel at a defined rate and culture broth is harvested continuously during the process. Adapted from X.-M. Sun *et al*. (2018); Dragosits and Mattanovich (2013).

ALE has been applied under different approaches aiming to: growth rate optimization, increasing tolerance, alternative substrate utilization, increasing product yield and general discovery (Sandberg *et al*. 2019). For the specific case of microalgae, the most common stress factors used for improving their performance include nutrient, environmental, oxidative and natural selection stresses (X.-M. Sun *et al*. 2018). Only one study was found for *Phaeodactylum tricornutum*, by which it was used the combination of 25 % blue light-emitting diode (LED) light and 75 % red LED light as selection pressure, under semi-continuous cultivation for 60 days (11 cycles). The results showed that ALE is an efficient method to promote both carotenoids accumulation and growth rate in this microalgae species, since the final fucoxanthin content and final growth rate were approximately two-fold higher (Yi *et al*. 2015). This study also suggests the combined use of UV mutagenesis and ALE to achieve a promising and effective strategy for the accumulation of value-added carotenoids.

UV light has been recognized as a potent genotoxic stimulus that induces deoxyribonucleic acid (DNA) damage, promotes mutations and can even be associated with cancer in animals. UV light can be generally divided into three categories based on its spectrum: UVA (from 320 to 400 nm), UVB (from 290 to 320 nm), and UVC (<290 nm). UV induced random mutagenesis advantage relates to not being classified as a genetically modified method, since in many countries, as the ones in EU, genetically modified organisms (GMOs) may encounter regulatory hurdles (Yi *et al*. 2015). Furthermore, it can be employed without clear genetic information from microalgae species and, when compared to chemical mutagenesis methods, UV mutagenesis can be flexibly more controlled to avoid secondary contamination. Previous studies have shown UV mutagen success on microalgae species, with EPA accumulation increasing in the diatom *P. tricornutum* (Fu *et al*. 2016).

Moreover, since UV light exposure has been established as an extrinsic aging factor (Ariede *et al*. 2017), it becomes understandable the importance to further explore this treatment effect on microalgae species. In fact, microalgae adaptability mechanisms may lead to the production of relevant compounds with cosmeceutical interesting properties. Some features may include superior antioxidant properties and radical scavaging activity, which allows an enhanced protection against UV-induced cellular damage (Pangestuti, Siahaan, and Kim 2018; Thiyagarasaiyar *et al*. 2020). In this manner, the lake of knowledge about the ALE effects on diatoms, allied with the UV stimuli potential as photo-oxidative stress, emphasis the need to underline this novel approach.

1.7. Microalgae extraction process: ohmic heating focus

Production of value-added products from microalgae biomass requires the growth and recovery of the algae biomass, extraction and downstream processing of the desired products (Abdel Ghaly 2015). The extraction process aims to improve compounds of interest separation from the matrix, in order to be further analyzed and employed (Grosso *et al.* 2015). Microalgae potential and exploitation is limited and dependent on the extraction techniques applied, since these can induce a negative influence in their bioactive compounds (Kadam, Tiwari, and O'Donnell 2013; Bleakley and Hayes 2017). The considerable interest in this sustainable natural resource for adding value to extracted biofunctional compounds is increasing. Thus the search for alternative extraction strategies aiming at high yields and recovery of bioactive compounds with its interesting activity preservation is enhanced (Wijesinghe and Jeon 2012). On the other hand, the final product cost displays as a critical aspect of the process viability, with an economical perspective needed in all its stages in order to achieve a competitive commercial alternative. With the extraction phase accounting for a major expensive concern, its analyses and optimization procedure should be exalted (Kempkes 2016). Therefore, the choice of the most adequate method presents of crucial importance to the final product quality, which should exceed costumer's demands as simultaneously enhance the offer of an economically appealing process.

Traditional methods include solvent solid-liquid extraction (SLE) that is frequently coupled with heat and/or agitation in order to increase the solute's solubility and mass transfer rate. SLE is based on the extraction of soluble components from a solid or semisolid matrix using suitable solvents. It involves five sequential steps: solvent penetration into the natural matrix; compounds solubilization; solute transport out of the natural matrix; migration of the extracted solute from the external surface of the natural matrix into the bulk solution; extract separation and discharge of the natural matrix (Grosso *et al.* 2015).

Several important factors must be taken into account when performing SLE: polarity and thermolability of the compounds of interest, solvent features (toxicity, volatility, polarity, viscosity and purity), artifacts' formation and the amount of biomass to be extracted (Grosso *et al.* 2015). Therefore, regarding the solvent selection, besides extraction yield, it should be easy to recover, with a viscosity sufficiently low to ensure high mass transfer efficiency and a melting temperature below ambient to guarantee ease of handling. In addition, it is also important that the solvent is thermally and chemically

stable to be easily recycled, while it is readily available at a reasonable cost and provides a minimal environmental impact of the whole extraction procedure (Ryckebosch *et al.* 2014).

The study and potential industrial applications of algae active components may be limited due to the cell wall firmness which may be responsible for strongly reducing the extraction efficiency during classical extraction methods application (Wijesinghe and Jeon 2012). Therefore, a pre-treatment is often performed, aiming for cell disruption and higher extract recovery yields, which include mechanical (bead milling and ultrasound), thermal (steam explosion, autoclaving and freeze-drying), electromagnetic radiation (microwave) and biological (enzymatic) treatments (Abdel Ghaly 2015).

SLE technique has some limiting aspects that decrease its global performance. In fact, it is highly dependent on the target's solute solubility and polarity to choose the correct solvents, which are mainly organic compounds and can interfere, by molecular affinity, with the solute properties (Rocha *et al.* 2018). On the other hand, traditional water or organic solvent extractions can be consider as time-consuming and energy intensive processes, as well as economic expensive, with lower or inaccurate selectivity. It is also often the requirement of high solvent amounts, leading to the formation of massive residues content. Considering the growing environmental preservation and protection problematics, as well as the increasing interest in modernize conventional processes to greener and safer approaches, the development of solvent-free methodologies becomes of greater significance (Chemat *et al.* 2015; Kadam, Tiwari, and O'Donnell 2013; Rocha *et al.* 2018). The green extraction definition is based on the discovery and design of processes that will allow to minimize energy consumption, which employ alternative solvents and renewable natural sources, assuring a safety handling and a high quality of the final product (Chemat, Vian, and Cravotto 2012). In this context, the search field has boosted to achieve an alternative ecological friendly extraction, aiming at fulfilling the green extraction definition requirements. Therefore, this operation strategy will decrease the system's energy and chemicals consumption, waste production and operating time, while promotes a superior overall yield and selectivity of the extract (Rocha *et al.* 2018; Kadam, Tiwari, and O'Donnell 2013).

Several techniques have been studied as potential alternative technologies, namely microwave, ultrasound and enzyme assisted extraction, supercritical fluid extraction, extrusion, subcritical water extraction, instant controlled pressure drop, electro-technologies, among others (Rocha *et al.* 2018; Chemat *et al.* 2015; R. N. Pereira and Vicente 2010). Solvent free extraction methods offer a multiplicity of advantages, since allows the manipulation simplification and downstream processes reduction, while a higher product purity outcome and energy consumption decrease is achievable (Chemat *et al.* 2015).

The several advantages, disadvantages and reviewed applications of each technique are more detailed described in Appendix 1.

The novel and emergent extraction and bioprocess methodologies focusing in biocompounds or fractions valorization based in electro-technologies presents itself as a particularly appealing strategy. This approach, which is based on electric current application in biomaterials, provides an interesting bioprocess assistant due to its versatile character, easy scale-up, short time procedure, membrane electroporation induction and energetic efficiency (Bleakley and Hayes 2017; Rocha *et al.* 2018). It can be classified taking into consideration different parameters, including electric flow type, electric field strength, pulses application based or not and heat deposition extension. The application of an external electric field (EF) can result in electroporation by membrane electropermeabilization, being induce when the formation of a transmembrane potential overcomes a value between 0.2 and 1 V. It is important to note that the EF intensity, exposure time and medium composition are determining factors to the permeabilization type, concerning to its degree and temporary or permanent nature (Rocha *et al.* 2018).

As previously reported, EF can be divided into pulsed and non-pulsed electro-technologies. While pulsed methodology uses electric pulses, usually of high intensity voltage (from 1 to 50 kV cm^{-1}) and shorter periods of time (1-20 μs), non-pulsed techniques are based in a continuous electric current flow system for a significant period of time (Rocha *et al.* 2018; Kempkes 2016). Their variation regarding the treatment mode and deliver electrical current mechanism leads to several appealing extraction electro-technologies (Poojary *et al.* 2016), as described in Table 3.

When EF is applied to a biological system to promote or assist its biocompounds extraction it leads to heat dissipation, due to the system's semi-conductivity (Rocha *et al.* 2018). This effect is frequently referred to as Ohmic Heating (OH), also called Joule heating, electrical resistance heating or electroconductive heating, which can be defined as the internal generation of heat in a material, phenomenon consequence of its ability to resist to the electrical current passage (R. N. Pereira and Vicente 2010; Sastry 2008; Varghese *et al.* 2014). An innovative and potential extraction strategy is based on ohmic heating by moderate electric fields (MEF), since its use has been reported as an advantageously approach by offering a high-quality product with minimal chemical interference. Having in consideration the increasing demand for process implementation and development towards an improved sustainability, efficiency and environmental performance, the gathering of these methodologies can enhance their individual potential and therefore present as a promising alternative (Rocha *et al.* 2018; Sastry 2008). In fact, in order to preserve and enhance the algae bioactivity potential, the non-pulsed methodology presents of further interest, since the resulting cellular damage is expected to be lower, based on the

lesser current flow intensity applied. On the other hand, despite pulsed electric fields (PEF) remains the most referred technology in the literature for extraction purposes, the use of MEF with the corresponding OH is gaining attention for bioresources valorization. This relates to the less demanding operational conditions and easier technology implementation, when compared with the more challenging high energy pulsed technologies (Rocha *et al*. 2018).

Table 3 – Main electro-technologies and its applications previously reported. Adapted from Sastry (2008); Rocha *et al*. (2018).

Electric fields	*Electro-technologies*	*Main applications*
Pulsed	Pulsed Electric Fields (PEF)	Non-thermal inactivation of microbial cells.
		Electroporation of cell membranes.
		Thermal labile biocompounds extraction.
		Softening tissues and peeling.
	Pulsed Ohmic Heating (POH)	Biocompounds thermal extraction.
	High Voltage Electric Discharges (HVED)	Biocompounds extraction.
Non-pulsed	Non-Pulsed Ohmic Heating (OH)	Biocompounds thermal extraction.
	Moderate Electric Fields (MEF)	Non-thermal inactivation of microbial cells.
		Eukaryotic cells permeabilization.
		Improvement of biocompounds extraction.

MEFs are based on the application of low to medium electric fields (commonly between 1 and 1000 V), using a defined wave shape which is mainly sinusoidal or square, with or without heating, to biomaterials placed between two electrodes (Poojary *et al*. 2016; Rocha *et al*. 2018). OH presents as a side effect of moderate electric fields application on a semi-conductive material, seen as an internal thermal energy generation technology (Rocha *et al*. 2018; Sastry 2008; Knirsch *et al*. 2010). Although OH leads to heat production, it may present mild non-thermal cellular damage, due to the presence of the electric field. Moreover, it induces permeabilization, which allows cells' walls to develop charges and form pores (Kaur and Singh 2016; Sastry 2008; Knirsch *et al*. 2010). Furthermore, OH can promote at

least reversible electroporation which has been reported to improve extraction efficiency, mainly due to assist in intracellular compounds diffusion (Poojary *et al*. 2016).

In this manner, OH technology is easily accomplished as long as the conditions leading to significant heat deposition are assured: medium with enough conductivity and proper exposure time. OH use appeal relies on the less demanding operational conditions and associated heating features and control systems, when compared to a more challenging high energy pulsed application. Its promising future can be establish by offering an easier and economic feasible implementation and scale-up (Rocha *et al*. 2018). In addition, the joule effect fundamental of OH allows a heating process not dependent of heat transfer mechanisms, leading to an uniform and extremely fast heating (Knirsch *et al*. 2010; R. N. Pereira and Vicente 2010; Kaur and Singh 2016; Rocha *et al*. 2018). Moreover, a precise temperature control is suitable for this method, offering, as consequence, a mild process capable of preserve the biosystem nutritional, functional and structural properties, with low maintenance costs and heat losses, resulting in high energy conversion efficiencies (R. N. Pereira and Vicente 2010; Kaur and Singh 2016; Rocha *et al*. 2018).

As a final mark, it is possible to enhance the OH technology as a potential strategy to achieve a more environmentally-friendly extraction system, while simultaneously preserving the algae bioactive potential compounds. This can be assembled as a determining aspect to microalgae exploration with enhanced quality production (R. N. Pereira and Vicente 2010; Rocha *et al*. 2018).

1.8. Algae biorefinery

The biorefinery concept presents of remarkable global acceptance and interest as a sustainable biomass processing into a large spectrum of desirable products and energy. It offers a facility capable of combining the biomass conversion with equipment to produce a wide range of bio-based compounds, biofuels, chemicals and valued added products (Chemat, Vian, and Cravotto 2012; Cherubini 2010). This approach relies in the use of several technologies to take advantage of the biomass fractioning into their main building blocks in order to improve each component value while generating its own power. This allows to maximize profitability and enhances the environment preservation (Cherubini 2010; Safi *et al*. 2014). Therefore, the main bottleneck of a biorefinery approach is the separation of different fractions without compromising their potential and further exploration. This can be overcome through the use of simple, low energy consumption, cost effective and scalable extraction and separation processes (Chew *et al*. 2017).

The biorefinery approach development and application should follow an integrative perspective to minimize non-renewable energy resources consumption and negative environmental impacts, while maximize a complete and efficient biomass conversion, and, thereby, offering a better advantageous strategy implementation. An important requirement that biorefinery system should fulfill is the renewable-based, consistent and regular supply of feedstock, term applied for the raw materials use. There are four different sectors that can provide renewable carbon-based raw materials: agriculture, forestry, industries and households and aquaculture (algae species) (Cherubini 2010).

In this manner, it is understandable that algae-based biorefinery concept has as fundamental basis a more efficient algae potential exploration through its fractionation. This results in different products, whose properties and bioactivities should be preserved, while promoting a higher sustainable methodology. In order to achieve this environmental and economic sustainability and viability conduct, an optimization procedure regarding the process inputs is required (Ibañez and Cifuentes 2013). Upstream processing (USP) and downstream processing (DSP) are the main stages of microalgae biorefineries. USP is related to cultivation growth phase, while DSP regards with extraction and purification methods to obtain valuable compounds (Chew *et al.* 2017).

Therefore, algae biorefinery presents of further importance and focus for the research community due to its expected additional environmental benefits insurance by allowing a synergetic coproduction of bioenergy and high value compounds (Cherubini 2010; De Bhowmick, Sarmah, and Sen 2019). A shift towards a multiple product parallel synthesis sustained by a biorefinery model has been reported, replacing the traditional single-centric product strategy. However, despite the evidence of several studies concerning algae biorefinery are available, this ecological and integrative approach is limited applied, being enhanced as a possible research opportunity (De Bhowmick, Sarmah, and Sen 2019; Chemat, Vian, and Cravotto 2012).

2. AIMS

The expanding consumers' interest on the implementation and development of bioactive formulations for cosmeceuticals obtained by natural sources, addressing a more sustainable and ecological product strategy conception results in an interesting market opportunity. Thereby, algae species are promising as a valuable source of structurally diverse biologically and technologically active compounds and present themselves as a potential anti-aging and skin degradation solution. One important factor to consider is the greener extraction methods applied in order to follow the increasing environmental preservation concern, while a higher yield and product quality is also envisaged. With OH assisted extraction as a promising strategy, the study of its implications in microalgae biocompounds becomes of greater importance, appearing as a crucial part of this scientific work.

Hence, this thesis work has as fundamental purpose to enhance the microalgae potential exploration, by ultimately providing important information for the formulation of an algae-based cosmeceutical anti-aging product with topical application. A more ecofriendly approach offered by OH will be used, possibly allowing the decrease of solvent consumption and downstream procedures requirements and thereby enhancing product viability by global cost reduction. Furthermore, a higher selectivity and energetic efficiency of the process may be attained. Seeking a more sustainable approach, microalgae extraction will be adressed to combine the health promoting effects possibly found with the potential structuring benefits acquired. For uniformity purposes, two designations will be hereby applied: bioactivity will be considered any direct health promoting effect, while functionality will be used when reporting to technological/supportive functions.

This thesis work arises as a follow-up of the results attained under the internship period, which aimed to explore microalgae potential as a source of novel, natural-based and sustainable antioxidant agents. Briefly, three microalgae species (*Chlorella vulgaris*, *Nannochloropsis oceanica* and *Phaeodactylum tricornutum*) were cultivated in Allmicroalgae facilities and submitted to different extraction solvent systems. *P. tricornutum* standed out for the highest antioxidant power. In this manner, several specific goals were sequentially fulfilled:

1) *Phaeodactylum tricornutum* cultivation aiming to achieve two different main fractions:
    a. UV exposure as adaptive laboratory evolution tool to enhance bioactive activity;
    b. mixotrophic growth followed by nitrogen limitation for functional properties.

2) Extraction tuning considering different solvents (water and ethanol), solvent ratio and different extraction technologies (OH and concentional heating) to attain at least two different extracts from *Phaeodactylum tricornutum* biomass, with bioactive and supportive functions.
3) Extracts characterization regarding their functionality properties.
4) *In vitro* characterization of the bioactive extracts obtained through their antioxidant activity and effect on normal and cancer cell lines.

3. MATERIALS AND METHODS

3.1. Biomass material

3.1.1. *P. tricornutum* growth for enhanced rich fractions

3.1.1.1. Microalgae and culture medium

Phaeodactylum tricornutum strain used in the present work was obtained from AlgaFarm (Pataias, Portugal) culture collection. A concentrated culture medium (Sameca, Porto, Portugal) based on Guillard's f/2 medium (Guillard 1975) was used in all trials, with nitrates' final concentration adjusted to 10 mM. It was further supplemented with iron chelated with EDDHSA (25 μM), as well as with sodium silicate (0.06 mM). Moreover, magnesium water and NaCl were added to achieve a final 30 g L^{-1} salinity. All cultures were sparged continuously with air at flow rate of 3.9 L min^{-1}, through sterilized pipes to assure culture constant aeration, using a 0.2 μm Midisart® 2000 PTFE air filter to sterilize the incoming air. The same filters were placed at the air outlet. Culture pH was maintained at 8.0 throughout a negative feedback response mediated by 0.33 % CO_2 supply into the aeration system. White light was continuously provided by fluorescence lamps (100 μmol photons m^{-2} s^{-1}) at 24 °C, with the distance between the photons source and the reactors being kept constant to assure similar light intensity conditions over the experiment. For the specific case of mixotrophic growth, glycerol was added to a final concentration of 10 mM.

3.1.1.2. Growth conditions

3.1.1.2.1. Adaptive laboratory evolution and UV exposure

The UV-C exposure was selected as the mutagenic agent. The germicidal tubular UV lamp used was Philips TUV T8 and the UV Output power was 30 Watts with a peak wavelength of 253.7 nm. 250 mL of *P. tricornutum* culture in exponential growth phase were placed in a 500 mL Erlenmeyer flask under constant magnetic agitation. The cultures were then directly exposed to the UV-C lamp at a distance of 20.0 cm for 10 min. Cultures after UV radiation were immediately kept at dark conditions overnight to prevent photo reactivation, at the same conditions as before. It was further used for culture renovation for a final volume of 1 L in 1 L glass bottles fitted with a bubbling aeration system.

Seed cultures for ALE were original wild type and taken under exponential growth phase. Before ALE cycle initiation, strains were cultivated under identical assay conditions in order to get preliminary adaptation and achieve desired cell density. ALE was applied with a three-day cycle by a semi-continuous

culture system. The initial biomass at beginning of each cycle was set at approximately 0.4 g L^{-1}. The initial and final optical density at 750 nm was measured for each cycle.

Biomass collection was performed for the cultures submitted to one and three UV-C stimuli. A Fiberlite™ F14-6x250LE Fixed Angle Rotor centrifuge (Thermo Scientific™, USA) was used until the total biomass required was attained.

3.1.1.2.2. Mixotrophic cultivation

Two cultures were grown in 2 L round glass flasks under fed-batch mode. The organic carbon source was sterilized by filtration through 0.45 μm FilterBio® PES syringe filter and added to the culture medium to achieve an initial concentration of 10 mM. The fed-batch system was carried out with successive additions of glycerol, when approximately 5 mM had been reached. Biomass collection was performed for one of the cultures arbitrarily selected during the exponential phase. When stationary growth phase was achieved, glycerol addition was stopped, and biomass was collected.

Glycerol concentration was determined as previously reported by Bondioli & Della Bella (2005). 1.6 M acetic acid stock solution, 4.0 M ammonium acetate stock solution, 0.2 M acetylacetone solution and 10 mM sodium periodate solution were prepared. Glycerol standards in ethanolic 50 % (v v^{-1}) solvent were prepared to attain a range of 1.0 mM to 0.125 mM. Glycerol quantification was performed by adding and mixing 1.2 mL of a 10 mM sodium periodate solution to 2 mL of each standard solution or sample, followed by the addition of 1.2 mL of a 0.2 M acetylacetone solution. After 1 min of incubation at 70 °C under agitation in a water bath, each standard solution or sample was immediately cooled in water to room temperature and 200 μL were used to measure absorbance at 410 nm by microplate spectrophotometry.

3.1.1.3. Biomass calibration curve

In order to establish the relationship between optical density (OD) and cell dry weight, a calibration curve was required. Different concentration solutions were achieved by a serial dilution process, with deionized water as diluent. 200 μL of each standard was pipetted in triplicate into a microplate well and measured at 750 nm. DW determination was performed by filtration. A 47 mm glass fiber filter (Frisenette, Denmark, Cat. No. GA.047) was dried overnight in a T6 Heraeus drying oven (Thermo Electron Corporation, USA) at 105 °C and weighed (AE200 analytical balance, Mettler Toledo, USA); this procedure was performed in triplicate. 10 mL of sample were filtered for each replicate, with a subsequent wash made by using an equal volume of 35 g L^{-1} ammonium formate. The washed sample was finally dried overnight at 105 °C and weighed. A blank test was performed by using 10 mL of water,

followed by a wash with ammonium formate solution, in triplicate. The standard curve achieved is represented in Appendix 2.1 and was used to determine biomass concentration (g L^{-1}) along time.

3.1.1.4. Growth evaluation

Microalgae cultures were sampled for cell density determination through OD at 750 nm (Griffiths *et al*. 2011; Sandnes *et al*. 2005; Lu *et al*. 2017). Cultures were diluted as required to achieve an OD value below 1.0 in order to fall within the linear range of the measurement (Griffiths *et al*. 2011).

Cultures were monitored by microscopic observation (Axio Scope.A1, ZEISS, Germany) to confirm cell integrity, regarding several parameters: bacterial presence, cell division occurrence, cell agglomerations, cell morphology and contamination degree.

3.1.1.5. Nitrate determination

Nitrate determination was performed when required as described by Armstrong (1963), using a standard curve as presented in Appendix 2.2. 1 mL of aliquots collected aseptically were centrifuged (EBA 20 centrifuge, Hettich®, Germany) at 10000 rpm for 10 min for total cell deposition. 9.575 mL of deionized water, 300 µL of hydrochloric acid 1 M and 125 µL of supernatant were measured, allowing a dilution of 1:80. After homogenization by vortex, OD values at 220 and 275 nm were measured. The difference between the OD at 220 nm and twice that at 275 nm gave the nitrate concentration. The results were expressed in mM.

3.1.2. *P. tricornutum* commercial biomass

P. tricornutum commercial gently spray dried powder (L201980035, AlgaFarm, Portugal) and *P. tricornutum* commercial frozen paste (Lot. 201985190, AlgaFarm, Portugal), hereby designated as powder and paste respectively, were used for different extraction purposes. It is important to consider the possible microalgae composition variability due to seasonality, what can mislead the conclusions taken. To mitigate this risk and attending its lot-dependence relation, all the microalgae assays performed will be made by using the same lots.

3.2. *P. tricornutum* extraction

3.2.1. Growth conditions impact on extracts fractions

The biomass paste collected in section 3.1.1.2.1. was submitted to two sequential solvent-based extractions at 40 °C, using 4 % solid solvent for a final volume of 5 mL. Briefly, ethanol 50 % was added

to 1 g microalgae paste, followed by vortex mix until total homogenization, in triplicate. The extraction was carried out under horizontal agitation (170 rpm) and darkness conditions in a shaking water bath (SW22, JULABO GmbH, Germany). The extracts obtained were submitted to centrifugation (EBA 20 centrifuge, Hettich®, Germany) for 15 min at 10000 rpm. The supernatant was passed through a 0.45 μm Midisart® 2000 PTFE filter and adjust to 5mL, being stored at -4°C for further analysis. The remain biomass paste was re-extracted with 5 mL of ethanol 96 %, following the procedure previously described.

An identical methodology was performed for the biomass paste collected in section 3.1.1.2.2., being submitted to three sequential solvent-based extractions at 40 °C: water, ethanol 25 % and finally to ethanol 50 %. The 4 % solid solvent was kept constant.

3.2.2. Preliminary assays: solvent based extractions

To gather information regarding cosmeceutical potential application of *P. tricornutum* extracts, two different rich-fractions were aimed: one accountable for the health promoting effects and another for its supportive role. Different extraction conditions were performed accordingly, as summarize in Table 4. Bioactive compounds are expected to be extracted using ethanol 96 % (as they are mainly related with carotenoids, lipids and phenolic compounds), while more aqueous solutions are applied aiming to achieve molecules of higher molecular weight such as proteins and carbohydrates. To establish if the two processing biomass methods have any significant bioactive impact, both commercial biomasses were initially tested using 45 °C and 30 min as extraction method. Extraction conditions impact were further acknowledged for the matrix with the best bioactive performance. For functionality purposes, only commercial powder was used.

Table 4 – Conditions for the extraction of *Phaeodactylum tricornutum* exposed to two different pretreatments, using several solvents, temperatures and extraction times.

Extract purpose	*Biomass pretreatment*	*Solvent*	*Solid solvent ratio*	*Temperature*	*Extraction Time*
Functionality	*Gently spray-dried*	Water Ethanol 25 %	2 % DW	40 °C, 60 °C and 80 °C	1 h
Bioactivity	*Frozen paste*	Ethanol 96 %	4 % DW	45 °C and 65 °C	15 min and 30 min

Briefly, for each extraction, 5 mL of the tested solvent or mixture were added to *Phaeodactylum tricornutum* biomass, followed by vortex mix until total homogenization. The extraction was carried out under horizontal agitation (170 rpm) and darkness conditions in a shaking water bath (SW22, JULABO GmbH, Germany). The extracts obtained were submitted to centrifugation for 10 min at 6000 rpm, with supernatant being adjusted to 5mL and stored at -20 °C for further analysis. For the specific case of bioactive extracts, the supernatant was also passed through by a 0.45 µm FilterBio® disposable syringe filter.

3.2.3. Ohmic heating

3.2.3.1. Ohmic heating equipment

Extractions were performed in a cylindrical glass reactor, double-walled water-jacketed (1 cm of inner diameter and 10.5 cm of total length) with two inox electrodes at each edge kept 5 cm apart. A Teflon cap was used to properly cover the reactor and to prevent solvent and temperature loss. The design of reactor is described in Figure 3.

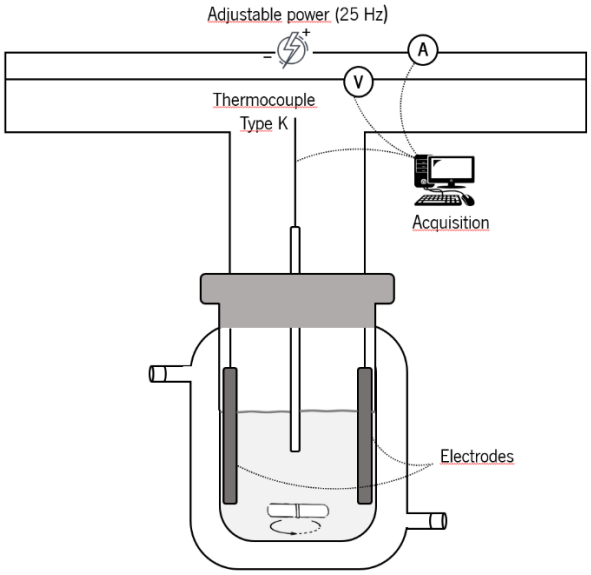

Figure 3 – Bench-scale batch ohmic heater and data acquisition system used for ohmic heating treatments.

The supplied voltage was adjusted when required to control the temperature, with the power source settled at a sinusoidal wave at 25 kHz (Protek 9205A, 0.03 Hz-3 MHz and Voltage Controlled Generator, Portugal). Temperature evolution was measured with a type-K thermocouple (± 1 °C, Omega, 709, U.S.A.), placed at the geometric center of the extractor's volume used. Temperature monitorization

was performed through a data logger (National Instruments, USB-9161, USA) working with Lab View 7 Express software (National Instruments, NI Data logger). A portable oscilloscope (ScopeMeter 125/S, Fluke, WA, USA) was used to measure nominal electric field parameters, namely, electrical frequency, voltage, and current intensity.

3.2.3.2. Conventional and ohmic heating extraction conditions

P. tricornutum commercial powder was used to achieve functional extracts. The extractions were performed distilled water and 25 % ethanol as solvents, in the reactor of 250 mL (previously described), with 2 % solids for a final volume of 100 mL, for 1 h. Two temperatures were selected: 40 °C and 80 °C.

For bioactive extracts, *P. tricornutum* commercial paste was used. Extractions were performed using 96 % ethanol in the reactor described above at 4 % solid solvent for a final volume of 100 mL. Extractions occurred over 15 min at 65 °C.

Conventional thermal experiments (0 V cm^{-1}) were carried on as negative control of the electrical effects. A thermostatic circulator water bath (1110 FrigoMix, B. Braun, Germany) was used as reactor temperature controller. Temperature profiles observed through sample heating phase, in both conventional and ohmic treatments, must be identical in order to assess the nonthermal effects of ohmic heating. A magnetic stirrer (size 2.0 cm) was used to promote solution homogenization, while improving heat transfer. Initial extracts conductivity was measured using a conductivity meter (HCO 304, VWR™, USA) at room temperature.

The extracts obtained were submitted to centrifugation for 15 min at 7000 *g* (Fiberlite™ F14-6x250LE Fixed Angle Rotor, Thermo Scientific™, USA). Afterwards, while functional extracts were stored at -20 °C for further analysis, 96 % ethanolic extracts were passed through 0.45 μm FilterBio® PES syringe filter and dried under nitrogen gas stream.

3.2.3.3. Extracts purification

Functional extracts were purified based in a precipitation method, by adding cold ethanol (-20 °C) to a final concentration of 80 % (v v^{-1}) (Xu *et al.* 2014). This purification step aimed to enhance protein and carbohydrate content (Bernaerts *et al.* 2019) and removing presence of salts. Precipitation was carried out for 2 h at -20 °C. Afterwards, the samples were centrifuged at 14000 *g* for 20 min at 4 °C. The pellet was left overnight drying at 40 °C (SI4-2-R Shaking Incubator, Sheldon, USA) and weighted for purification yield determination.

3.3. Biomass and extracts characterization

The previous work completed under the internship period was further explored through *P. tricornutum* potential analysis. When required, a blank assay was performed based on each extract diluted with the respective solvent, in order to disregard pigment color-related interferences. At least two replicates were performed for each sample.

3.3.1. Moisture and ash content

Moisture content in microalgae biomass and in each extract were determined according to the convection oven method published by National Renewable Energy Laboratory (NREL) Laboratory Analytical Procedures (LAP). Briefly, 100 mg of biomass (AE200 analytical balance, Mettler Toledo, USA) or a known extract volume were placed into crucibles and dried overnight using a T6 Heraeus drying oven (Thermo Electron Corporation, USA) at 105 °C. The crucibles were cooled to room temperature in a desiccant chamber and weighed (Laurens *et al.* 2012; Sluiter, Hames, Hyman, *et al.* 2008). The difference in weight before and after drying was expressed as a percentage, representing the biomass moisture content (Bernaerts, Gheysen, *et al.* 2018). For extracts, the difference in weight was multiplied by the conversion factor to achieve total extract weight.

Ash determination was performed by placement of approximately 100 mg of biomass in a muffle furnace (ECF 12/6, Lenton, UK) operating for 24 h at 575 °C. The crucibles were further cooled to room temperature in a desiccant chamber and weighed (Sluiter, Hames, Ruiz, *et al.* 2008a).

3.3.2. Lipid content

Biomass lipid content was determined according to Bligh and Dyer (1959) with some modifications and improved with ultrasound-assisted extraction, based on higher extraction yields attained by Araujo *et al.* (2013). For this, 2 mL of chloroform/methanol (2/1, v v^{-1}) was added to 50 mg of *P. tricornutum* powder, followed by 2 min vortex homogenization (Vortex 2, IKA, Germany) and incubation at 30°C (PURA 10 water bath, JULABO GmbH, Germany) for 25 min. After, the mixture was centrifuged (EBA 20 centrifuge, Hettich®, Germany) at 2000 rpm for 10 min to allow the organic phase collection to a pre-weighed glass tube, while biomass residue was re-extracted with 1mL solvent until the solvent had no color. All resulting organic phases were pooled together, and the total lipid content was determined gravimetrically after evaporation of organic solvent using a nitrogen atmosphere. For microalgae extracts, 1 mL of extract was dried under a nitrogen atmosphere.

Afterwards, lipid quantification was carried out by re-dissolving the dried lipidic residue (of biomass and extracts) in 3 mL chloroform/methanol (2/1, v v^{-1}), vortexed and sonicated (24 °C, 3 min). 0.75 mL milli-Q water were added, and the mixture was vortexed during 2 min, followed by phase separation by centrifugation (EBA 20 centrifuge, Hettich®, Germany) at 2000 rpm for 10 min. The organic phase was collected to a new pre-weighed tube and dried under a nitrogen stream and weighted. The lipid content in the extracts was calculated using Equation 1.

$$\text{Total lipid content (\% DW, w/w)} = \frac{\text{lipidic extract weight (g)}}{\text{biomass or total extract weight (g)}} \times 100 \qquad \textbf{Equation 1}$$

3.3.3. Protein content

In order to establish the relationship between protein colorimetric methods, such as Bradford and Lowry, and another method, namely Kjedahl, three different extractions were performed at 40 °C using water, ethanol 50 % and ethanol 96 % (Appendix 3). Briefly, the tested solvent or mixture (200 mL) was added to 8 g of *Phaeodactylum tricornutum* commercial spray dried powder followed by vortex mix until total homogenization, in duplicate. Each extraction was carried out under horizontal agitation (150 rpm) and darkness conditions in a shaking water bath (SW22, JULABO GmbH, Germany) over 1 h. The extracts obtained were submitted to centrifugation for 20 min at 4000 rpm (Fiberlite™ F14-6x250LE Fixed Angle Rotor, Thermo Scientific™, USA), the supernatant was passed through an 8 µm Whatman™ filter paper (Maidstone, UK) and the final volume was adjusted to 200 mL. 2 mL were used for dry weight determination and 1.5 mL was stored at -4 °C for further analysis.

3.3.3.1. Bradford and Lowry assays

Protein content determination was performed by Bradford (1976) and Lowry *et al.* (1951) methods optimized for microplate analyses. A bovine serum albumin (BSA) 10 % (100 mg mL^{-1} distilled water) solution was used as standard. Calibration curves were established using different BSA standard solutions between a range of 1250 to 62 µg mL^{-1}. Different solvents were screened, namely distilled water, 10 % and 50 % (v v^{-1}) ethanol and a 30 g L^{-1} solution (Appendix 2.3). Moreover, catequin influence on both methods was assessed by testing a known BSA standard solution with different catequin concentrations within a range of 0 to 180 mg mL^{-1}, aiming at analyzing the possible phenolic compounds interference with both methods (Appendix 2.4).

For Bradford analyses, 20 µL of extract or standard was added to each well plate with 200 µL of Bradford solution. After 30 min incubation in the dark, at room temperature, the plate was read at 595 nm (Synergy™HT, BioTek®, USA) after 10 s of shaking. Lowry method was performed by adding 20 µL of

extract or standard to each well plate with 200 µL of Lowry solution and 20 µL of Folin-Ciocalteu reagent. After 4 h incubation in the dark, at 42 °C and 180 rpm, the plate was read at 750 nm after 10 s of shaking. Results were expressed as BSA equivalents per 100 g of extract or biomass.

3.3.3.2. Kjeldahl method

For protein content determination by the Kjeldahl method, approximately 500 mg of biomass or dry extract were transferred to glass containers. Total nitrogen content was determined after acid digestion using a digestor™ 2508 autorack (FOSS Tecator Line, Denmark), followed by Kjeltec™ 8400 analyser (FOSS, Denmark). Protein content was estimated from total nitrogen content, multiplied by an overall conversion factor of 4.89, which is specific for the microalgae species studied (Lourenço *et al.* 2004).

3.3.4. Carbohydrate content

Determination of structural carbohydrates and acid insoluble residue in biomass was performed according to Sluiter *et al.* (2012). Briefly, 300 mg of *P. tricornutum* powder was subjected to a two-set acid hydrolysis: i) 72 % sulfuric acid at 30 °C for 1 h, under manual stirring followed by ii) dilution to 4 % sulfuric acid for 1 h at 121 °C in the autoclave. Acid insoluble residue was gravimetrically determined after vacuum filtration (Gooch crucibles porosity grade 3) and dried overnight at 105 °C. The remaining autoclaved hydrolysis solution was filtered (0.22 µm FilterBio®) and analysed by high-performance liquid chromatography (HPLC).

For extracts, the oligosaccharide analysis was based on a quantitative posthydrolysis (121 °C, 20 min, 4 % H_2SO_4), which followed the method published by NREL (Sluiter, Hames, Ruiz, *et al.* 2008b). Autoclaved extracts were filtered through 0.22 µm membranes (FilterBio® PES syringe filter) and analyzed by HPLC. The conditions used in the HPLC analysis were as follows: Refractive Index (RI) detector, Aminex HPX-87H column at 60 °C with a mobile phase of 0.005 M sulfuric acid at a flow rate of 0.6 mL min^{-1}. A standard curve comprising arabinose fucose, galactose, galacturonic acid, glucose and mannitol was used (0.2 to 1.0 g L^{-1}).

Phenol-sulfuric acid (PSA) method (Masuko *et al.* 2005) was also performed for total carbohydrates determination, which was optimized for this specific matrix. Aqueous and 10 % ethanolic solutions of known glucose concentration were used to establish calibration curve within a range of 0.025 to 1 mg mL^{-1}. Briefly, 600 µL of acid sulfuric (>95 %) and 120 µL of a 5 % phenol solution were added to 200 µL of each sample or standard solution. After, the mixture was placed in boiling water

(100 °C) for 45 min and the absorbance was read at 490 nm after 10 s of shaking. Carbohydrate content was expressed as glucose equivalents per 100 g of extract or biomass.

3.3.5. Photosynthetic pigment analyses

Photosynthetic pigment determination was performed based on microplate spectrophotometry analysis (Synergy™HT, BioTek®, USA) with 200 µL in each well plate, in triplicate. A scan spectrum from 230 to 700 nm, with a resolution of 10 nm, was digitally recorded. Furthermore, specific equations were used to quantify chlorophyll and carotenoids content for ethanolic extracts, after spectrophotometric measurements (V-560 UV/Vis Spectrophotometer, Jasco, UK) at wavelength of 470, 649 and 664 nm (Amin *et al.* 2018; Sumanta *et al.* 2014). Results were expressed in mg g^{-1} DE.

$$[\text{Chlorophyll a}] = 13.36 \times OD664 - 5.19 \times OD649 \quad \textbf{Equation 2}$$

$$[\text{Chlorophyll b}] = 27.43 \times OD\ 649 - 8.12 \times OD\ 664 \quad \textbf{Equation 3}$$

$$[\text{Total carotenoid}] = \frac{1000 \times OD470 - 2.13[\text{chla}] - 97.63[\text{chlb}]}{209} \quad \textbf{Equation 4}$$

3.3.6. Total phenolic content determination

The total phenolic content (TPC) was quantified by the Folin–Ciocalteau method optimized for 96- well plates by Teixeira-Guedes *et al.* (2019). Gallic acid was used as the reference standard compound, with the results being expressed as gallic acid equivalents (mg GAE g^{-1} DE). Standards were prepared to achieve a range of 5 to 250 mg mL^{-1} gallic acid, with ethanol 50 % as diluent. 20 µL of each standard or sample was pipetted into a microplate well. To each well, 100 µL of Folin-Ciocalteu reagent, previously diluted (1:10) in deionized water, was added, followed by 80 µL of Alkaline Working Solution (Na_2CO_3 7.5 %). After an incubation period of 30 min, at 42 °C and protected from light, measurement at 750 nm was performed.

3.3.7. Total flavonoid content determination

The total flavonoid content (TFC) was determined by the aluminum chloride colorimetric method as described by Teixeira-Guedes *et al.* (2019). Catechin was used as the reference standard compound, with results being expressed as catechin equivalents (mg CE g^{-1} DE). Catechin standard solutions were prepared to achieve a range of 10 to 250 mg mL^{-1}, using ethanol 50 % as diluent. To each of well of 96-wells plate, 24 µL of the sample or standard were added, followed by 28 µL of sodium nitrite (5 %), and 5 min later, 28 µL of aluminum chloride (10 %). The mixture was left to react for 6 min and then 120 µL

sodium hydroxide (1 M) were added. Every new reagent addition was followed by individual well mixing. Absorbance was read at 510 nm after 30 s shaking.

3.4. Functional properties analyses

Functional properties analyses were only performed for the purified extracts. At least two replicates were performed for each sample.

3.4.1. Intrinsic fluorescence analyses

Intrinsic fluorescence of extracts solutions was followed by means of soluble tryptophan/tyrosine fluorescence value at excitation wavelength of 286 nm (Moreira *et al.* 2019). Samples were prepared to a final concentration of 1 mg mL^{-1} in water, to avoid effects of light scattering, and then assayed in a spectrofluorometer (Aqualog 800, HORIBA, Japan) with a 1.0 cm path length quartz cuvette. Emission spectra were recorded between 300 and 400 nm.

3.4.2. SDS-PAGE electrophoresis

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed to determine the molecular weight (MW) distribution of proteins as underlined by Laemmli (1970). The purified extracts were dissolved in deionized water at 1 % (m v^{-1}). Samples were prepared by mixing 16 µL of 1 % purified extract solution with 4 µL of buffer (0.313 M Tris-HCl (pH 6.8), 10 % SDS, 50 % of glycerol, 10 % of 2-mercaptoethanol and 0.05 % bromophenol blue), followed by 5 min incubation at 100 °C. 15 µL of mixture were loaded into the gel (4 % acrylamide stacking gel and 12 % acrylamide separating gel). The electrophoresis was accomplished at 30 mA using a semi-dry blot apparatus (Bio-Rad) with 500 mL of running buffer (25 mM tris, 192 mM glicin and 0.1 % (w v^{-1}) SDS). After separation, the gel was stained with a protein staining reagent (BlueSafe, nytech®). The approximate molecular weights were determined using MW standards ranging from 5 to 250 kDa.

3.4.3. Thermal behavior

The thermal properties of functional samples were assessed through Differential Scanning Calorimetry (DSC) using a PerkinElmer DSC 6000 (Norwalk, CT, USA). Per sample, 3–6 mg was weighed in an aluminum pan (PerkinElmer) and sealed. The temperature program started with a 1 min hold at 0 °C, followed by a temperature ramp with a heating rate of 10 °C min^{-1} until 250 °C.

3.4.4. Rheological analyses

The rheological tests were performed using a controlled stress rheometer HR-1 (TA Instruments, New Castle, USA) fitted with a parallel plate geometry (40 mm diameter, gap 500 μm). The solution was centrifuged and placed onto the plate of the rheometer, with liquid paraffin oil being used to prevent water loss. In order to accurately determine the liquid–gel transition behavior, a temperature ramp from 20 to 90 °C at the rate of 1 °C min^{-1} was applied. The samples were equilibrated for 10 min at 90 °C, followed by frequency sweep tests in the frequency range of 0.1–100 Hz. The samples were cooled from 90 to 20 °C with the same rate of 1 °C min^{-1}. The samples were equilibrated for 10 min at 20 °C before the frequency sweep tests. Storage (G') and viscous (G'') moduli were recorded as a function of temperature at a frequency of 1.0 Hz and 1.0% strain. Preliminary strain sweep tests were made to ensure that the selected strain was within the linear visco-elastic region. The gelling and melting temperature were determined.

3.4.5. Chemical and structure analyses

Functional groups and possible chemical interactions of purified extractd samples were characterized by Fourier Transform Infrared (FTIR) Spectroscopy using an ALPHA II- Bruker spectrometer (Ettlingen, Germany) with a diamond-composite attenuated total reflectance (ATR) cell. The FTIR spectra were recorded between 400 and 4000 cm^{-1}, by acquiring 24 scans cycles per samples with 4 cm^{-1} resolution.

3.4.6. Gel permeation chromatography

The molecular weight distribution of polysaccharides was evaluated by HPLC gel permeation chromatography (GPC) on a PolySep-GFC-P Linear column (300 x 7.8 mm, Phenomenex®, USA). The purified extracts were dissolved in deionized water at 1 mg mL^{-1}. The samples were eluted with ultrapure water, using a flow rate of 0.8 mL min^{-1} at 40 °C with RI and UV detection. Linear regression calibration was performed using standard pullulan kit P-82 (Shodex™, Japan).

3.4.7. Emulsification activity

Both conventional and ohmic purified extracts were analyzed regarding their emulsification index (EI), using soybean oil as a hydrocarbon (Lovaglio *et al.* 2011; Shimizu, Saito, and Yamauchi 1985). For each extraction condition, 1 % solutions were prepared. 750 μL of soybean oil were added to 750 μL of each sample, following five minutes stirring using a vortex. The mixture was allowed to stand for 15 h. The emulsification index was measure over a 25 days period to approach the emulsifying stability provided by each extraction condition. A cloudy and pale white phase is indicative of emulsion formation

(Yamashita, Miyahara, and Sakamoto 2017). EI was calculated by dividing the height of the emulsion layer by the mixture total height and multiplying by 100 (Morales-Jiménez *et al.* 2020; Fontes *et al.* 2010).

3.5. Bioactive properties analyses

3.5.1. Antioxidant assays

3.5.1.1. Ferric reducing antioxidant power assay

The ferric reducing antioxidant power (FRAP) was determined as previously reported (Teixeira-Guedes *et al.* 2019; Benzie and Strain 1996). 300 mM acetate buffer pH 3.6 (prepared with glacial acetic acid), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM hydrochloric acid and 20 mM ferric acid were prepared. Working FRAP reagent was prepared as required by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL ferric chloride ($FeCl_3$) solution. 50 % ethanolic solutions of known Fe^{2+} concentration were used for calibration using 5mM Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) as stock solution, in a range of 0.039 mM to 1.250 mM (1.250, 0.9, 0.625, 0.313, 0.156, 0.078, 0.039 mM). 20 µL of sample and 280 µL of FRAP working solution were incubated at room temperature for 1 h, followed by microplate spectrophotometry measure (Synergy™HT, BioTek®, USA) at 593 nm. Results were expressed in µmol Trolox equivalent per g of biomass (µmol Teq g biomass^{-1}) or dry extract (µmol trolox eq.g $^{-1}$ DE ^{1}).

3.5.1.2. DPPH radical scavenging activity assay

The method used for measuring the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability of the algae extracts was carried out as in Teixeira-Guedes *et al.* (2019). Briefly, 8.87 mM DPPH solution was prepared. Trolox standards in ethanolic 50 % (v v^{-1}) solvent were prepared to attain a range of 1.250 mM to 0.039 mM. A 0.5 mL aliquot was used to achieve an absorbance of DPPH near 1.000 at 520 nm. 10 µL of each sample or standard and 190 µL of DPPH were added to each well to complete 200 µL as total volume reaction, followed by incubation at room temperature, protected from light, for 30 minutes and analysis at 520 nm. The absorbances measured at 520 nm were plotted against sample concentration, with lower absorbance values indicating higher radical scavenging potential. A reagent blank test was also performed. % inhibition was calculated as described in Equation 5. Results were expressed in µmol Trolox equivalent per g of biomass (µmol Teq g biomass^{-1}). Moreover, the sample concentration (mg mL^{-1}) required to inhibit 50% of the activity (IC_{50}) was also determined from a dose response curve using GraphPad software.

$$\% \text{ inhibition}_{sample} = \frac{100 \text{ x } (OD520_{Blank} - OD520_{Sample})}{OD520_{Blank}} \qquad \textbf{Equation 5}$$

3.5.1.3. ABTS radical scavenging activity assay

2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolorization assay for antioxidant activity determination was assessed according to Teixeira-Guedes *et al.* (2019). This assay comprises the direct production of the blue/green ABTS· chromophore through the reaction between ABTS and potassium persulfate (CAS: 7727-21-1, Sigma-Aldrich, USA). 6.88 mM ABTS concentrated solution was produced by reacting ABTS solution with potassium persulfate solution, allowing the mixture to stand protected from light at room temperature, for 12-16 h before use. ABTS·solution was diluted in acetic acid buffer (20 mM, pH 4.5) to show an absorbance of 0.70±0.02 at 734 nm. Trolox standards in ethanolic 50 % (v v^{-1}) solvent were prepared to attain a range of 1.0 mM to 0.039 mM. Extracts antioxidant capacity was assembled by adding 12 µL of each standard solution or sample to 188 µL of ABTS working solution to microplate well and reading at 734 nm, after an incubation period of 30 min., protected from light, at room temperature. All determinations were carried out three times. The percentage of inhibition indicating the ABTS radical scavenging capacity was calculated and plotted as a function of Trolox concentration for the standard reference data, following equation 5. Results were expressed in µmol Trolox equivalent per g of biomass (µmol Teq g $biomass^{-1}$). Moreover, IC_{50} (mg mL^{-1}) was also determined from a dose response curve using GraphPad software.

$$\% \text{ inhibition}_{SAMPLE} = 100 \text{ x } \frac{OD734 \text{ ABTS Blank} - OD734 \text{ sample}}{OD734 \text{ ABTS Blank}} \qquad \textbf{Equation 6}$$

3.5.2. Cell Viability

In vitro cell metabolic activity of the bioactive extracts was evaluated using different cell lines: normal mouse fibroblast (L929 ATCC® CCL-1), human lung cancer (A549 ATCC® CCL-185), human epithelial adenocarcinoma (Hela ATCC® CCL-2) and human epithelial colorectal adenocarcinoma (Caco-2 ATCC® HTB-37). The metabolic activity of each cell line was assessed by the resazurin reduction assay (Präbst *et al.* 2017). Cells were grown Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin, at 37 °C in a humidified atmosphere with 5 % CO_2. When a 70–80 % of confluence was reached, cells were trypsinized and seeded in a 96-well plate at a density of 1×10^5 cells per well. The different cell lines were incubated with supplemented DMEM and the extracts in a concentration ranging from 16 to 2000 µg mL^{-1} for 24 h. After incubation, cell viability was measured using the resazurin assay (7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium

salt). Supernatant was replaced by 200 µL culture media containing resazurin (0.5 mM in Phosphate Buffer Saline), following 2 h incubation at 37 °C. Afterwards, 150 µL of supernatant was transferred to a new 96-well microplate and the resorufin pink fluorescent was measured at 560 nm (λex) and 590 nm (λem) by a microplate reader (Cytation 3, BioTek Instruments, Inc., Winooski, VT, USA). The % cell viability was determined by correcting blank values (cell-free medium) and untreated controls (0.5 % dimethyl sulfoxide solution). Half-maximal inhibitory concentration (IC_{50}) values were inferred from a dose response curve using GraphPad software.

3.6.Statistical analyses

All experimental analyses were carried out at least in duplicate (n=2), and the data were presented as mean value ± standard deviation (Mean ± SD). The statistical analyses were performed using GraphPad Prism 8.3.0 at $p \leq 0.05$. Principal component analysis (PCA) was used to assemble differences between extracts using the software RStudio.

4. RESULTS AND DISCUSSION

This thesis' work follows the results attained under the internship period in AlgaFarm facilities, in which *Phaeodactylum tricornutum* showed a superior antioxidant ability (Appendix 4). The results are presented in line with the primary goals previously established, comprising *P. tricornutum* cultivation aiming to achieve two main fractions with enhanced bioactivity and functionality properties; *P. tricornutum* biomass pretreatment selection and extraction tuning with traditional and OH technologies to attain at least two different extracts, with bioactive and supportive functions.

4.1. Adaptive laboratory evolution and UV exposure

In order to evaluate *Phaeodactylum tricornutum* ability to adapt and endure UV exposure, a total of three 10 min-long UV stimuli were applied in a three day cycle period to the culture in exponential phase. An initial adaptation period to culture conditions was performed, in duplicate, in which OD was recorded and converted to biomass concentration using a calibration curve (Appendix 2.1). The data obtained were used to build *P. tricornutum* growth curve, as shown in Figure 4.

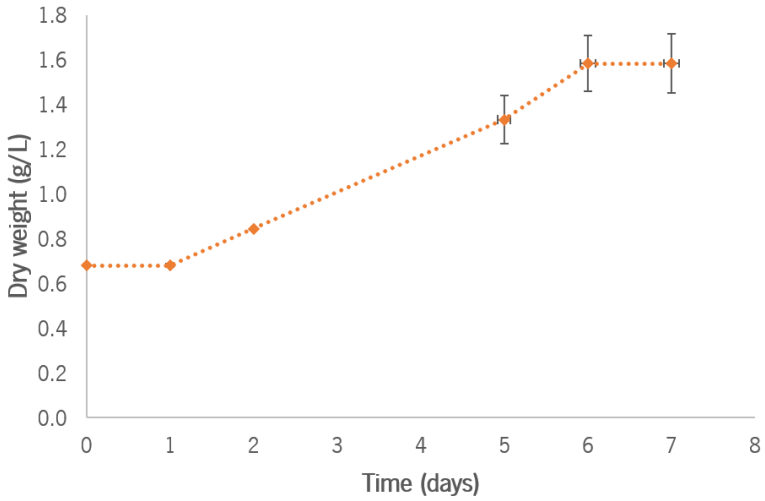

Figure 4 – *Phaeodactylum tricornutum* growth curve in 1 L photobioreactor, for a maximum of 7 days. Results are the average of a duplicated experiment. Cultures were maintained using Guillard's f/2 medium at 24 °C, under continuously white light (100 µmol photons m^{-2} s^{-1}) and aeration with 0.33% CO_2.

P. tricornutum presents a typical microorganism growth curve behavior, showing three different growth phases: lag, log and stationary. Lag phase relates to culture adaptation to new conditions (i.e. an acclimation process), during which biomass concentration is mostly kept constant. This phase lasted one

day. Cultures showed a 5-day growth period, with stationary phase being reached at day 6. After microalgae growth curve was established, it was possible to determine the respective kinetic parameters, which are summarized in Table 5.

Table 5 – Kinetic parameters of *P. tricornutum* cultures grown in 1 L reactors under batch conditions. Results are the average of a duplicated experiment, and a measurement of the dispersion of the data is provided by ploting the maximum and minimum values obtained.

	Maximum dry weight (g L^{-1})	*Volumetric productivity (g L^{-1} d^{-1})*		*Growth rate (day^{-1})*	*Doubling time (day)*
		Global	*Maximum*		
1-L batch culture	1.58±0.09	0.129±0.013	0.251±0.011	0.217±0.006	3.20±0.09

Several studies regarding *P. tricornutum* cultivation are reported in literature, but to our knowledge none of them report cultivation conditions identical to the ones presented in our work. The closest conditions relate to a study performed in 5-L glass flasks, which evidenced a growth rate of 0.131 ± 0.007 day^{-1} and a maximum volumetric productivity of 0.086 ± 0.006 g L^{-1} d^{-1}. The better growth performance achieved in our study appear to be related to scale disparities, since all the other variables were maintained constant. Several scale-up variables have been reported as important growth-determining factors, since they may have an impact on photosynthetic activity, biomass productivity, physiological and biochemical composition of microalgae (Zarrinmehr *et al.* 2020). The main factors comprise mixing type and efficiency, surface-to-volume ratio and light path and attenuation. Generally, algal productivity decreases with the increase of the volume of the bioreactor, which may be related to higher light and nutrient limitation, as well as a more inefficient mixing (Granata 2017). In fact, higher cell densities in a culture lead to a higher light attenuation due to the shading effect (Gupta, Lee, and Choi 2015) and, consequently, to a lower exposure time of the cells to the light regime, which may have a negative impact on cell growth (Granata 2017). Moreover, air lift mixing presents as a cheap and an efficient way to deliver dissolved inorganic carbon to cultures, while keeping cells mixed. However, bubbles may also cause a high attenuation of light and high shear when breaking. In this manner, the interplay between biomass homogenous mixing to optimize light levels, air mixing to enhance inorganic carbon uptake and high surface-to-volume ratio may explain the disparity of the results attained when compared to a higher scale production (Granata 2017).

Regarding UV-treated cultures, several cultivation indicators such as cell morphology, medium pH, salinity and nitrate concentration, were monitored through time, as represented in Table 6.

Table 6 – Maximum dry weight and maximum volumetric productivities of *P. tricornutum* cultures grown in 1 L reactors under batch conditions and submitted to different ultraviolet radiation stimuli. 1-UVR – After first UV stimulus; 3-UVR – After third UV stimulus.

Phaeodactylum tricornutum culture		*Maximum dry weight ($g\ L^{-1}$)*	*Maximum Volumetric productivity ($g\ L^{-1}\ d^{1}$)*
1-UVR	Control	1.214	0.250
	UV stimulated	1.184	0.261
3-UVR	Control	0.914	0.173
	UV stimulated	0.514	0.039

Generally, the results obtained in this study suggest a decrease in maximum dry weight and volumetric productivity over cultivation time, regardless of UV stimulation. This may be explained based on a higher stress on microalgae culture due to three day period renovation. In this manner, considering the weaker growth performance, a higher adaptation time might be required, enhancing the importance to adjust renovation scheme depending on culture growth evolution. On the other hand, UV treated cultures evidenced a poorer growth performance over time than control. This is in line with the conclusions presented by (Yi *et al.* 2015), in which a long UV-C exposure appears to induce serious growth suppression on diatoms cells. However, this was only reported for cultures exposed to UV-C radiation for more than 10 min. An explanation for the results obtained may rely on the output power of the lamp used, which was more than two times higher, causing a negative impact on microalgae growth identical to a longer UV-C exposure period. On the other hand, a study by Rech *et al.* (2005) reported a decrease by about 80 % in *P. tricornutum* growth rate when expose to UV, as well as its inability to acclimate when the highest UV treatment was applied, foreseen by almost a complete growth inhibition. An identical behavior was registered in this study. Therefore, time and intensity of UV-C treatment should be further explored and optimized, in order to understand its impact on *P. tricornutum* cells as a mutagenic agent, while ascertain if it is achievable the endorse of both microalgae growth and potential interesting bioproducts.

As previously underlined, *P. tricornutum* presents different morphologic conformations as a response to environmental conditions variation (Zhao *et al.* 2015; Ovide *et al.* 2018). Despite control being maintained unchanged over time, the same was not reported for the UV stimulated cultures. In fact, a microalgae phenotype change was visible after one treatment with UV-C radiation, with oval cells appearing in microscopic analysis, as evidenced in Figure 5. It has been recognized that oval cells are

enhanced under stressed conditions, while fusiform cells occur when favorable growth conditions are assured, which supports the results obtained (Zhao *et al*. 2015; Ovide *et al*. 2018).

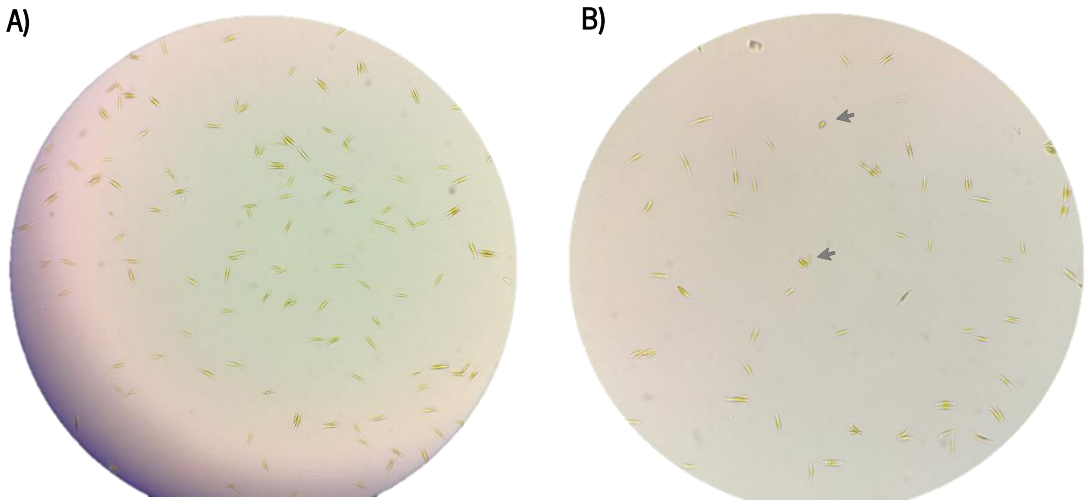

Figure 5 – Microscopic observation of *Phaeodactylum tricornutum* cultures at 60x. (A) Control; (B) Culture after one UV-C treatment of 10 min.

Molecular differences in oval morphology when compared to the other *P. tricornutum* morphotypes have been identified, such as promotion of triglyceride, nucleotide and glucuronomannan biosynthesis. As far as glucuronomannan overproduction is concerned, one hypothesis may rely in the advantage of a thicker cell wall layer to achieve a more efficient microalgae protection, as well as in the superior ability to repair cell wall damage (Ovide *et al*. 2018). In fact, glucuronomannan has been previously identified as the major polysaccharide component of *P. tricornutum* cell wall (Bernaerts, Gheysen, *et al*. 2018; Ovide *et al*. 2018). Genes encoding key enzymes involved in purines and pyrimidines biosynthesis are also overexpressed in oval cells (Ovide *et al*. 2018), which in this particular case may be a response to DNA damage caused by UV treatment. In fact, it has been previously reported that UV-C is responsible for direct DNA damage through absorption of photons by DNA bases, resulting in chemical quenching and pyrimidine dimers formation in the sequence (Sydney *et al*. 2018). Furthermore, the activation of key primary metabolism pathways that has been described in the oval morphotype such as glycolysis, pentose phosphate pathway and triglycerides biosynthesis can be related to a widely common response to stress conditions, namely energy storage through lipid accumulation (Xue *et al*. 2017; Ovide *et al*. 2018). In fact, this is identical to what has been described for the fusiform morphotype when analyzing lipid accumulation mechanisms following nitrogen deprivation (Ovide *et al*. 2018). Altogether, these metabolic adaptations may present as a great advantage to microalgae indulge UV radiation negative impact, allowing a higher cell resistance to sustain harsh growth conditions.

On the other hand, oval cells are characterized for their higher sedimentation rates (Song, Lye, and Parker 2020; Yi *et al.* 2019). This phenomenon was intensified with the increasing UV-C stimuli applied, sustaining the hypothesis of a higher density of this morphotype proportional to stress treatment intensity. However, microscopic analysis through time did not confirm this conclusion, with the fusiform morphotype remaining as the predominant culture phenotype. Thus, the UV-C treatment applied appears to not allow a general acclimation of *P. tricornutum* cells, leading to serious cellular damage and higher death rates that may explain the greater sedimentation level. In fact, Hull *et al.* (2017) reported that DNA repair of microalgae cells occurs within 24 h after UV light treatment, while Kumar *et al.* (2018) evidenced growth rate increasing after 4 days of UV radiation exposure, suggesting an adaptation in the long run. This was not possible to observe in the present study, which may relate to the high intensity of the UV treatment applied.

Further studies should investigate whether UV-C treatment influenced bacterial and fungal growth profile in the culture, since it may have had a negative impact on microalgae growth and acclimation process. Microalgae culture microenvironment has been gaining attention as an important factor of microalgae growth, with recent studies recognizing beneficial effects to it when specific bacteria strains are added to axenic cultures. Although it has not been well established how or if the microbioma interacts with microalgae cells, it is known to be a crucial aspect for the achievement of more robust algal cultures that can better withstand environmental stresses. On the other hand, besides to their more direct ecophysiological role, microbioma may also present as an important tool for environmental conditions adaptation, providing a gene reservoir for horizontal gene transfer (Lian *et al.* 2018). Therefore, a better understanding of the adaptation of both microalgae and microbial symbionts may potentially lead to a better knowledge of UV stimuli outcome on microalgae cultures.

Growth performance decrease observed along the greater amount of UV-C treatments applied go towards cell lysis induction due to UV radiation absorption by DNA, ribonucleic acid (RNA), protein and lipids, which can lead to structural damage and signaling/metabolic disorder. After UV exposure, DNA replication may be flawed if the correct repair does not take place, which may induce transcription and translation mistakes, and, consequently, result in protein synthesis with incorrect sequencing and misfolds. It has been acknowledged that DNA damage can be photorepaired after initial exposure, if the culture is placed back into natural light, through the enzyme cyclobutane pyrimidine dimer photolyase. However, microalgae cultures were left in dark conditions following irradiation stimuli in order to maximize disruption and prevent UV impact mitigation. Additionally, other DNA repair methods such as excision repair and recombination repair may occur, since they can not be prevented through dark storage (Sydney

et al. 2018). On the other hand, it has been reported the ability of *P. tricornutum* to withstand UV-A radiation, as well as it higher sensibility to UV-B radiation (Jokiel and York 1984). Altogether, despite high intensity UV radiation appears to have a negative impact on *P. tricornutum* cells' viability, it may be especially interesting for extraction purposes.

Biomass characterization regarding ash and lipidic content was achieved. The percentage of ash in biomass was identical for UV stimulated and non-stimulated cultures, accounting for 23.96 ± 0.26 % of total dry matter. This goes towards the conclusions previously reported by other authors, since medium composition was kept constant for all assays and a directly proportional correlation has been recognized between ash content and culture medium salt concentration (Quelhas *et al.*, 2019). Lipidic content was determined for all paste collected, by which it was possible to acknowledge a lower lipidic biomass content related to a higher UV-stimuli degree, as shown in Figure 6. This does not follow what has been recognized in literature, since stress conditions often lead to microalgae lipid accumulation. However, UV radiation high oxidizing power results in the formation of powerful oxidants, which leads to fatty acids peroxidation and assembles one of the main types of cell damage. Therefore, this outcome could be one of the reasons behind lipid accumulation reduction over UV increasing treatment (Guihéneuf *et al*. 2010).

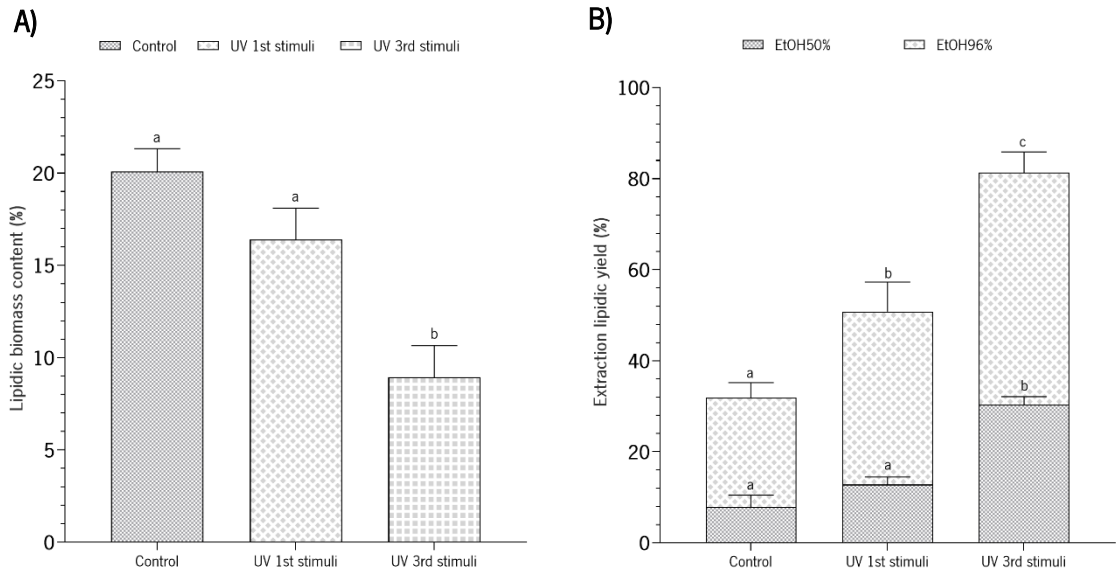

Figure 6 – Lipidic content determined on (A) *P. tricornutum* biomass and (B) ethanolic extracts. Cultures were maintained using Guillard's f/2 medium at 24 °C, under continuously white light (100 µmol photons m^{-2} s^{-1}) and 0.33 % CO_2 aeration. Different letters indicate significant differences ($p \leq 0.05$) between microalgae expose to different UV-C treatments within the same extraction solvent used.

Nevertheless, when analyzing extraction lipidic yield, a reverse behavior occurs, with the extractions performed in the culture submitted to three UV stimuli (3-UVR) allowing the recovery of more than 80% of the total lipidic biomass content. Therefore, UV stimuli might have changed *P. tricornutum* lipidic profile or induced cell wall damage, leading to a superior lipidic content extracted. In fact, a study showed that UV exposure resulted in an increase of PUFA and a reduction of saturated fatty acids in *P. tricornutum* (Liang, Beardall, and Heraud 2006). However, this effect is not uniformized in the scientific community, with numerous authors often reporting contradictory results regarding UV radiation effect on microalgae lipidic content. Results variability seem to be highly dependent on microalgal species and UV treatment (Guihéneuf *et al.* 2010). On the other hand, it has been reported that microalgae exposure to UV radiation may considerably affect their structural integrity and general biological function. UV damaging effects on nucleic acid, proteins and lipids has been previously described for microalgae, with alteration of important cell mechanisms such as motility, photo-orientation, cellular differentiation, growth, membrane permeability, productivity, pigmentation, photosynthesis, CO_2 and N_2 fixation, etc (Rastogi *et al.* 2020). Therefore, this study sustains what has been previously highlighted by Sydney *et al.* (2018), with UV treatment presenting as an useful tool for increasing microalgae lipidic extraction. This may be explained based on lipidic membrane oxidation and degradation which leads to the formation of free fatty acids (Guihéneuf *et al.* 2010).

Pigment content in all extracts was analyzed by a wavelength sweep, the results of which are presented in Figure 7. Generally, all extracts are characterized by a peak at 665 nm, which has been reported as the most accurate wavelength to determine chlorophyll-a content in ethanolic solvents (Ritchie 2018). On the other hand, the most obvious peak occurred at approximately 440 nm, which profile is different depending on the solvent used, with 96 % ethanolic extracts showing a greater pigment amount, except for 3-UVR cultures. Several studies also assigned the peak absorption of chlorophyll-a at about 440 nm (Fujiki 2002; Agberien and Örmeci 2019; Israsena Na Ayudhya *et al.* 2015). Moreover, it has been established that carotenoid content should be quantified at 470 nm, which peak does not clearly appear. However, diatoms are phylogenetically closer to brown algae than to the green lineage, leading to a different carotenoid profile by which fucoxanthin presents as the most abundant carotenoid. Chlorophyll-a and chlorophyll-c form with fucoxanthin the fucoxanthin-chlorophyll protein complex, which is involved in the light-harvesting mechanism. The visible spectrum scan of *P. tricornutum* total pigments previously presented by Manfellotto *et al.* (2020) highlighted a broad peak centered at 445 nm, identical to the one found for 96 % ethanolic extracts. This absorption peak has contributions from both fucoxanthin and Chl a (Manfellotto *et al.* 2020). The results attained evidence a greater pigment extraction when higher ethanol

percentage is used as solvent, which may be sustained based on both chlorophyll (A. S. Fernandes *et al.* 2017) and carotenoid (Y. Sun *et al.* 2016) hydrophobic nature. These results sustain the conclusions infer through a study underwent with *P. tricornutum* and different solvents for fucoxanthin extraction, where the increasing ethanol percentage in water led to higher fucoxanthin yields (S. M. Kim *et al.* 2012).

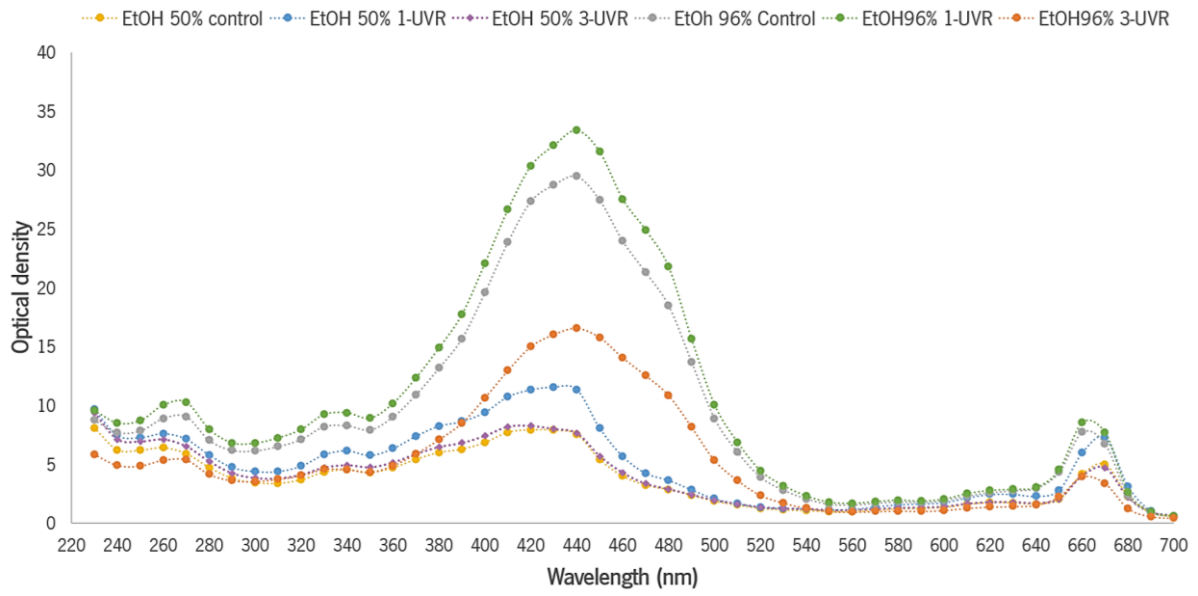

Figure 7 – Scan spectrum of the different extracts within 230 and 700 nm, with a resolution of 10 nm.

In addition, it is possible to recognize that the first UV stimulus allowed both extracts to present the higher pigment content, regardless of the ethanolic percentage used. This may be a consequent of UV exposure, since it has been acknowledged both photoprotective and light-harvesting functions to carotenoids. In fact, certain carotenoids accumulate out of thylakoid membrane of the cellular system only after exposure to specific environmental stress like UV radiation to protect cells against oxidative damage. Despite the exact carotenoids role in UV photoprotection remains controversial, they may play a crucial role in photoprotective mechanisms, either directly by quenching chlorophyll triplet states and scavenging ROS or indirectly by thermal dissipation of excess light energy (Rastogi *et al.* 2020). Moreover, increasing evidence highlights photoreceptors key role in regulation of the dynamic responses of microalgae photosynthetic system, as well as molecular cross-linking between photoreceptors and chloroplast-mediated signaling (Jaubert *et al.* 2017). It has been also underlined photoreceptors major importance for diatoms photoacclimation (Schellenberger Costa *et al.* 2013). In this manner, it becomes understandable that one of the first acclimation responses to UV radiation exposure relay on photoreceptors adaptation. A study previously performed by Yi *et al.* (2015) showed that UV radiation enhanced fucoxanthin accumulation in most selected mutants, despite the decrease of β-carotene and chlorophyll *a* contents. This may also have occurred, allowing to explain the higher difference observed

at 445 nm when comparing 1-UVR cultures with control samples. In fact, fucoxanthin plays an essential role in managing photo-oxidative stress and could be directly induced with light stress treatment, since its up-regulation might offer a greater protection against photo-damaging effects. Therefore, further studies should consider analyze fucoxanthin content in order to access if the UV-C treatment leads to its enhancement (Yi *et al.* 2015).

Despite the first UV-C treatment resulted in higher pigment content extracted, 3-UVR cultures showed a significant lower amount. This may be related to the viability loss caused by the long exposure to UV radiation. It is known that *P. tricornutum* does not contain UV radiation absorbing compounds such as mycosporine-like amino acids (MAAs), which might not have decreased the negative UV radiaton impact (Halac, García-Mendoza, and Banaszak 2009). Hence, UV stimulation appears to entail higher pigment content after only one UV treatment, but UV exposure in the long-term resulted in serious cell damage. Thus, in order to establish if the total pigments content reduction after the third UV treatment is related to the loss of viable cells, it should be quantified in the respective biomass.

A PCA was performed to establish extracts different composition based on solvent extraction used and number of UV-C stimuli applied (Figure 8). The first two principal components (PC1 and PC2) accounted for 97.3 % of the total variance, with 87.3 % and 10 %, respectively.

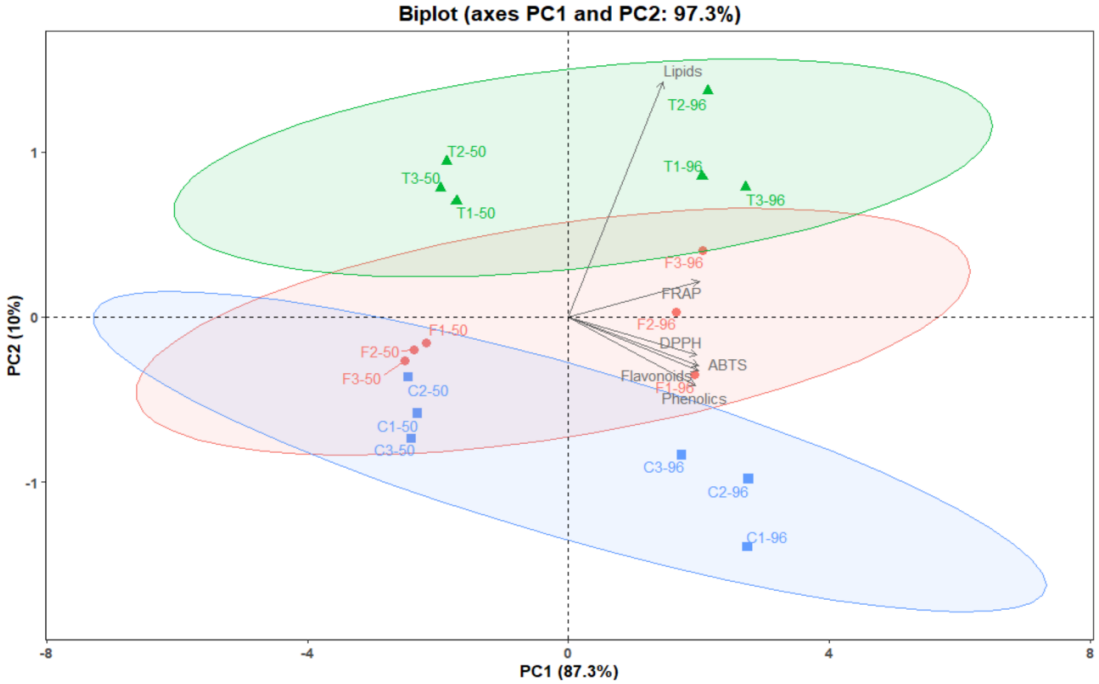

Figure 8 – Principle component analysis (PCA) on the ethanolic 50 % or 96 % extracts obtained from *P. tricornutum* cultivation, with cluster segregation assemble by the number of UV stimuli applied: C – control culture; F – culture after the first UV treatment; T – culture after the third UV treatment.

Generally, PCA analysis demonstrated a good discrimination among samples. PC1 component allowed sample distribution according to ethanol percentage used as solvent, while the number of UV stimuli was responsible for the variance in PC2. This highlights solvent importance to selectively extract different biocoumpounds. The highest antioxidant activity achieved, as well as the upper phenolic, flavonoid and lipidic content occurred for the 96 % extracts. This follows the results reported by Rodriguez-Garcia and Guil-Guerrero (2008), with ethanol being acknowledged as the most effective solvent for antioxidant compounds extraction yield maximization. To what UV treatment concerns, it is possible to recognize that extracts' profiles change throughout the number of stimuli applied. Higher UV radiation exposure resulted in richer lipidic extracts, as previously underlined, as well as a higher ferric to ferrous ion reduction ability. Concurrently, a decrease in phenolic and flavonoid content was observed, along with radical scavenging reduction. This suggests an adjustment of the antioxidant activity spectrum. UV-C radiation might have actively induced oxidative stress defensive responses, such as synthesis of astaxanthin, docosahexaenoic acid (DHA) and antioxidant enzymes as superoxide dismutase (SOD), glutathione reductase (GR), and ascorbate peroxidase (APX) (Yi *et al*. 2015). However, phenolics and flavonoids reduction does not go towards what was expected, since several studies have reported a significant increase of those two components for different algae species when exposed to UV radiation, being recognized as a defense mechanism against light-stress conditions (Cruces *et al*. 2018; Al-Rashed *et al*. 2016; Cotas *et al*. 2020). This might have occurred due to the higher cellular damage induced by UV-C stress performed in this study. On the other hand, a study using *Scenedesmus quadricauda* evidenced that neither total soluble phenols or flavonoids were influenced by UV-A or by UV-C exposure (Kováčik, Klejdus, and Bačkor 2010), which might suggest that this response is species-dependent. Thus, despite UV-C approach has been identified as potentially useful in developing strains with higher antioxidant systems protection (Yi *et al*. 2015), it was not possible to sustain this claim in the present study.

4.2. *Phaeodactylum tricornutum* mixotrophic growth

P. tricornutum mixotrophic cultivation was not possible to accomplish, due to microorganisms contamination. This may be related to the non-axenic conditions of the inoculum used, which microbiome showed a superior glycerol consumption and subsequent growth ability, ultimately enabling diatom to subsist. A multiplicity of studies report *P. tricornutum* ability to grow under a mixotrophic regime, with all specifying the aseptic cultivation conditions (García *et al*. 2000; Cerón-García *et al*. 2013; García *et al*. 2005; Villanova *et al*. 2017). Limited research has been conducted on non-axenic algae cultures grown

under mixotrophic conditions, with microbial contamination impact being poorly understood (Higgins and VanderGheynst 2014). However, several studies report algae-bacteria symbiosis, either by bacteria secretion of hormones and co-factors with growth-promoting effects, or by vitamins synthesis (de-Bashan, Antoun, and Bashan 2008; Higgins and VanderGheynst 2014; Bajguz and Piotrowska-Niczyporuk 2013). A study underlining *Chlorella minutissima* co-cultivation with *Escherichia coli* showed that co-cultures achieved higher cellular densities and growth rates, while enhanced lipid and starch productivity (Higgins and VanderGheynst 2014). Although microorganisms co-presence with *P. tricornutum* cells showed resource competition with negative impact on microalgae survival rate, further studies should aim to assess possible microalgae-microbiome interactions. In fact, in light of mixotrophic growth challenges regarding organic carbon supplementation, a commitment between organic-rich wastewaters and algae cultivation may be of potential interesting to explore (Higgins and VanderGheynst 2014).

4.3. Biomass pretreatment selection

Pretreatment and microalgae cell wall interactions determine their compositional and structural changes (Singh *et al.* 2015), which ultimately impact the extent of their bioactive and functional properties. Moreover, compounds susceptibility to endure physical or chemical processes may vary, resulting in different modification profiles. In order to fully perceive the biomass *P. tricornutum* potential applications, the selection of the more adequate pretreatment for the purpose aimed becomes of major importance. Biochemical composition of *P. tricornutum* commercial dry powder and paste can be found in Fig. 9.

Significant differences were observed between all molecule fractions, except for lipidic content. Higher protein and carbohydrate content was registered for commercial dry powder, which may likely result in superior mechanical properties due to its important contribution for foaming, emulsifying and gelling properties (Tako 2015; Zayas 1997; Burgos-Díaz *et al.* 2016). As a result, the following analysis aiming to enhance and characterize extracts functional application will only be performed using this biomass matrix. Moreover, when compared to reported biochemical characterization for typical *P. tricornutum* photoautotropic growth, namely 34.8-39.6 % of crude protein, 16.8-26.1 % carbohydrate and 16.1-18.2 % lipid (Barkia, Saari, and Manning 2019; S. M. Kim *et al.* 2012; Tibbetts, Milley, and Lall 2015), an identical compounds distribution was observed. The minor disparities and the wide range of values found in literature are common and may be related to growth conditions, strain specifications and differences in the quantification methods. The drying process may induce higher cellular damage than biomass paste collection, ultimately resulting in weaker antioxidant performance due to compounds

negative impact. However, this could not be foreseen by the results attained, requiring further evaluation assays.

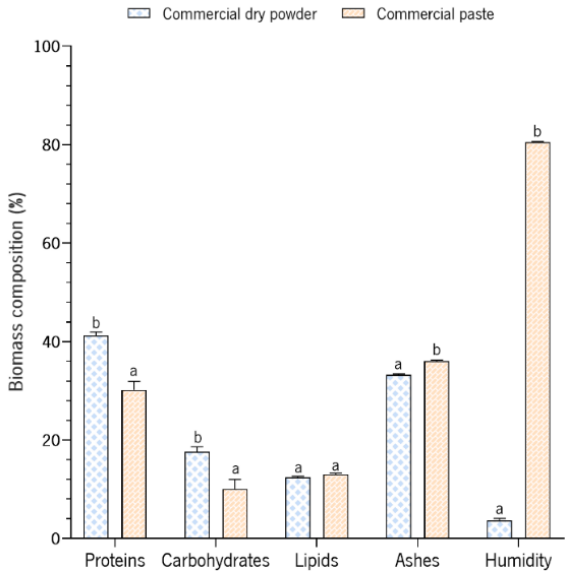

Figure 9 – *Phaeodactylum tricornutum* biochemical composition on a dry weight basis when exposed to different pretreatments, resulting in gently dry spray powder and frozen paste. Different letters indicate significant differences ($p \leq 0.05$) between pretreatments, within the same compound fraction.

4.4. Functional extracts

Microalgae generally contain large amounts of structural biopolymers which might possibly display interesting technological functionalities. In order to explore *P. tricornutum* potential role as texturizer, stabilizer, or emulsifier, different extraction conditions were evaluated. The use of specific solvents is responsible for the selectivity of the extract composition, determining intracellular compounds dissolution of the raw material. The solvent selection, namely water and ethanol, relied on their natural, environmentally friendly, nontoxic and food grade character (Ferreira-Santos *et al.* 2020). Moreover, water has been commonly applied in the extraction of polysaccharides (Zhang *et al.* 2010; W. Chen *et al.* 2012), and many proteins (Kazir *et al.* 2019; Phong *et al.* 2016), while water and ethanol mixture allow to explore different optima of extractability of compounds with different polarities (Getachew, Jacobsen, and Holdt 2020). Preliminary assays allowed to distinguish the solvent and temperature impact on protein and polysaccharide extract content (Table 7). The highest protein fraction was achieved using water at 80 °C, while the best condition to enhance polysaccharide content was 80 °C ethanolic extraction. The following work did not comprise 60 °C extractions, since it showed the lowest global extraction yield to what concerns to protein and polysaccharide fractions, regardless of the solvent applied.

Table 7 – Preliminary assays results to establish extraction conditions impact on protein and polysaccharide content on the extracts attained. Extractions were carried out under 170 rpm agitation with 2 % as solid solvent ratio.

Extraction solvent	Extraction temperature (°C)	Extraction yield (%)	Protein content (%)	Carbohydrate content (%)	Total (%)
Water	40	42.8 ± 4.0	14.4 ± 1.8	16.9 ± 0.3	32.5 ± 1.5
	60	44.4 ± 1.0	14.3 ± 0.2	14.1 ± 0.5	29.3 ± 1.3
	80	41.6 ± 2.1	17.2 ± 1.6	13.9 ± 1.0	31.7 ± 1.3
Ethanol 25 %	40	32.4 ± 2.9	12.4 ± 0.6	10.8 ± 1.9	21.4 ± 1.4
	60	38.5 ± 2.4	9.29 ± 0.07	8.70 ± 0.16	17.7 ± 0.2
	80	41.4 ± 1.1	10.8 ± 0.7	21.8 ± 1.2	32.7 ± 1.9

Extracts yield can be found in Fig. 10. Significant higher extraction yields were reported when higher temperatures were applied, showing similar values between different solvents. This may be related to the widely known positive temperature effect on reaction kinetics, resulting in an increased molecules movement and solubilization promotion.

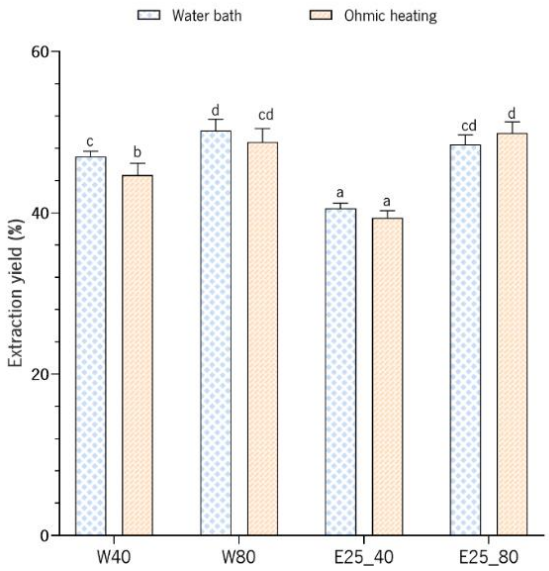

Figure 10 – *Phaeodactylum tricornutum* global extraction yield when exposed to different extraction conditions: temperatures of 40 and 80 °C by control treatments (without the presence of OH) and OH at 25 Hz, using water (W) and ethanol 25% (E). Different letters indicate significant differences ($p \leq 0.05$) between extraction conditions.

Moreover, under high temperatures cell walls are weakened, causing easier extractability of the compounds into the solvent. Sometimes, higher temperature exposure may result in the extraction yield decreasing, due to chemical modification of the compounds related to oxidation or degradation processes (Che Sulaiman *et al*. 2017). This phenomenon was not observed for the tested temperatures.

The extracts present as a mixture of proteins and polysaccharides (Fig. 11A), which might enhance techno-functionality features (especially solubility and emulsifying properties), and are consistent with the results reported in literature (Ebert *et al*. 2019). Protein solubility is influenced by several intrinsic factors as molecular weight, amino acid composition, and α-helix propensity, as well as by extrinsic factors such as pH, ionic strength, and temperature (Grossmann, Hinrichs, and Weiss 2019). Total polysaccharide content was identical for all extraction conditions, despite it has been previously acknowledged a higher polysaccharide solubility in water than in ethanol mixtures (Bouchard, Hofland, and Witkamp 2007). This may be related to composition and structure variation, explaining the different water-solubility properties observed (Gügi *et al*. 2015). As a result, distinctive protein-polysaccharides interactions might have occurred.

In addition, temperature impacts the extent of hydrogen bonding and hydrophobic interaction in protein-polysaccharide stabilization, as well as in protein conformation, which may affect technological properties (K. and Bandyopadhyay 2012). Moreover, hydrophilicity protein variation may justify the results attained, since a highly diverse protein profile has been recognized for *P. tricornutum* cells (Grossmann, Hinrichs, and Weiss 2019). Higher temperature treatment when using ethanol allowed a higher protein extractability, suggesting a higher exposure of hydrophobic groups to the solvent. It has been well recognized temperature major impact on protein conformation and folding (Lapidus 2017). Thus, thermal denaturation may explain the higher protein solubility in less polar solvents, namely ethanol. Taking into account protein and carbohydrate fractions extracted from dry matter (Fig. 11B), it is possible to highlight the positive outcome of electric fields on protein solubility for 40 °C aqueous extraction. This suggests that OH may lead to protein conformation variation in some extent. The major extracts' fraction, representing more than 50 %, refer to minerals. The high ash content in microalgae is consistent with other findings, and relate to mineral important role in DNA synthesis, enzymatic reactions and counterbalance of osmotic pressure (Ebert *et al*. 2019).

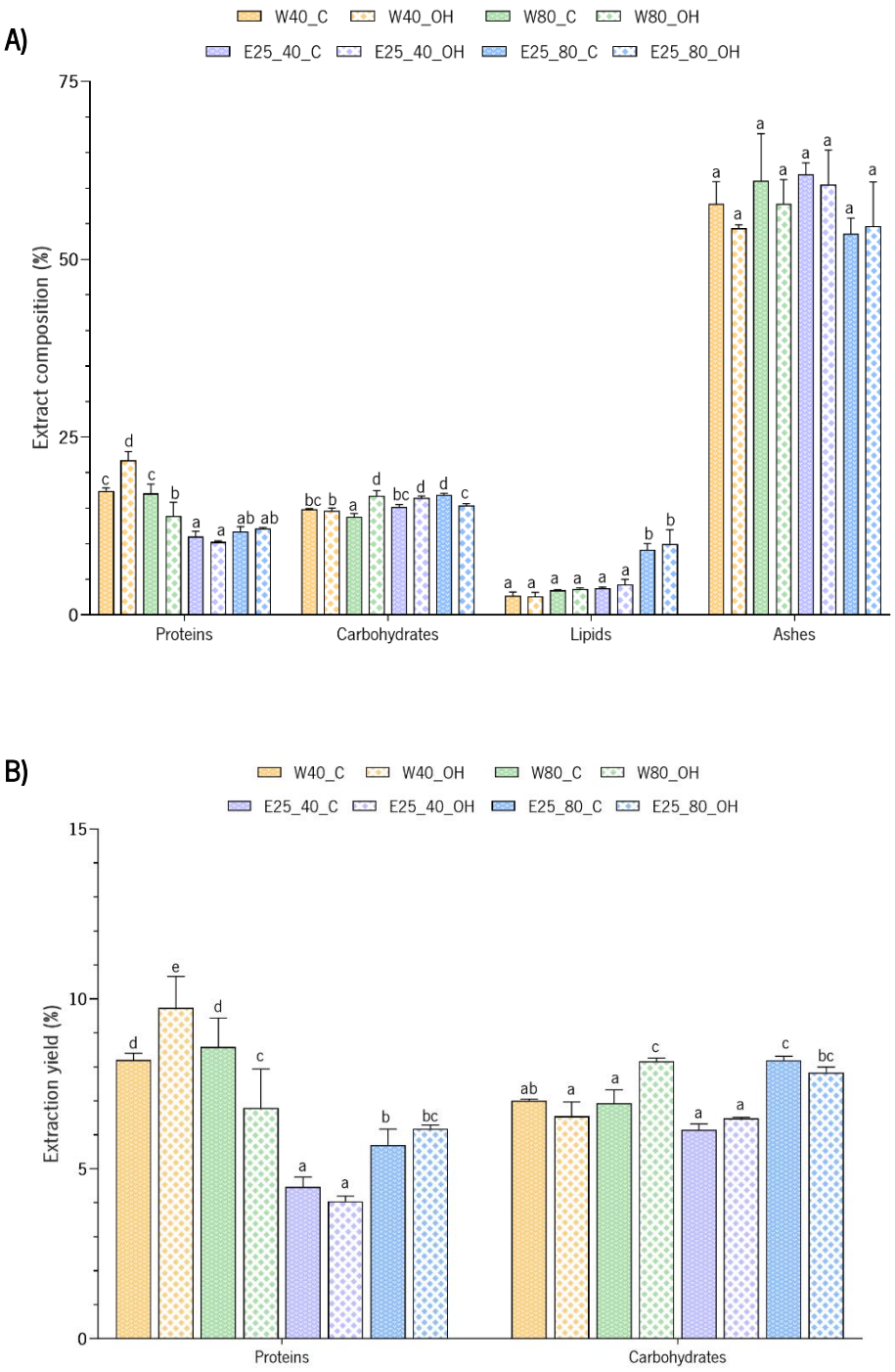

Figure 11 – *Phaeodactylum tricornutum* (A) extracts composition and (B) extraction yield on dry weight microalgae basis. Different extraction conditions were performed: temperatures of 40 and 80 °C by control treatments (without the presence of OH) and OH at 25 Hz, using water (W) and ethanol 25% (E). Different letters indicate significant differences ($p \leq 0.05$) between extraction conditions, within the same molecule fraction.

Ethanol precipitation was performed as a purification strategy, to enhance polysaccharide and protein fractions technological performance, facilitate analyses and partially remove salts and other contaminants. The precipitation yield achieved (Fig. 12) evidences significant differences between temperature treatments, with 80 °C extractions appearing to allow the attainment of molecules with superior molecular weight. In fact, molecular size has been recognized as a determine factor in precipitation yield, with the smaller polysaccharides not being able to be purified extract using 80 % ethanol. Moreover, polysaccharides with different structural features, even though present similar molecular weights, exhibit significantly different precipitation behaviors (Xu *et al.* 2014).

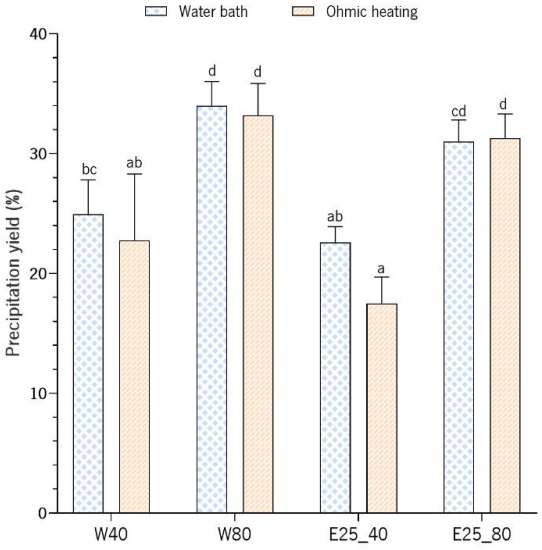

Figure 12 – *Phaeodactylum tricornutum* precipitation yield when exposed to different extraction conditions: temperatures of 40 and 80 °C by control treatments (without the presence of OH) and OH at 25 Hz, using water (W) and ethanol 25 % (E). Precipitation was performed by adding cold ethanol to a final concentration of 80 % (v v^{-1}). Different letters indicate significant differences ($p \leq 0.05$) between extraction conditions.

Purified extracts proximate composition was determined (Fig. 13) in order to acknowledge precipitation effectiveness to fulfill its purpose. It is possible to recognized different precipitation profiles depending on the extraction condition applied. This may be due to the variability of the compounds extracted (different extracts composition), to their solubility and/or to structure changes in the same polymers. Generally, ethanolic precipitation enhanced both protein and polysaccharide content, while salt decreased. This is in line with the separation of polysaccharides from lower molecular weight ethanol-soluble compounds (A. Alves, Sousa, and Reis 2013), with protein co-precipitation (Bernaerts *et al.* 2019). Mineral presence may be potential interesting in a nutritional point of view, since *P. tricornutum* has been

recognized to possess high amounts of calcium and iron. Given that iron deficiency is considered as the most prevalent single nutritional deficiency in the world, *P. tricornutum* by-products may arise as an important component in human diet (Bernaerts, Gheysen, *et al.* 2018). Moreover, the mineral presence might have an impact on physicochemical properties of polysaccharide suspensions, as specific cations play an important role in the stabilization of electrostatic repulsions and in the gelation of negatively charged polysaccharides (Bernaerts, Kyomugasho, *et al.* 2018).

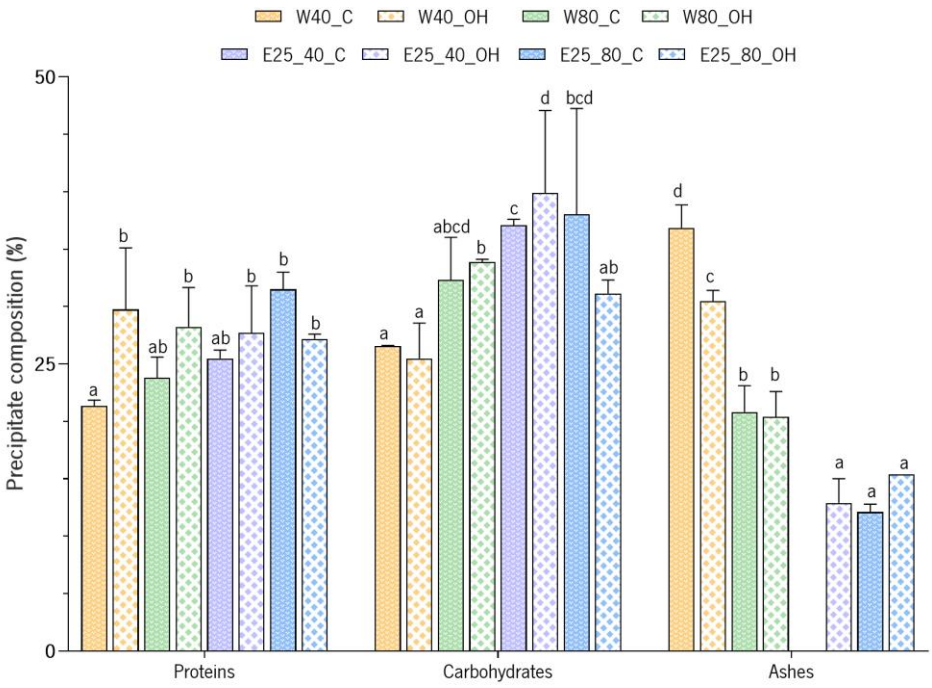

Figure 13 – *Phaeodactylum tricornutum* purified extracts composition when exposed to different extraction conditions: temperatures of 40 and 80 °C by control treatments (without the presence of OH) and OH at 25 Hz, using water (W) and ethanol 25 % (E). Different letters indicate significant differences ($p \leq 0.05$) between extraction conditions, within the same molecule fraction.

The fluorescence emission spectra of proteins present in the several purified extracts were analyzed (Fig. 14), since it may reveal protein conformation and solvent-exposure variation. The maximum of the fluorescence emission spectra (λ_{max}) of many fluorophores are sensitive to the polarity of their surrounding environment. A shift in emission spectrum to shorter wavelengths (blue shift) occurs as the solvent polarity is decreased (Lakowicz 1983; Duy and Fitter 2006). The emission of tyrosine (Tyr) in water occurs near 303 nm and is relatively insensitive to solvent polarity, while the emission maximum of tryptophan (Trp) appears at 350 nm and is highly dependent upon details of its surrounding (Lakowicz 2006).

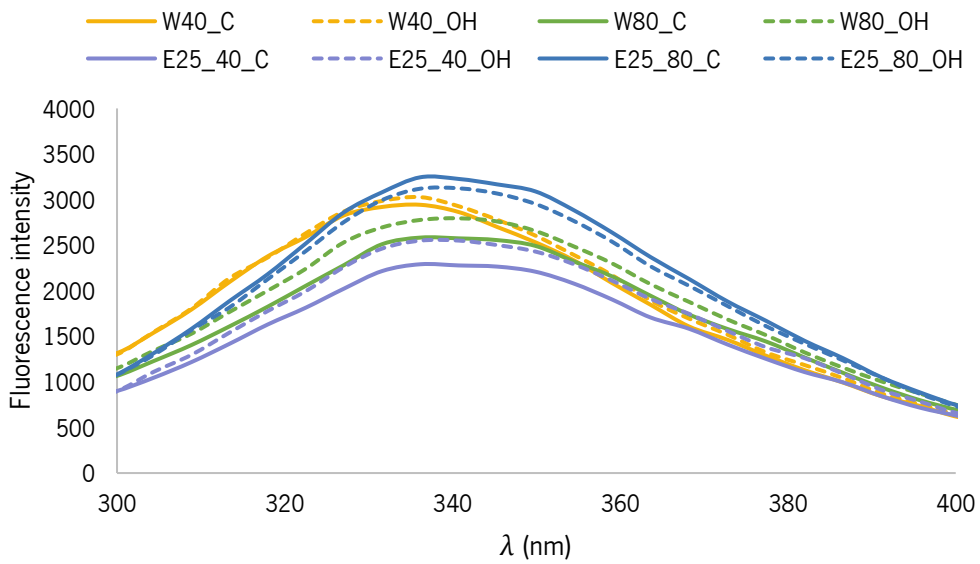

Figure 14 – Fluorescence emission spectra (excitation at 286 nm) of *P. tricornutum* purified extracts when exposed to different extraction conditions: temperatures of 40 and 80 °C by control treatments (without the presence of OH) and OH at 25 Hz, using water and ethanol 25 %.

Exposure to more aqueous solvents did not result in a blue shift, possible due to the polarity similarity of the solvents analyzed. Generally, all purified extracts evidenced a flatted emission peak around 330-350 nm, while a more well-defined peak at 336 nm is observed for 40 °C treatment in aqueous solvent. No differences between OH treatment and conventional extraction were detected. The variation in the emission spectrum shape suggests the presence of heterogeneity, which is a result of emission from different fluorophores (e.g., Tyr and Trp) or from the same fluorophore in different environments (e.g., buried and solvent-exposed Trp residues) (J. Chen *et al.* 2008). Moreover, changes in the tryptophan emission spectra often arise in response to conformational transitions, subunit association, substrate binding, or denaturation (Lakowicz 2006). λ_{max} is a valuable parameter to characterize the unfolded state properties (Duy and Fitter 2006). The red shift observed for 80 °C treatment in aqueous extracts, as well when ethanol 25 % was the selected solvent, may be explained based on denatured and structurally unfolded states. In fact, unfolded protein conformations display higher solvent-exposure of Trp residues, resulting almost often in red shifts (Ghisaidoobe and Chung 2014; Eftink 1994). Depending on the kind of denaturing conditions, the ensemble of unfolded states possess diverse structural and conformational properties (Duy and Fitter 2006), which is reflected in spectra differences. The emission response observed for aqueous 40 °C extraction suggest that the residues are shielded from water to some extent in the native protein structure (Lakowicz 2006). Furthermore, the fluorescence decrease observed must be the result of an increased quenching from the

neighbor amino acids due to small conformation rearrangements or higher structural dynamics. Concurrently, exposure to 80 °C using ethanolic mixture resulted in higher Trp fluorescence with a broader spectrum. These results suggest the presence of substantial structural changes between different extraction treatments (Rodrigues *et al.* 2020). It has been reported that mixtures of folded and unfolded would produce broader spectral widths (Moon and Fleming 2011). On the other hand, fluorescence intensity disparities may be correlated to protein concentration differences. In fact, purified extract analyses previously underlined follows a similar profile to the results attained. In this manner, a combined effect among the aspects highlighted may be occurring.

The electrophoretic profile of the proteins present in the several purified extracts are represented in Fig. 15. Generally, five clear bands of different molecular weight are visible, regardless of the extraction conditions: one <20 kDa, two around 30 kDa and other two near 40 kDa.

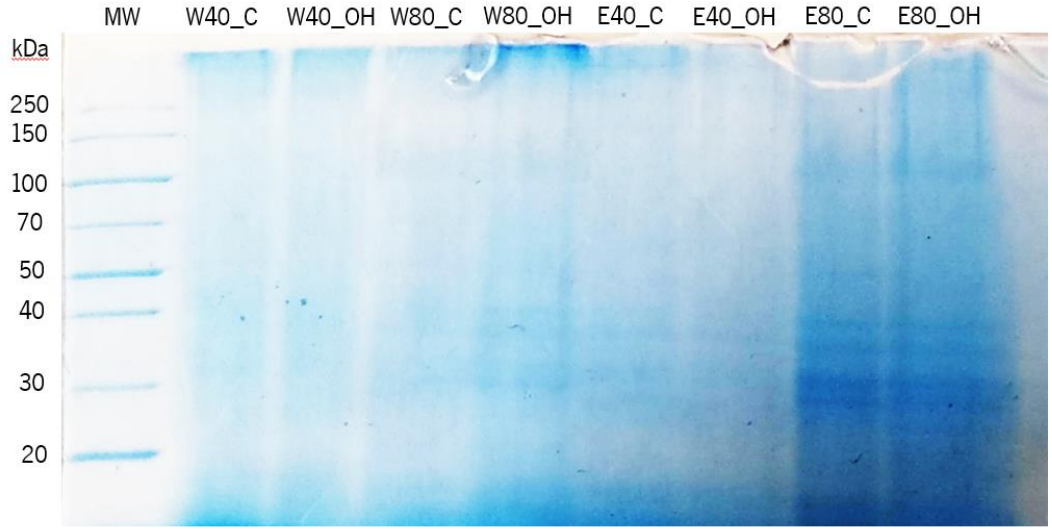

Figure 15 – Gel electrophoresis of proteins present in the *P. tricornutum* purified extracts when expose to different extraction procedures to establish molecular weight distribution: temperatures of 40 and 80 °C by control treatments (without the presence of OH) and OH at 25 Hz, using water (W) and ethanol 25 % (E).

The very intense area of staining at <20 kDa may be explained based on diatom light harvest complexes presence. In fact, fucoxanthin-chlorophyll proteins have molecular mass values ranging from 17 to 23 kDa (Stack *et al.* 2018). A band near 100 kDa is also present for 80 °C treatments, which may indicate protein denaturation and aggregation or interaction with polysaccharides in the matrix, affecting its molecular weight. This may have a direct impact on proteins functionality that should be considered depending on the intended application (Rodríguez-Restrepo *et al.* 2020). Moreover, the stronger smearing effect and fuzzy bands observed for aqueous extraction conditions may be due to salt interference (Dunbar

1987), since these purified extracts show superior ash content. The bands are more intense for 80 °C ethanolic purified extracts, suggesting a higher protein content (in accordance with the results described above for composition and fluorescence).

Monosaccharide profile of purified extracts were observed by HPLC-RI (Table 8). *P. tricornutum* purified extracts show differences depending on solvent and temperature exposure, with no major impact of OH treatment.

Table 8 – Carbohydrate quantification in *P. tricornutum* purified extracts performed by HPLC-RI. Values are the mean ± SD. Different extraction conditions were performed: temperatures of 40 and 80 °C, using water (W) and ethanol 25 % (E). Glu – glucose; Xil – xilose; Man – mannose; Gal – galactose; Fuc – fucose. Different letters indicate significant differences ($p \leq 0.05$) between extraction conditions, within the same fraction.

Extraction conditions	Acids	Monosaccharides composition (%)			Total carbohydrate (%)
		Glu	Xil/Man/Gal	Fuc	
W40_C	10.50 ± 0.25d	3.86 ± 0.88a	3.07± 0.69a	1.93 ± 0.75a	19.4± 2.6de
W40_OH	10.48 ± 0.16d	3.95 ± 0.69a	2.85 ± 0.55a	1.34 ± 0.01a	18.6 ± 1.1cde
W80_C	6.42 ± 0.06c	4.85 ± 0.78a	3.11 ± 0.39a	2.11 ± 0.43a	16.5 ± 1.5bd
W80_OH	6.41 ± 0.27c	4.11 ± 0.95a	2.76 ± 0.36a	1.99 ± 0.08a	15.3 ± 1.1ab
E40_C	2.62 ± 0.29a	6.29 ± 0.76ab	3.96 ± 0.36a	2.59 ± 0.13a	15.5 ± 1.5abc
E40_OH	3.48 ± 0.09a	8.69 ± 2.06b	5.36 ± 0.72a	3.17 ± 0.17a	20.7 ± 3.0e
E80_C	3.66 ± 0.18a	5.68 ± 0.11ab	4.33 ± 0.18a	2.92 ± 0.28a	16.6 ± 0.5b
E80_OH	2.36 ± 0.21a	4.6 ± 1.2a	3.49 ± 0.51a	2.44 ± 0.19a	12.9 ± 1.7a

Despite information about the original polysaccharides structure not being conceivable from this analysis, the individual monosaccharides recognition can give some indication of the origin polymer. As follows, acids major contribution in aqueous extraction may refer to glucuronic acid presence, which adds for approximately 4 % of the total biomass and may be derived from an alginate-like polysaccharide (Templeton *et al.* 2012). In fact, this suggests the extraction of the *P. tricornutum* cell wall prominent polysaccharide, namely, a sulfated glucuronomannan (Bernaerts *et al.* 2019). The fewer acid content on 80 °C aqueous extraction possible means that some hydrolysis took placed, which may counteract its mechanical properties. For ethanolic extractions, a different profile is registered, with the main fractions reporting to glucose and xylose/mannose/galactose. Mannose has been previously recognized as the

major monosaccharide in *P. tricornutum* hydrolysate, being derived from the structural mannan in the cell wall (Bernaerts *et al.* 2019; Templeton *et al.* 2012). The fucose content on these purified extracts is likely due to a fucoidan-like polysaccharide. Galactose, mannose and glucose presence is consistent with phylogenetic reports for diatoms (Templeton *et al.* 2012). In fact, the high glucose content may be related to the presence of chrysolaminarin, which has been recognized as a typical reserve carbohydrate in diatoms and comprises glucose monomers linked by a β-1,3 bond with limited β-1,6 branching (Caballero et al., 2016). Therefore, the disparity of monosaccharides profile obtained between solvents, suggest the extraction of different polysaccharides in different proportions, as well as corroborate solvent impact on polymer extraction.

A typical thermogram achieved for DSC is shown in Fig. 16. Four main endothermic peaks were detected in the heating cycle performed: one around 130°C, another around 140 °C, the biggest one near 160 °C and a last one at approximately 200 °C.

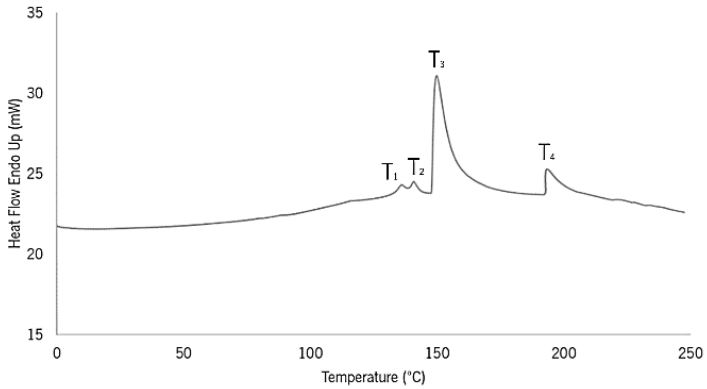

Figure 16 – Typical DSC thermogram for *P. tricornutum* purified extracts during heating cycle.

The first peak is usually assigned to residual water evaporation present in the sample (Rocha *et al.* 2019). The latest peak is not present in all samples, possible due to a concealed effect caused by the major peak observed at 160 °C. DSC characteristic peaks may be observed in more detail in Table 9. Generally, *P. tricornutum* purified extracts show three characteristic endotermic peaks. Despite it has been previously recognized OH possible effect in thermal properties (Kaur and Singh 2016), this was not observed.

Table 9 – Thermal properties attained by DSC analyses for *P. tricornutum* purified extracts. Values are the mean ± SD. Different extraction conditions were performed: temperatures of 40 and 80 °C, using water (W) and ethanol 25 % (E). n.d. – not discernible; *T* – temperature. Different letters indicate significant differences ($p \leq 0.05$) between extraction conditions, within the same peak and parameter.

Extraction conditions	Peak 2		Peak 3		Peak 4	
	T (°C)	ΔH (J g $^{-1}$)	T (°C)	ΔH (J g $^{-1}$)	T (°C)	ΔH (J g $^{-1}$)
W40_C	141.03 ± 0.83a	3.164 ± 0.007a	157.3 ± 2.2a	113.0 ± 8.2b	205.87 ± 0.11b	1.71 ± 0.15a
W40_OH	141.31 ± 0.46a	1.75 ± 0.42a	156.7 ± 9.7a	87.2 ± 6.2a	n.d.	n.d.
W80_C	141.0 ± 2.1a	2.7 ± 1.4a	163.9 ± 3.5a	118.9 ± 21.8b	n.d.	n.d.
W80_OH	142.9 ± 3.0a	1.70 ± 0.46a	163.1 ± 5.1a	107.3 ± 1.3ab	197.5 ± 2.1a	9.1 ± 3.5b
E40_C	141.2 ± 1.6a	2.83 ± 0.96a	164.56 ± 0.59a	177 ± 19c	n.d.	n.d.
E40_OH	144.1 ± 1.0a	3.15 ± 0.49a	164.7 ± 1.6a	130.4 ± 4.2b	n.d.	n.d.
E80_C	140.6 ± 2.7a	4.9± 3.1a	164.7 ± 4.4a	111 ± 3.0ab	n.d.	n.d.
E80_OH	139.55 ± 0.36a	1.87 ± 0.32a	159.68 ± 0.93a	113 ± 27b	n.d.	n.d.

Rheological parameters for solutions of the various purified extracts prepared at 5 % are presented in Table 10. The storage modulus (*G'*) is a measure of the solid nature of a sample, indicating the resistance to stretching, while the loss modulus (*G''*) gives a measure of viscous flow (Rocha *et al*. 2019; Curnutt *et al*. 2020). *G'* is expected to be positively correlated with the gel strength (Rocha *et al*. 2019). Analysis of *G'* and *G''* change as a function of temperature allowed to distinguish different sample behavior, depending on the extraction solvent and temperature applied. For 40 °C aqueous purified extracts, the elasticity of the solution is the dominant factor, with *G'>G''* regardless of the temperature, indicating always a gel nature throughout the test. It is possible to observe that *G'* remains constant until around 50 °C, from which it increases. This may possibly occur due to the thermal denaturation of proteins and subsequent aggregation, resulting in the reinforcement of gel network structure (Chronakis 2001; Andrade *et al*. 2010; Zheng, Matsumura, and Mori 1993), explaining the higher *G'* achieved after the heat treatment, as well as at the end of the analysis. These results are consistent with the findings achieved in fluorescence analysis. The same rheologic behavior was not reported for 40 °C ethanolic purified extracts. Despite a *G'* increasing was observed around 80-85 °C, temperature of melting (T_m) and gelling (T_g) were measurable, indicating a gel-temperature dependence. However, the lower values attained, as well as the disparities observed between OH and conventional extraction may be related to working with concentrations near the gelling limit. Therefore, T_m values are not considered trustworthy. Moreover, the ability of water extracts at 40 °C to form a gel at 20 °C may be related to the presence of alginate-like polysaccharides. In fact, at lower temperatures, the reactivity of ionic cross-linker is reduced, allowing a cross-linkage network structure more ordered with enhanced mechanical properties (K. Y. Lee and Mooney 2012; Augst, Kong, and Mooney 2006). It has been reported that ethanol percentage has an important impact on rheologic properties of alginate gels, with 24 % ethanol reducing its gel strength. This may be explained based on the observed polymer network, which is more compact, with larger pores and less connected (Hermansson *et al*. 2016). These findings go towards the monosaccharide profile previously recognized for these purified extracts, with uronic acids major content (possibly related with the alginate-like behaviour) being referred to aqueous 40 °C extraction condition. On the other hand, the weaker elastic behavior observed for ethanolic 40 °C purified extracts may be related to the lower alginate-like polysaccharide presence, as suggested by HPLC analysis. The enhanced *G'* observed at 20 °C after the heat treatment is also explained based on the protein denaturation that occurred at higher temperatures. The purified extracts whose extraction occurred at 80 °C present a typical behavior for the presence of a water-soluble polysaccharide, with viscosity decreasing as temperature increases (Zaim *et al*. 2020; Wu *et al*. 2015). Despite protein may be already partially denaturated due to the extraction heat

treatment, the superior *G'* observed after the heat cycle suggests that a further denaturation may have occured, as a result of the higher temperature (90 °C) exposure over rheological analysis. High temperature may intensify molecules thermal motion, resulting in the enlargement of intermolecular distance and weaker interactions (Wu *et al*. 2015). Moreover, some bonding forces (e.g. hydrogen, electrostatic and hydrophobic bonds) that can occur between polymer molecules may be weakened (V. D. Alves *et al*. 2010). In fact, gels whose helices primarily contain hydrogen-bonded crosslinks melt with the temperature increasing (Ji *et al*. 2017). T_m and T_g were different between the solvent extraction used, suggesting different extraction composition. In fact, during heating of 80 °C aqueous purified extracts the increased thermal energy destabilises the structure of the second transition observed on cooling at higher temperatures (with near 30 °C of difference), producing an extensive thermal hysteresis (Papageorgiou, Kasapis, and Gothard 1994). This phenomenum was reported in minor extent for ethanolic extracts, with aqueous polymer interactions appearing to be more difficult to break. Moreover, a simultaneous contribution from polysaccharide and protein polymers undergoing a gel-sol transition may be occurring, leading to more complex rheologic behaviors (Panouillé and Larreta-Garde 2009). In this manner, different temperature and solvent treatments during extraction process, appear to affect both protein and polysaccharide conformation and distribution composition, as well as their final ratio. This translates into distinct rheological properties, giving a wide range of texturizing features that can be used in the cosmetic formulations.

Table 10 – Rheological data obtained through dynamic rheological measurements in a stress-controlled rheometer for 5 % *P. tricornutum* purified extracts. Values are the mean ± SD. Different extraction conditions were performed: temperatures of 40 and 80 °C, using water (W) and ethanol 25 % (E). *G'*– storage modulus; *G''*– viscous modulus; T_g– gelling temperature; T_m – melting temperature. Different letters indicate significant differences ($p \leq 0.05$) between extraction conditions, within the same parameter.

Extraction conditions	T_m	T_g	20 °C				90 °C	
	(°C)	(°C)	*G'* initial (Pa)	*G'* final (Pa)	*G''* initial (Pa)	*G''* final (Pa)	*G'* (Pa)	*G''* (Pa)
W40_C	-	-	12.31 ± 0.80b	22.0 ± 6.6e	8.74 ± 0.40a	8.04 ± 0.49a	14.3 ± 5.1c	5.55 ± 0.57a
W40_OH	-	-	13.1 ± 2.1b	18.4 ± 2.4de	8.99 ± 0.83a	9.3 ± 1.5a	8.1 ± 2.7b	5.218 ± 0.089a
W80_C	90b	59.5 ± 2.1b	6.61 ± 0.13a	10.16 ± 0.35bc	5.157 ± 0.078a	4.954 ± 0.073a	1.66 ± 0.32a	1.91 ± 0.21a
W80_OH	90b	66.5 ± 5.9b	7.50 ± 0.91a	14.86 ± 0.30cd	5.95 ± 0.50a	5.91 ± 0.30a	2.07 ± 0.58a	2.29 ± 0.29a
E40_C	-	32.12 ± 0.44a	4.49 ± 0.55a	5.09 ± 0.80a	4.44 ± 0.29a	4.56 ± 0.43a	1.210 ± 0.085a	1.58 ± 0.17a
E40_OH	42.26 ± 0.31a	39.6 ± 1.0a	7.50 ± 0.12a	9.0 ± 1.7ab	6.334 ± 0.030a	6.13 ± 0.31a	0.914 ± 0.023a	1.58 ± 0.18a
E80_C	82.7 ± 3.2b	76.3 ± 4.1c	13.8 ± 1.8b	16.48 ± 0.12d	8.03 ± 0.67a	7.018 ± 0.070a	1.78 ± 0.57a	2.13 ± 0.53a
E80_OH	84.4 ± 4.8b	75.9 ± 6.6c	12.4 ± 2.5b	18.52 ± 0.60de	7.4 ± 1.2a	7.18 ± 0.40a	1.80 ± 0.34a	2.16 ± 0.30a

FTIR spectra are presented in Figure 17. Generally, all samples evidence the typical bands for polysaccharide and protein presence.

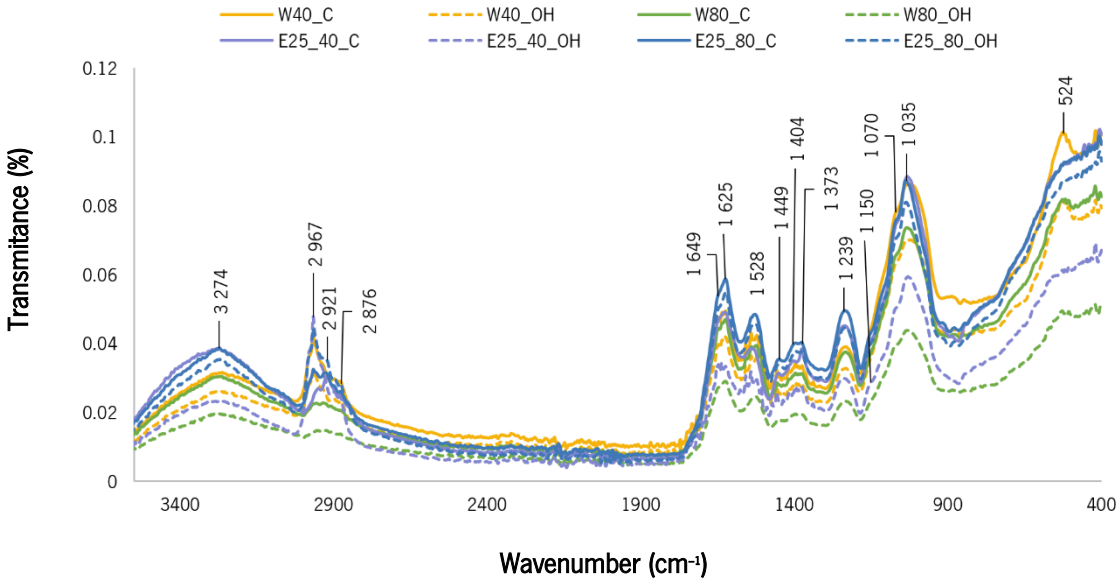

Figure 17 – FTIR spectra for *P. tricornutum* purified extracts when exposed to different extraction conditions: temperatures of 40 and 80 °C by control treatments (without the presence of OH) and OH at 25 Hz, using water and ethanol 25 %.

The broadened peak around 1280-1180 cm^{-1} may be assigned to the presence of sulphate ester groups (S=O) which is a characteristic component in fucoidan and sulphated polysaccharides other than alginate in brown seaweeds (Fernando *et al.* 2017; L. Pereira, Gheda, and Ribeiro-Claro 2013; Gómez-Ordóñez and Rupérez 2011). Ester sulfates presence is evidenced by 1370 cm^{-1} band (Rocha *et al.* 2019). The intense peak centered at 1035 cm^{-1}, as well as the band at 1150 cm^{-1} are common to all polysaccharides and could be assigned to C–O–H bending and to C–O and C–C stretching of the glycoside bridge (Fernando *et al.* 2017; Rocha *et al.* 2019; L. Pereira, Gheda, and Ribeiro-Claro 2013). The shoulder observed at 1070 cm^{-1} is due to the mannose units (Kacuráková 2000), which has been recognized as the major monosaccharide of *P. tricornutum* (Templeton *et al.* 2012). The band at 2920 cm^{-1} is related with the C–H, being a good measure of total sugar content (Rocha *et al.* 2019). The 1700-1600 cm^{-1} and 1575–1480 cm^{-1} peaks can be assigned to amide I and II absorption, respectively. Amide I band arises due almost entirely to the C=O stretching vibration of the peptide linkages (about 80 %), while amide II absorption has contributions from NH bending (40-60 %) and CN stretching vibration (18-40 %) (Jackson and Mantsch 1995; Kong and Yu 2007). The bands occurring at 2967 cm^{-1} and 2875 cm^{-1} can be assigned to the asymmetric and symmetric CH stretch of the methyl group, respectively. The asymmetric and symmetric vibrations of the methylene group may be responsible for the 2930 cm^{-1} and

2855 cm^{-1} absorption bands, respectively (Coates 2006). Differences between different extraction conditions are visible in this absorption region (3000–2850 cm^{-1}), as well as at 550–500 cm^{-1}. The peak observe for aqueous extractions around 520 cm^{-1} relates to S–S stretch (Nyquist 2001). The 3700–3000 cm^{-1} broad absorption peak relates to stretching vibrations of the OH group in water (Warren *et al.* 2013; Fernando *et al.* 2017).

In order to further explore purified extract composition, polysaccharide segregation was conceivable by gel permeation chromatography (Fig. 18).

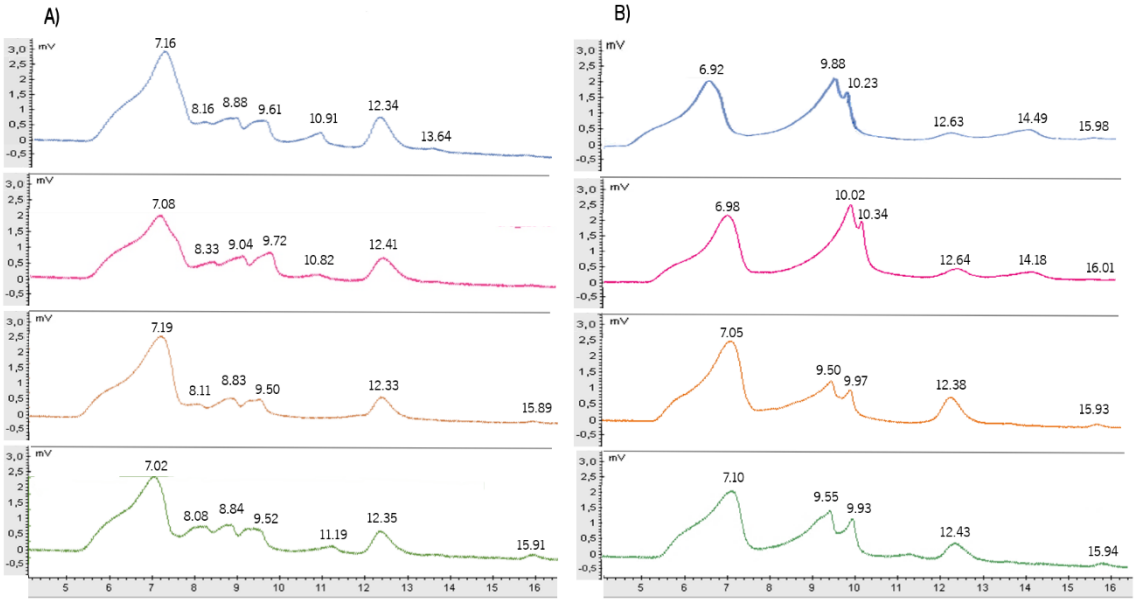

Figure 18 – Gel permeation high-performance liquid chromatography profile of polysaccharides present on *P. tricornutum* purified extracts (1 mg mL^{-1}), using RI detection: effect of using (A) ethanol 25 % and (B) water as solvent extraction. Temperature and OH effect were analyzed, from top to bottom: 40 °C conventional extraction; 40 °C extraction with OH treatment; 80 °C conventional extraction; and 80 °C extraction with OH treatment.

The peaks around 7 and 12.5 min are common to all purified extracts. The major differences between extraction conditions occur within 8 to 11 min, with ethanolic extracts resulting in a superior molecular weight dispersion, suggesting different polysaccharides extracted. Aqueous extracts present only two peaks within this range, while ethanolic extraction led to four peaks of lower intensities. Molecular weight values were possible to be determined, using a pullulan standard curve in the range of 22 – 642 kDa. The results are in agreement with the evidences reffered before that all aqueous extracts appear to possess an alginate-like polymer. In fact, a previous study underwent with brown seaweed alginate recognized its molecular weight within 213–277 kDa (Gómez-Ordóñez, Jiménez-Escrig, and Rupérez 2012). A hydrolysis of the peaks around 10 min appear to exist in all extracts except for 40 °C aqueous

purified extracts, explaining the higher intensities observed for the peak near 12.5 min. This may have occurred due to higher temperature exposure, as well as a result of the ethanolic mixture impact. In fact, high temperatures are responsible for significant modifications in the adopted conformation of polymer chains (M. Raposo, De Morais, and Bernardo de Morais 2013). Rheological behavior sustain this hypothesis, with enhanced elastic properties being confirmed for 40 °C water extracts.

On the other hand, the results attained by UV detection (Fig. 19) show concurrent peaks when higher intensities are observed, implying a strong interaction between polysaccharides and proteins. Moreover, higher intensities at the lowest retention time for 80 °C extractions were observed, verifying the electrophoresis band of higher molecular weight previously identified. Considering the spectra attained by RI and UV detection it appears that different polysaccharide-protein interactions are occurring under solvent and temperature treatment variation. Protein denaturation followed by protein or polysaccharide interaction and aggregation appear to have occurred for all extract conditions except when 40 °C water was used, causing the peak around 7 min. This is in line with the findings attained in fluorescence spectra.

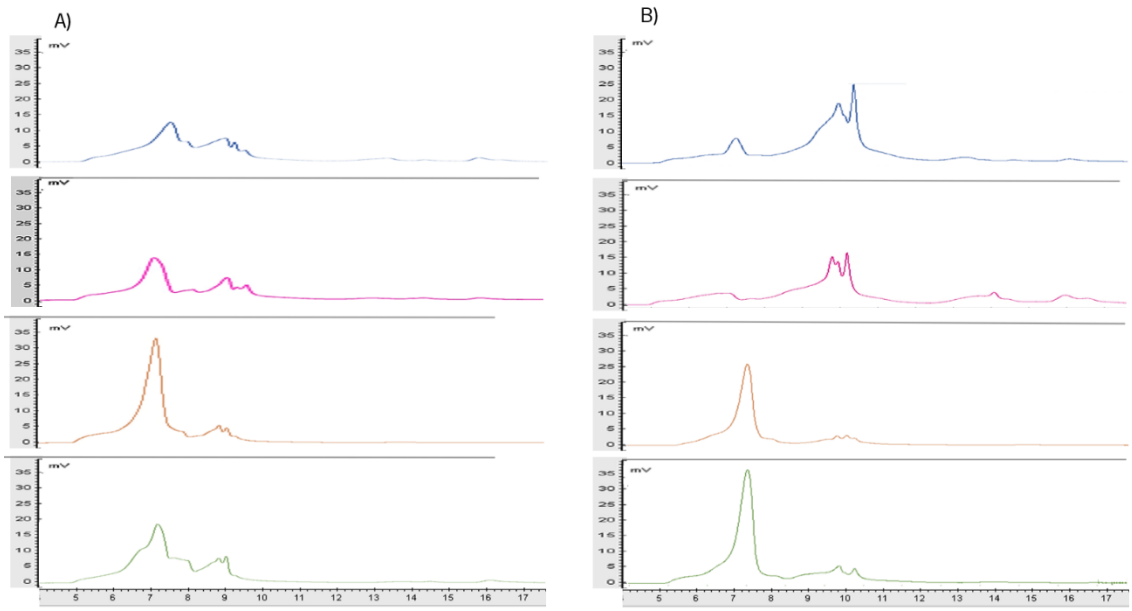

Figure 19 – Gel permeation high-performance liquid chromatography profile of proteins present on *P. tricornutum* purified extracts (1 mg mL^{-1}), using UV detection: effect of using (A) ethanol 25% and (B) water as solvent extraction. Temperature and OH effect were analyzed, from top to bottom: 40 °C conventional extraction; 40 °C extraction with OH treatment; 80 °C conventional extraction; and 80 °C extraction with OH treatment.

To access *P. tricornutum* purified extracts further potential in terms of their technological properties, emulsification capacity and stability presents as an important parameter in product formulation that should be evaluated. The emulsion layer height was monitored along time (Fig. 20).

Overall, *P. tricornutum* purified extracts were able to form emulsions with soybean oil. 80 °C ethanolic purified extracts showed the greatest emulsifier potential, by presenting the highest emulsification index, as well as the greater emulsion stability over the time evaluated. This goes towards the results previously attained by both electrophoresis and rheologic analysis, with these purified extracts showing bands more pronounced of higher molecular weight, as well as superior mechanical properties. Proteins are often used as emulsifiers agents, since they can adsorb at the oil-water interface and increase emulsion stability (Bouyer *et al*. 2012; Ebert *et al*. 2019), while most polysaccharides emulsion stabilizing mechanism relay in increasing viscosity of the aqueous phase, leading to droplet movement inhibition (Bouyer *et al*. 2012; Melanie *et al*. 2020).

In this manner, the use of 80 °C as extraction temperature allowed protein denaturation to occur, leading to a higher surface hydrophobicity exposure. It has been ascertained that surface hydrophobicity plays an important role as trigger of emulsification, enhancing the functional properties (Kato *et al*. 1983). Hence, a protein-polysaccharide interaction appears to be responsible for the increased emulsifying performance (Bouyer *et al*. 2012; Ebert *et al*. 2019). An identical behavior was not reported for 80 °C aqueous purified extracts, possible due to solvent related affinity and extraction of proteins with a more hydrophilic character. On the other hand, the mechanical energy applied might have been insufficient to promote the homogenization of emulsion droplet sizes (Maphosa and Jideani 2018). After 24h, it can be observed that, among the purified extracts assayed, those obtained from 40 °C aqueous extraction evidenced the second best emulsion forming capacity, which follows the superior gel strength achieved under rheologic analysis. However, after 90h, the emulsification index decreased in more than half, highlining its low emulsion stability. This may be explained based on the previous gel formation of the aqueous phase at low temperatures due to alginate-like polymers, allied with an insufficient homogenization, leading to a lower interaction oil-water and emulsion droplets embedded (Dickinson 2012). Despite the formation of a hydrogel network can immobilize droplets within the gel structure, thus preventing flocculation and coalescence (Farjami and Madadlou 2019; Dickinson 2012), it was not possible to observe. This suggests that gel formation should had occurred during or after the emulsification process, or alternatively applying a higher mechanical energy to the system, capable of reversely breaking down the gel network. Moreover, these purified extracts possess the higher ash content between the extraction conditions evaluated. In this manner, this might have had a negative impact on emulsion stability since the main driving force stabilizing protein-polysaccharide complexes is electrostatic and, consequently, an increase in ionic strength can influence the complexes formation and stability (Ebert *et al*. 2019). The weaker emulsifying properties observed for 40 °C ethanolic purified extracts goes

towards the rheology findings, as well as the minor protein content previously observed in UV spectrum. Altogether, it is possible to recognize the potential use of *P. tricornutum* purified extracts as emulsifiers, with further insights on droplet size and distribution; pH, temperature and homogenization method impact; and molecular composition being required.

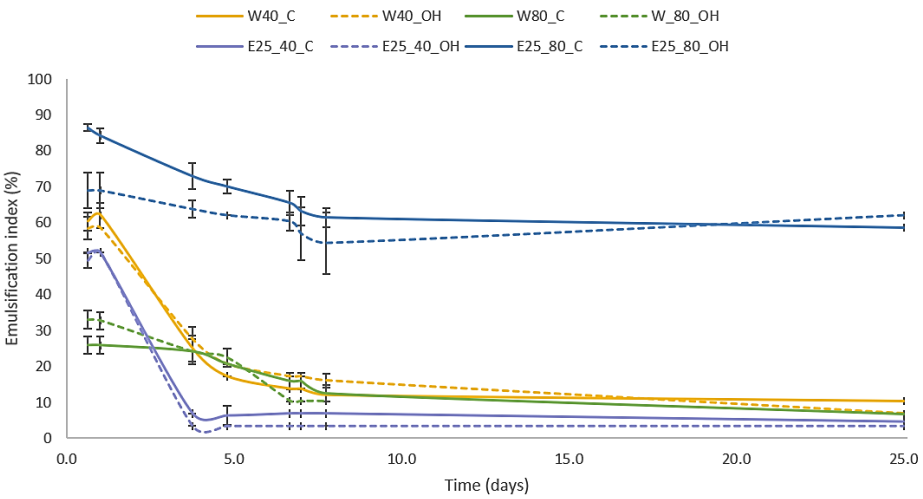

Figure 20 – Emulsification index over a 25 days period registered for 1 % *P. tricornutum* purified extracts, using soybean oil as a hydrocarbon. Different extraction conditions were analyzed to establish emulsifying formation and stability impact: temperatures of 40 and 80 °C by control treatments (without the presence of OH) and OH at 25 Hz, using water (W) and ethanol 25 % (E).

4.5. Bioactive extracts

Extractions from different *P. tricornutum* biomasses, namely commercial powder and paste, were performed using ethanol 96 % as solvent at 45 °C for 30 min, to establish pretreatment impact on bioactive properties and ultimately select the more advantageous matrix for the current purpose. Different antioxidant compounds present in microalgae ethanolic extracts may act through different mechanisms. Moreover, each assay relies in an approximation of the total extract antioxidant capacity, depending on reaction time, method applied, and reaction kinetics complexity (Assunção *et al.* 2017). In this sense, several quantification assays were performed to allow a more accurate evaluation, analyzing different mechanisms of extracts action. The ferric reducing antioxidant power (FRAP) method is based on the reduction of an iron complex (Fe^{3+} to Fe^{2+}), and DPPH and ABTS are the most widely used methods for determining the free radical scavenging capacity (Ferreira-Santos *et al.* 2020). Antioxidant profile of both

extracts (Fig. 21A), as well as lipidic and total phenolic content (TPC) (Fig. 21B) allow to significantly distinguish between the two extracts attained.

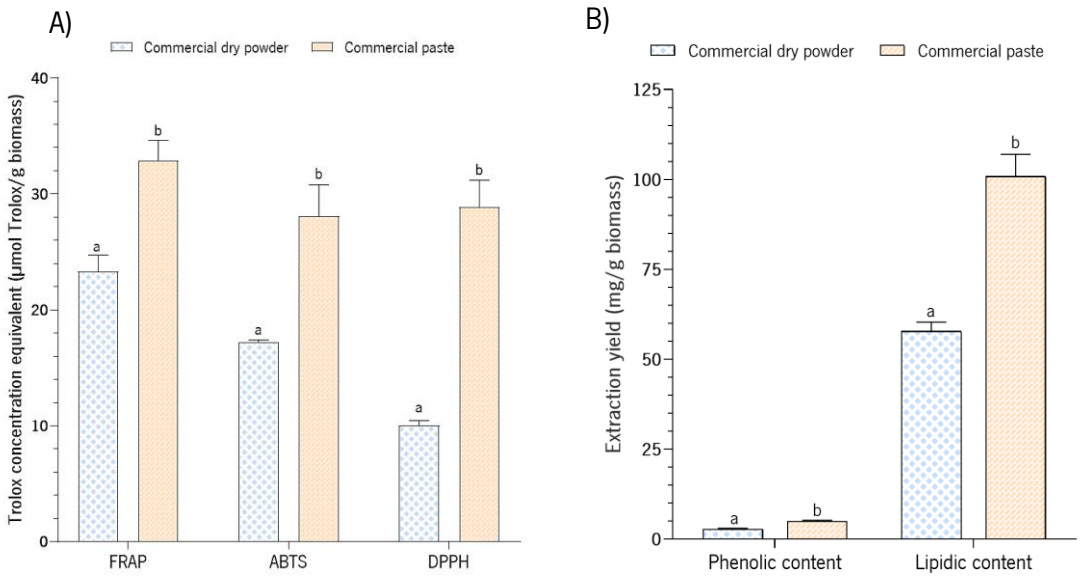

Figure 21 – Pretreatment impact on *P. tricornutum* extracts: (A) antioxidant (ABTS, DPPH and FRAP) activities and (B) phenolic and lipidic content.

Spray drying has been widely applied in food and pharmaceutical industries (T.A. Tran and V.H. Nguyen 2018), being a highly appropriate process for heat sensitive components such as carotenoids, flavonoids and phenolic compounds, since it allows their preservation (Kha, Nguyen, and Roach 2010). However, when compared to biomass paste, a weaker antioxidant performance was reported. These results may be explained based on the superior solvent exposure of molecules in more hydrated matrices. In fact, dried samples impose a minor solvent accessibility to compounds, encumber solvent penetration, which presents as one critical step of solvent extraction (Grosso *et al.* 2015). Moreover, frozen-thaw process may be considered as a physical disruption cell wall method, being frequently employed to damage cell wall structures without completely disrupting the walls (Teuling *et al.* 2019). Frozen-thaw impact on cell wall integrity, could have enhanced cell rupture efficiency, ultimately increasing compounds bioavailability. In this manner, the more suitable matrix for the following analyzes is commercial frozen paste, due to the promising bioactive properties registered.

Preliminary assays were conducted in order to ascertain the impact of independent variables, such as extraction time and temperature, on antioxidant activity and related compounds (Fig 22). All extracts present similar composition and antioxidant profiles, with the major differences referring to FRAP analysis, in which 65 °C extracts showed greater antioxidant ability. Accordingly, only the extraction at

65 °C throughout 15 min was selected to evaluate ohmic heating treatment impact and determine extracts bioactive and cytotoxic activities.

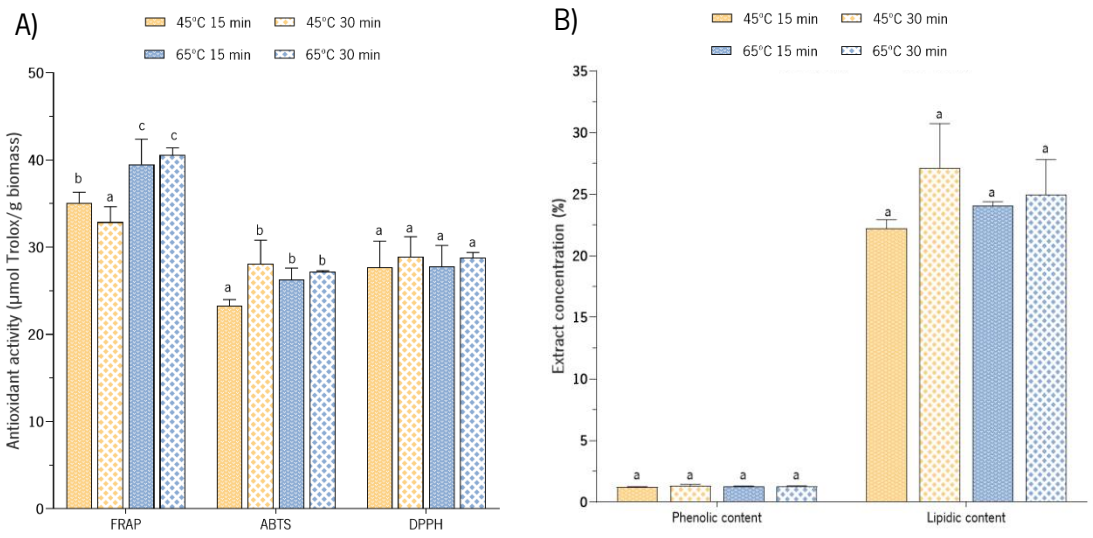

Figure 22 – Impact of different extractions conditions, namely, temperature (45 and 65 °C) and time (15 and 30 min) on *P. tricornutum* frozen paste: (A) antioxidant (ABTS, DPPH and FRAP) activities and (B) phenolic and lipidic content.

Microalgae are widely known for producing a variety of different biological active components with great interest for their antioxidant and health beneficial effects (Banskota *et al.* 2019). Both extracts were characterized regarding the main fractions more likely to be responsible for their bioactive properties (Table 11). These include phenolics (Ferreira-Santos *et al.* 2020) and flavonoids (Sansone and Brunet 2019) compounds, carotenoids (Goiris *et al.* 2012), and fatty acids (e.g. PUFA) (Banskota *et al.* 2019). It has been acknowledged that microalgae are a rich source of protein enzymes, peptides, and amino acids, which are recognized for their important role for normal physiological activities, as well as for their strong health-protecting effects (Sansone and Brunet 2019). However, protein quantification was not performed, since the methods more commonly used, namely Bradford and Lowry, prove to be inadequate for this specific matrix (Appendix 2.3). Several compounds may interfere with both Lowry and Bradford methods, causing inaccurate quantifications. Lowry quantification showed to be influence by the ethanol percentage in the solution, as well as by the phenolic compounds concentration. Moreover, the reactivity of both methods to a specific protein is strongly dependent by its amino acid composition, since not all amino acid residues can oxidate equally the Folin phenol reactive or bind to the Coomassie dye (Barbarino and Lourenço 2005). In fact, Bradford's method led to lower protein values, which is consisting with the findings by other authors (Barbarino and Lourenço 2005). Protein quantification by Lowry's method tend

to be greater, since it interacts with all peptide bonds and with some amino acids (Barbarino and Lourenço 2005). The results show that Lowry method provides similar protein quantification as Kjeldahl method for aqueous and 50 % ethanolic *P. tricornutum* extracts (Appendix 3). This suggest that Lowry method should be used for *P. tricornutum* extracts only when ethanolic concentration is not a conditional factor to be considered. To obtain a more reliable measurement of protein, it would be useful to identify the predominant proteins in the specific working matrices. However, this is not of practical value due to the time consuming and high demanding work (Barbarino and Lourenço 2005). Alternately, Kjeldahl quantification should be performed. However, this was not possible to ensure due to the lack of sufficient extracts material.

Table 11 – Ohmic heating impact on antioxidant (ABTS, DPPH and FRAP) activities and biochemical characterization of ethanolic extracts from *Phaeodactylum tricornutum*. Extractions were carried out at 65 °C for 15 min. Values are expressed as mean ± SD of 3–4 experiments (half-maximal inhibitory concentration, IC_{50}).

		Conventional extraction	*Ohmic heating extraction*
IC_{50} (mg mL^{-1})	ABTS	12.2 ± 1.8	12.3 ± 2.8
	DPPH	42.0 ± 6.8	42.5 ± 9.8
FRAP (µmol trolox eq.g^{-1} DE)		35.8 ± 3.1	32.2 ± 2.4
Chlorophyll content (mg. g^{-1} DE)		7.81 ± 0.74	7.14 ± 0.24
Carotenoid content (mg. g^{-1} DE)		0.99 ± 0.26	1.326 ± 0.068
Phenolic content (mg GAE.g^{-1} DE)		7.67 ± 0.37	7.34 ± 0.36
Flavonoid content (mg CE.g^{-1} DE)		7.56 ± 0.86	8.55 ± 0.36
Lipidic content (g g^{-1} DE)		0.1281 ± 0.0043	0.1238 ± 0.0025

No significant differences were induced by ohmic heating in comparison to conventional extraction, except for carotenoid content. The inhibition percentages of the radicals DPPH and ABTS by the extracts were found to be concentration-dependent, as expected. The deduced values of IC_{50} can also be found in Table 11. Lower IC_{50} values indicate superior antioxidant activities. The IC_{50} value attained for ABTS assay is almost 4–fold lower than the IC_{50} determined for DPPH test. DPPH has been regarded as a limited evaluator of antioxidant capacity in living systems due to its radical superior stability. Moreover, the major drawback of DPPH assay in the present study relates to carotenoid presence, which leads to a dark purple color. In this manner, this interference with the absorbance readings may explain the differences in the antioxidant power observed between the two methods (Assunção *et al.* 2017).

Concurrently, different compounds with distinctive antioxidant mechanisms of action may be presence. While the $ABTS^{\cdot+}$ nitrogen atom quenches the hydrogen atom, causing solution decolorization; DPPH scavenging assay depends on the presence of hydrogen-donating compounds (Ferreira-Santos *et al*. 2020). The concentration required to inhibit 50 % of $DPPH^{\cdot}$ radical by *P. tricornutum* extracts (≈ 42 mg mL^{-1}) is comparable to the value attain for *Characiopsis* sp. ethanolic extract (44.27 ± 0.39 mg mL^{-1}), which showed the highest scavenging activity within a multiplicity of microalgae strains (Assunção *et al*. 2017). The FRAP analysis also revealed high antioxidant power, which agrees with a previous study performed by Goiris *et al*. (2012), with ethanolic extracts of *P. tricornutum* being within the higher antioxidant capacities observed. Both phenolic and carotenoid compounds have been recognized for their important contribution in total FRAP antioxidant activity (Goiris *et al*. 2012). The total phenol and flavonoid content do not follow the results attained by Safafar *et al*. (2015) – with 3.16 ± 0.04 mg GAE g^{-1} and 0.84 ± 0.12 mg quercetine equivalent g^{-1}, respectively –, with our work showing greater extraction of these compounds. The reported study highlights *P. tricornutum* lowest phenolic content when compared to other microalgae species in methanolic extracts. However, the highest phenolic content was observed for *Desmodesmus* methanolic extracts (7.72 ± 0.08 mg GAE.g^{-1}), which is similar to the values achieved in the present work for *P. tricornutum* ethanolic extracts. Flavonoids are known to be the largest group of naturally occurring phenolic compounds (Sulaiman and Balachandran 2012), comprising a major fraction of total phenolic content in *P. tricornutum* (Rico *et al*. 2013). In this manner, the results suggest that an overestimation and/or underestimation of the analytical data might have occurred. Phenolic compounds analysis is determined by their source, the extraction and purification techniques employed, the storage conditions, and the presence of interfering substances (non-phenolic reducing compounds) in extracts such as fatty acids or pigments (Cotas *et al*. 2020). This ultimately hampers assays accuracy (Sánchez-Rangel *et al*. 2013). Thus, several aspects as solvent concentration, standard compound and complexation time should be evaluated and adjusted to this specific matrix (Ramos *et al*. 2017).

Lipids with higher unsaturation degrees enhance the antioxidant potency of microalgal extracts. In this manner, considering the high lipidic content of the extracts attained in comparison with others previously described for this diatom (Banskota *et al*. 2019), further comprehensive studies should be performed. This should pass through underlining *P. tricornutum* extracts lipidic profile, in order to assess its relation to the antioxidant behavior presented.

Altogether, it is reasonable to highlight this diatom extracts antioxidant activity when compared to other microalgae extracts, being in line with the previous statement that ethanol has an important role

in the extraction of compounds with strong antioxidant activities (Rodriguez-Garcia and Guil-Guerrero 2008; Ferreira-Santos *et al.* 2020).

To evaluate the effects of *P. tricornutum* ethanolic extracts on cell viability, *in vitro* cell metabolic activity was assess using four different cell lines: normal mouse fibroblast (L929), human lung cancer (A549), human epithelial adenocarcinoma (Hela) and human epithelial colorectal adenocarcinoma (Caco-2). The extracts impact on cell proliferation was measure by resazurin assay and the results are presented in Fig. 23.

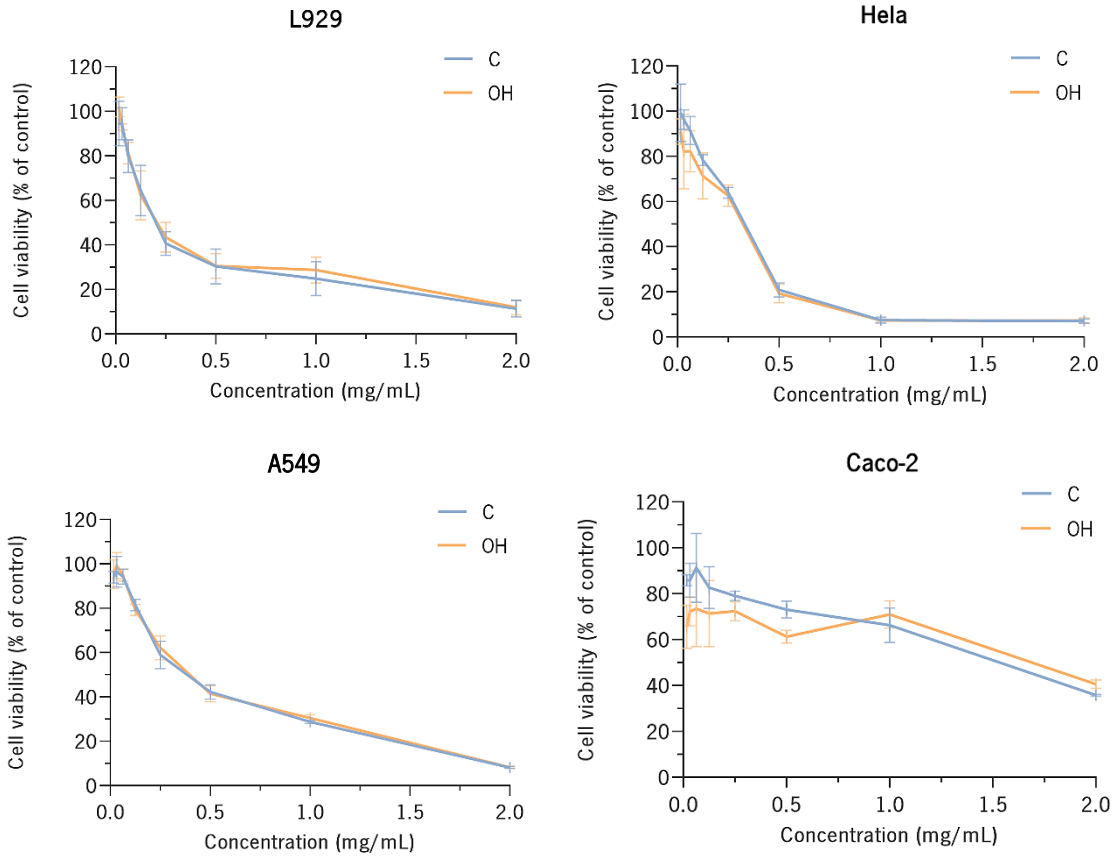

Figure 23 – Effect on cell viability of *P tricornutum* extracts using conventional (C) and ohmic heating (OH) treatment in four different cell lines. Values are expressed as mean and standard deviation of three independent experiments.

Generally, both extracts exhibited a dose-response suppression in all cell lines used, with no significant differences between conventional and OH extraction. The IC_{50} value determined for normal fibroblast cells (Table 12) underlines extracts concentration range that can be safely used for their health-promoting properties. In this manner, further studies exploring *P. tricornutum* extracts potential application in cosmeceutical formulations should work with concentrations lower than 0.19 mg mL^{-1}.

Caco-2 cells have been recognized as a reliable intestinal cell model for nutrient uptake studies, drug absorption and toxicity analysis (Caron *et al.* 2017; Glahn 2009). In this manner, the slight variation observed in Caco-2 dose-response curve suggest lower toxicity levels in intestinal absorptive cells, which is verified by the high IC_{50} values attained. These results highlight the potential interesting application of *P. tricornutum* extracts for nutraceutical purposes. Moreover, a negative impact on carcinogenic cells viability was observed, suggesting a dose-dependent anticancer activity of *P. tricornutum* extracts. However, the American Cancer Institute (NCI) establishes that crude extracts should only be considered as interesting antitumor agents when IC_{50} was less than 30 μg mL^{-1} (Saadaoui *et al.* 2020). The IC_{50}values attained were much higher than the ones found for other microalgae extracts (Martínez Andrade *et al.* 2018), rejecting *P. tricornutum* extracts therapeutic use in lung and epithelial cancer pathologies.

Table 12 – Half-maximal inhibitory concentration (IC_{50}) of *P. tricornutum* extracts on proliferation in four different cell lines.

IC_{50} *(mg* mL^{-1}*)*	*Conventional extraction*	*Ohmic heating extraction*
L929	0.19 ± 0.05	0.19 ± 0.05
Hela	0.31 ± 0.01	0.29 ± 0.06
A549	0.40 ± 0.06	0.41 ± 0.07
Caco-2	1.5 ± 0.2	1.7 ± 0.1

5. CONCLUSIONS AND FUTURE WORK PERSPECTIVES

Microalgae species have been gaining increasing attention in the last few years due to the multiplicity of advantages that they can offer in several fields. The adoption of more sustainable and environmental-friendly approaches in product development is of growing importance, with microalgae exploration providing a novel path towards this purpose. This study provided insights into a more integrated strategy to enhance *Phaeodactylum tricornutum* properties, comprising an overlook from cultivation conditions to extracts production and characterization. Thus, the present work focused on achieving two different main fractions accountable for bioactive and technologic functions, underlying *P. tricornutum* growth conditions impact and establishing OH technology extraction tuning.

Even though UV treatment applied to *P. tricornutum* cultures as a natural selection agent did not provide a clear impact on total antioxidant ability of ethanolic extracts, it presented as a potential useful tool to enhance lipidic extraction. The combined use of specific growth conditions that can simultaneously favor both extraction and production of biocompounds of interest, might ultimately lead to a higher efficiency of the process and provide a more competitive production. Therefore, further comprehensive studies regarding UV impact on carotenoid, phenolic and lipidic profiles, as well as on astaxanthin content should be addressed.

The features of this diatom extracts demonstrate a promising potential to be used in product formulation, either for their technologic function or as a bioactive ingredient. In fact, extracts showed different biochemical profiles, depending on the solvent used, allowing to attain a broader spectrum of compounds activities. Generally, no statistically significant ($p \leq 0.05$) effects were observed for electric fields application, suggesting that the replacement of conventional solvent-based extraction for this technology is feasible, as a more energetically favourable alternative.

Regarding techno-functionality purposes, the applied procedures resulted in extracts with a main polysaccharide composition, with substantial co-presence of proteins and minerals. These combined extracts may be applied as functional food components, providing a more economical and environmental friendly production in line with the “zero waste” vision. Moreover, the distinctive rheologic behavior and emulsifying properties attained may be of great value for further product development studies. Extraction with ethanol 25 % at 80 °C provided the best emulsifying characteristics, which appear to be due to protein-polysaccharides synergetic effect and higher protein surface hydrophobicity exposure. Emulsifiers have been frequently used in cosmetic and food industries as additives to enhance organoleptic features and extend shelf-life (Partridge *et al.* 2019). They are known to assist in stabilization of the dispersed

phase against coalescence and to decrease interfacial tension between both liquids (Moravkova and Filip 2013). Aqueous extraction at 40 °C conferred superior mechanical properties, suggesting its possible application as thickening and gelling agent. Thickeners can partially inhibit the reaction between the chemicals in the emulsion and simultaneously modify in higher extent the rheological characteristics (Moravkova and Filip 2013). Thus, future work may rely on exploring these purified extracts' potential under coadjuvant conditions, as well as underlining other important features for cosmetic application, such as moisture-absorption and retention ability (J. Wang *et al.* 2013) and microbial charge.

Bioactive extracts showed promising antioxidant activities when compared to other microalgae extracts, enhancing ethanol important role in promoting phenolic and lipid content. Considering the high phenolic and lipid content of the extracts attained, further studies should pass through underlining *P. tricornutum* extracts profiles, in order to assess its relation to the antioxidant behavior presented. Cell viability assays suggest lower toxicity levels in intestinal absorptive cells, unravelling the potential use of this extracts for the nutraceutical and food sectors. In order to assess these extracts usefulness in cosmetic field, further studies should be adressed regarding their antimicrobial effects and ability as antioxidant preservative agents (Halla *et al.* 2018).

The present work brings new perspectives on *Phaeodactylum tricornutum* which revealed to be a valuable source of structurally diverse biologically and technologically active compounds, with high potential for further application in nutraceutical and cosmetic industries. Furthermore, this study also proved that an integrated strategy of nutrient bioextraction is feasible. In fact, the combined use of the extracts responsible for the greater technologic behavior and antioxidant performance may be useful to address a synergetic effect on the final product. This strategy could also be employed to simplify the formulation, while providing an alternative to the use of external commercial agents, which is likely to result in decreased production costs. This follows the biorefinery approach, by which an integrative perspective is aimed to minimize non-renewable energy resources consumption and negative environmental impacts, while maximize a complete and efficient biomass conversion.

In conclusion, the major goals of the present work were successfully accomplished, highlighting *P. tricornutum* extracts potential not only for the cosmeceutical industry, but also for functional foods development. Generally, further comprehensive studies are required to unravel the feasibility of this diatom extracts in product formulation.

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7. APPENDIX

APPENDIX 1 – EXTRACTION TECHNOLOGY EXTRACTIONS

Table A1 – Extraction technology extraction used in algae species with the advantages, disadvantages and reviewed applications.

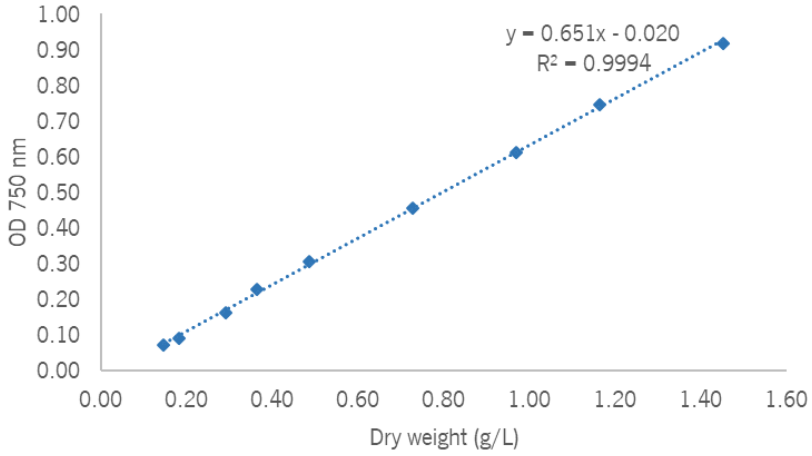
Extraction technology extraction	Technology fundamental	Advantages	Disadvantages	Reviewed applications	Ref.
Ionic liquids	Application of liquids consisting in cations and anions entirely based.	Greener; efficient; low cost.	Downstreaming processes required, difficult separation from target.	Lipid extraction and carbohydrate rich extract.	(Rocha *et al*. 2018; To *et al*. 2018)
Microwave-assisted extraction (MAE)	Nonionizing electromagnetic radiation with a frequency from 300 MHz to 300 GHz.	Rapid and volumetric heating; improved extraction rate and yield; lower use of solvents, economically feasible; high reproducibility and purity product; less energy consumption; shorter procedures.	Not suitable for thermolabile compounds extraction.	Algae fucoidans, carotenoids minerals and sulfated polysaccharides extraction.	(Kadam, Tiwari, and O'Donnell 2013; Pangestuti, Siahaan, and Kim 2018; R. N. Pereira and Vicente 2010; Chemat *et al*. 2015)
Ultrasound-assisted extraction (UAE)	High frequency sound waves above 20 kHz.	Increased extraction yield; simple, cost-effective; efficient; fast processing time; low solvent consumption; higher purity product; reduced downstreaming processes required.	High power consumption; difficult scale up; clean up step required.	Omega 3-fatty acids and algae isoflavones, minerals and sulfated polysaccharides extraction.	(Cravotto *et al*. 2008; Kadam, Tiwari, and O'Donnell 2013; Pangestuti, Siahaan, and Kim 2018; R. N. Pereira and Vicente 2010; Chemat *et al*. 2015)

Enzyme assisted extraction (EAE)	Application of cell wall degrading enzymes (e.g. carbohydrases and proteases).	Ecofriendly, relatively low-cost nontoxic, mild, high bioactive yield and selectivity; enhanced antioxidant activity preservation.	Highly dependent on temperature and treatment time conditions; careful enzyme selection; enzymatic optimization needed; further processes required, high enzymes cost.	Fucoxanthin and antioxidant rich extraction.	(Kadam, Tiwari, and O'Donnell 2013; Pangestuti, Siahaan, and Kim 2018)
Supercritical fluid extraction (SFE)	Use of a fluid whose temperature and pressure are above its critical limit.	Fast extraction rate, high yield, ecofriendly and more selective than UAE.	High investment cost and low CO_2 supercritical polarity.	Nutraceuticals and bioactive compounds extraction (carotenoids, pigments, polyunsaturated fatty acids, polyphenols).	(Bleakley and Hayes 2017; Kadam, Tiwari, and O'Donnell 2013)
Subcritical water extraction (SWE)	Use of hot water (100–374˚C) under high pressure (~10 bar) to maintain water in a liquid state.	Reduce extraction time; simple; ecofriendly; high yield; low solvent consumption.	High investment costs and temperature.	Algae lipids extraction.	(Pangestuti, Siahaan, and Kim 2018; Bleakley and Hayes 2017)
Instant controlled pressure drop	High temperature short-time (HTST) process based in a fast transition from high steam pressure to vacuum.	Short-time process; no solvent use; rapid metabolite release; low thermal degradation.	–	Essential oils extraction	(Chemat *et al.* 2015)
Electro-technologies	Application of electric fields to a system.	Improve mass-transfer processes; versatility, easy scale-up, fast; membrane	Conductivity medium dependent.	Biocompounds extraction.	(Rocha *et al.* 2018; Chemat *et al.* 2015;

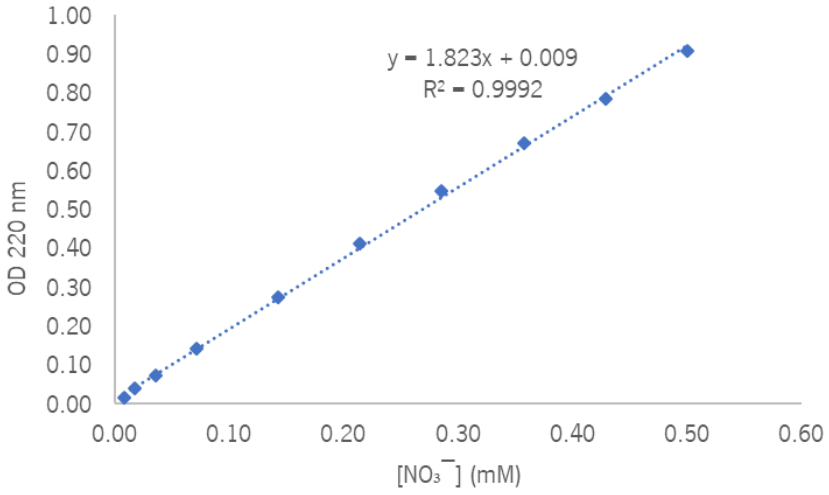
electroporation effects; green; energetic efficiency.	Bleakley and Hayes 2017)

APPENDIX 2 – CALIBRATION CURVES

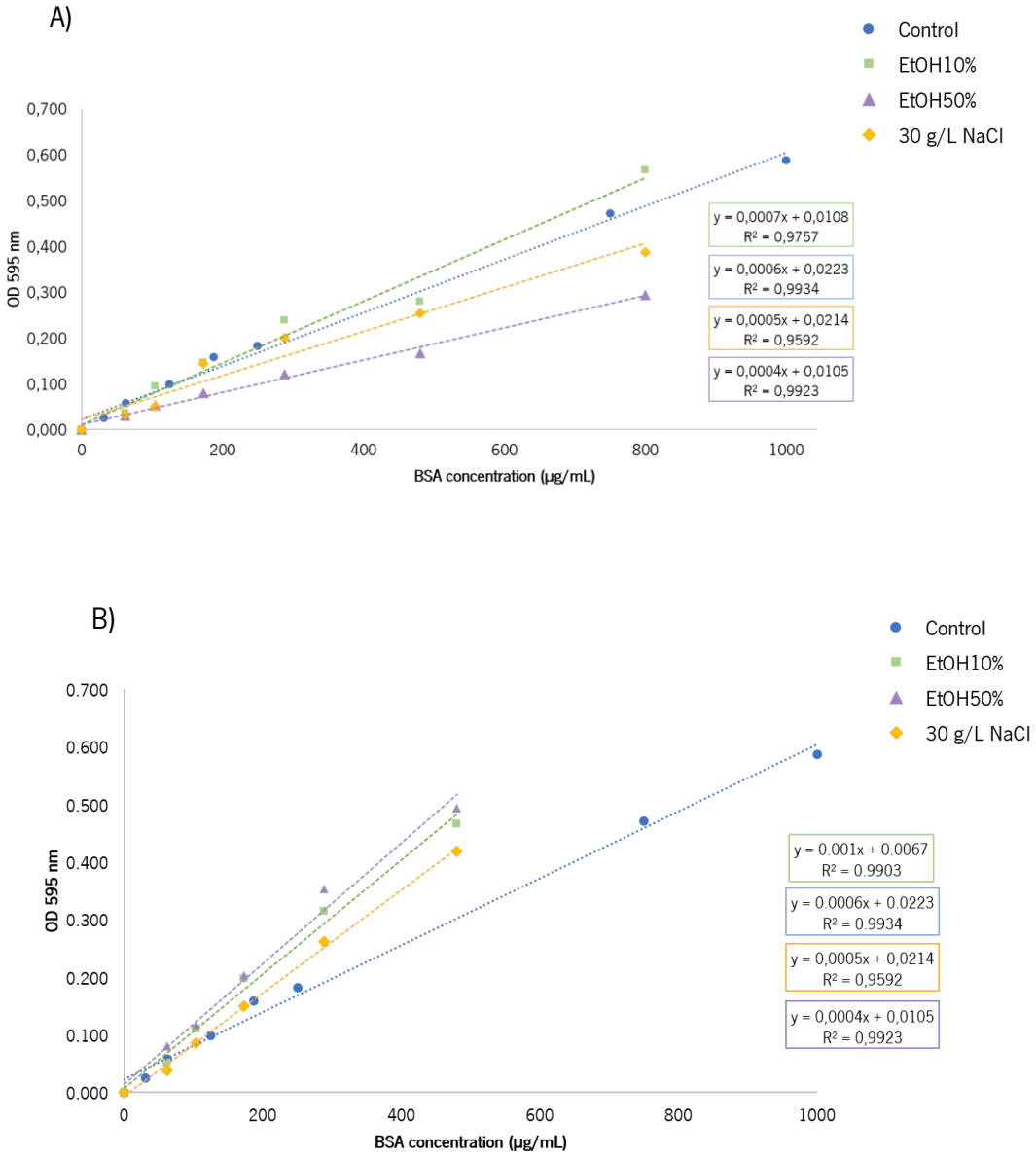
A2.1– Calibration curve for *P. tricornutum* concentration determination expressed in g L^{-1}, with a linear equation of OD750nm= (0.651±0.014) x Concentration (g L^{-1}) + (-0.020 ±0.011) for a 95 % confidence level.



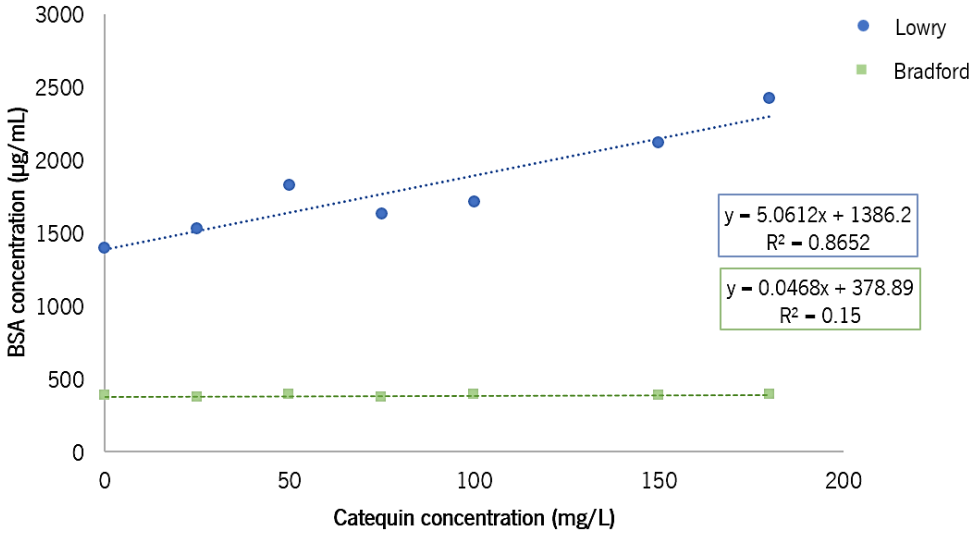
A2.2– Calibration curve for nitrate determination expressed in mM, with a linear equation of OD220nm= (1.823 ±0.043) x Concentration (mM) + (0.009 ±0.011) for a 95 % confidence level.



A2.3– Ethanol percentage and salt interference on (A) Lowry and (B) Bradford quantification methods.



A2.4– Catequin influence on Lowry and Bradford quantification methods.



APPENDIX 3 – PROTEIN QUANTIFICATION METHODS

Table A3 – Protein content of *Phaeodactylum tricornutum* extracts determined by different quantification methods (Bradford, Lowry and Kjedahl) under different solvent systems: water, ethanol 50 % and ethanol 96 %.

Extraction conditions	Bradford (%)	Lowry (%)	Kjedahl (%)
Water 40 °C	2.14 ± 0.29	8.85 ± 0.14	16.03 ± 0.07
Ethanol 50% 40 °C	1.37 ± 0.03	10.88 ± 0.33	9.83± 0.50
Ethanol 96 % 40 °C	6.77 ± 0.83	21.41± 0.55	9.61 ± 0.33

APPENDIX 4 – MICROALGAE EXTRACTS ANTIOXIDANT PROFILE

A4.1– Antioxidant activity determined by (A) FRAP, (B) ABTS, (C) DPPH methods determined for the different extraction conditions tested for the three microalgal studied, namely, *Chlorella vulgaris*, *Nannochloropsis oceanica* and *Phaeodactylum tricornutum*. Cultures were maintained using Guillard's f/2 medium at 24°C, under continuously white light (100 µmol photons m^{-2} s^{-1}) and aeration with CO_2 pulses. Extractions were carried out using 4 % solid solvent for a final volume of 5 mL. Different letters indicate significant differences ($p \leq 0.05$) between extraction conditions within the same microalgae species (water; H_2O; ethanol, EtOH; dichloromethane:ethanol 50%, DE).