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Evaluation of Ohmic Heating processing on extraction of bioactive compounds from microalgae biomass

Dezembro de 2020

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School of Engineering

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Trabalho efetuado sob a orientação do
Doutor Ricardo Nuno Correia Pereira
e do
Professor Doutor António Augusto Martins de Oliveira Soares Vicente

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A todos um muito obrigado

STATEMENT OF INTEGRITY

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RESUMO

Avaliação do processamento de aquecimento óhmico na extração de compostos bioativos da biomassa de microalgas

As microalgas são uma fonte privilegiada de compostos bioativos, sendo microrganismos importantes em termos industriais numa ampla gama de segmentos industriais, como as indústrias farmacêuticas, agroalimentar e de biodiesel. O maior desafio na comercialização de produtos de microalgas é a redução do custo geral de extração de frações enriquecidas. As metodologias tradicionais de extração apresentam diversos problemas ao nível da aplicabilidade industrial e sustentabilidade e por isso é necessário encontrar novas formas de realizar a extração de bioprodutos. Uma nova abordagem para este problema consiste na utilização de eletrotecnologias, como é o caso do processamento por aquecimento óhmico (OH, abreviação em inglês de Ohmic Heating). Este método permite a sinergia natural de efeitos térmicos e elétricos nos processos de extração de forma a aumentar a sua eficiencia. O objetivo principal deste trabalho consistiu em realizar uma avaliação multi-exploratória da tecnologia de OH como metodologia para a extração de bioprodutos de uma microalga. Para este propósito foram realizados um conjunto de ensaios por aplicação de OH com diferentes campos elétricos, temperaturas de tratamento e cinéticas térmicas. Através deste estudo foi possível demonstrar que aplicação de OH e os seus campos elétricos moderados (MEF, abreviação em inglês para moderate electric fields) contribuiu para aumentar a extração de compostos bioativos em comparação com técnicas de extração térmica convencionais. A condição de tratamento "Pulsos de Aquecimento Óhmico (OHP, abreviação em inglês para Ohmic heating pulses) sob um campo elétrico de 217 V/cm apresentou a melhor taxa de extração de clorofila a (50.5 %) e proteínas (20.4 %). Por sua vez, Lipídios (40.1 %) e Clorofilas b (38.9 %) foram extraídas com mais eficiência sob um campo elétrico de 133 V/cm. Carotenoides, carbohidratos e compostos de caracter antioxidante foram extraídos de forma igualmente eficaz por OH, independentemente da intensidade do campo elétrico aplicado.

Palavras-chave: Microalga, Aquecimento Óhmico, Campos Elétricos, Taxa de extração, Compostos Bioativos.

ABSTRACT

Evaluation of Ohmic Heating processing on the extraction of bioactive compounds from microalgae biomass

Microalgae are a privileged source of bioactive compounds being industrially important microorganisms in a wide range of industrial segments such as as pharmaceutical, agro-food, and biodiesel industries. The greatest challenge in commercialization of microalgae products is the reduction of the overall cost of extraction of enriched fractions. The traditional methodologies of extraction have several problems due to poor industrial applicability and sustainability issues, and because of that there is a need to find new ways to accomplish the extraction of bioproducts. A new approach for this problem consists in the utilization of electrotechnologies, such as the case of ohmic heating processing (OH), a methodology that allows a synergy between thermal and electric fields as a way to increase extraction efficiency. The main goal of this work was to perform a multi exploratory evaluation of OH as a methodology for the extraction of bioproducts from a microalgae. For this purpose, a set experiments using OH were developed in which different electrical fields, treatment temperatures and thermal kinetics were evaluated. Through this study it was possible to demonstrate that the application of OH and its moderate electric fields (MEF) contributed to an increase extraction of bioactive compounds when compared with traditional thermal treatments. Results from this study have shown that Ohmic Heating Pulses (OHP) at 217 V/cm resulted in the best extraction rate of Chlorophyll a (50.5 %) and proteins (20.4 %). While other compounds such as lipids (40.1 %) and chlorophyll b (38.9 %) were extracted more efficiently under an electric field of 133 V/cm. Carotenoids, carbohydrates and antioxidant compounds were equally extracted by OH, independently of the electric field applied.

Keywords: Microalgae, Ohmic Heating, Electrical Fields, Extraction rate, Bioactive Compounds.

GRAPHICAL ABSTRACT

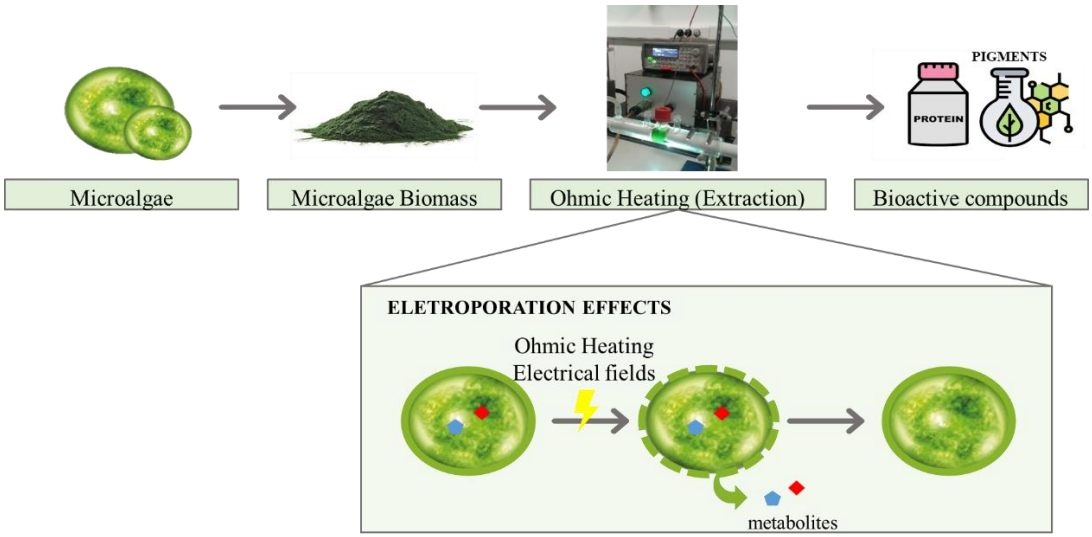

TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND ACRONYMS

ABTS 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid

BHT Butylated hydroxytoluene

BSA Bovine serum albumin

CO_2 Carbone dioxide

COV Conventional Heating

DPPH 2,2-diphenyl-1-picrylhydrazyl

EF Electrical Fields

FRAP Fluorescence recovery after photobleaching

HTST High temperature short-time processing

IOMR Intracellular organic matter release

kV Kilovolt

LFR1 Lichen foliose rock 1

mL Milliliter

MEF Moderate Electrical Fields

M Molar concentration

nm nanometers

NO_x Nitrogen oxides

OH Ohmic Heating

OHP Ohmic Heating Pulses

PAR Photosynthetically active radiation

PCA Principal component analysis

PEF Pulsed electric fields

PUFAs Polyunsaturated fatty acid

RPM Rotations per minute

SD standard deviation

SPV sulfo-phosphovanillin

SO_x Sulfur Oxides

TPTZ Tris(2-pyridyl)-s-triazine

UV Ultraviolet radiation

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

Society faces an exponential population growth and foresees a global food crisis in the mid of 2050 that can be anticipated by SARS-CoV-2 and pandemic COVID19 disease. This context turns even more critical the need to find more sustainable and functional ways to meet human nutritional needs. A potential solution is the use of microalgae as source of biocompounds namely for food industry. Microalgae biomass is a potential source of high value biocomponents with potential in some industrial applications as in biofuels, pharmaceuticals, nutraceuticals, food additives, cosmetics, among others [1,2]. They are a rich source of carbohydrates [3], proteins [4], lipids and pigments [5]; besides this, many vitamins, antioxidants and minerals are abundantly found in microalgae [1]. This characteristic makes microalgae an excellent food additive. Their growing conditions and bulk biomass production are easy to achieve on a wide range of environments even under extreme conditions (i.e., temperature, pH, light intensity and salinity), discarding the need of arable land. Microalgae is then envisaged as an emergent source of non-animal protein source for human consumption [1,6], and thus as sustainable way of food production. Microalgae are usually aquatic microorganisms but in some cases, they can also live in harsh terrestrial environments. In this particular work the used microalgae was isolated from a terrestrial lichen. The liquen are the symbiotic relationship between fungi and microalgae, we are just interested in the microalgae. The choice for this microalgae is because lichens live in extreme environments and this may be an indicator of unusual bioproducts.

The capacity to generate compounds of interest by microalgae is not a factor that contributes to the valorisation of microalgae. It becomes necessary to access these compounds so that the whole process can be viable [7]. Most of the products resulting from the metabolism of microalgae are intracellular, which is a disadvantage [5–7]. Disruption or alteration of cell wall structure can improve digestibility of intracellular nutrients of microalgae. In reason of this a set of cell disruption methodologies are currently available [2,6].

The disruption methods can be divided in two groups: mechanical and non-mechanical. The mechanical disruption methods such as bead milling, microwave, ultrasonication are responsible for a non-specific destruction of the cell wall. The non-mechanical disruption methods such as chemical agents, enzymes or osmotic shock are responsible for the cell lysis[6]. Disruption method should facilitate the access to the metabolite of interest, guarantee a significant yield of extraction

(when needed), but at the same preserve its biological value and include less number possible additional downstream steps thus representing a low operating cost. Methods that can offer an eco-friendly character and high energy efficiencies during operation bring also competitive advantages [2,5]. Based on these assumptions, there is today a growing interest in the application of electric fields based technologies as a method of cell disruption. The extraction of compounds through the application of external electric fields become known by electroporation effect. Electroporation consists in the formation of membrane pores on the cell surface that can be temporary or permanent depending on the intensity of the electric field applied [1,2,8]. Several studies have already shown the efficiency that the application of pulsed electric fields (PEF) in the extraction of proteins [4], and pigments [5,9] of microalgae biomass. Although the application of PEF at high field strength may result in some electroporation effects on cell membranes and allow extraction of some small molecules, the presence of a thick cell wall in microalgae limits PEF performance by conferring protection to internal organelles and avoiding removal of large intracellular molecules [7,10]. In reason of this, other treatment strategies have been exploited by combining several effects such as physical, enzymatic and chemical ones – i.e. high pressure, microwave, ultrasounds, use of solvents, among others - aiming an effective cell disruption[11]. One promising approach consists in the combination of controllable electrical and thermal effects through Ohmic Heating (OH) technology. OH is caused due to the resistance that a material offers to the passage of the electric current and this resistance will result in an internal energy dissipation in the form of heat [12,13]. OH as an extraction method allows synergism between thermal and electrical effects, enhancing each other effects [13]. There is evidence that points to a higher permeability increase for situations where OH is applied instead of conventional heating [14]. Jaeschke et. al. have shown that moderate electric fields (MEF) of approximately 40 V/cm, when combined with mild temperatures (< 35 °C) and solvent effects (i.e. ethanol) can yielded up to 73% and 83% of carotenoid and lipid extraction, respectively [9] . However, to the best of our knowledge, the full potential of OH, and possibility of applying high-temperature short-time (HTST) protocols in combination with MEF has not been applied or understood regarding extraction of macronutrients and molecules from microalgae. OH allow a fast-thermal process that contributes to a higher integrity of the biomolecules that are extracted [13]. OH offer operational advantages such as the fine-tune controllable heating rate, no residual heat generation/transfer after power shut-off and its volumetric heating, which can greatly reduce the occurrence of an excessive thermal load and

denaturation of the extracted biomolecules. The objective of this work is for the first time to carry out a multi exploratory evaluation of the combination of OH effects – i.e. intensity of electric field, temperature, and type of heating (continuous or pulsed) as a pre-treatment methodology in the weakening of microalgae cell structure.

2. STATE-OF-ART

Currently, the world is highly globalized, and this reality directly interferes with our daily lives, without us often realizing it. In a fast-paced, highly consumerist society, ordinary people do not concern themselves with the provenance of the products they consume on a daily basis. However, those who are more attentive understand that a great quantity of the products that are acquired come from the most varied origins (yeasts, bacteria or microalgae) and it is to be emphasized that such phenomenon would not be observable without the existence of a growing technological development.

The greatest difficulty modern society faces is the exponential population growth observed during the last century, where the world population has doubled. This has contributed to the need to find more affordable ways to meet human needs. Thus, several industries had to reinvent themselves to continue to satisfy the necessities of their consumers but now in an ecological and sustainable way, namely the food, pharmaceutical, cosmetic, biofuels and agricultural sectors.

Based on this problem, there is a need to find new and ecological ways of obtaining products of interest, in which the use of microorganisms as precursors for their production is highlighted. The use of these methodologies constitutes a great advantage for the overpopulation paradigm, where in a short time, with relatively simple procedures and in small spaces, high yields can be obtained in the production of important macronutrients and bioactive compounds. The conjugation of all these makes usage of microorganisms a sustainable basis to be explored to produce compounds like antibiotics, sugars like erythritol, proteins, lipids and others.

There is a huge variety of microorganisms that are used both in industrial and laboratory terms, such as fungi, yeasts or bacteria, but one of the most prevalent in recent times are the microalgae.

2.1 Lichen, the symbiotic organism

A lichen is not a single organism. Rather, it is a symbiosis between different organisms (**Figure 1**), a heterotrophic fungus (mycobiont) and a photosynthetic partner or photobiont, which can be either cyanobacteria or unicellular green algae. The lichen symbiosis involves close morphological and physiological integration between mycobiont and photobionts giving rise to the

lichen thallus, which is a unique entity or holobiont [15]. While most lichen partnerships consist of one mycobiont and one photobiont, that is not universal for there are lichens with more than one photobiont partner. When looked at microscopically, the fungal partner is seen to be composed of filamentous cells and each such filament is called a hypha. These hyphae grow by extension and may branch but keep a constant diameter. Amongst the photobionts there are those that are also filamentous in structure while others are composed of chains or clusters of more-or-less globose cells [16].

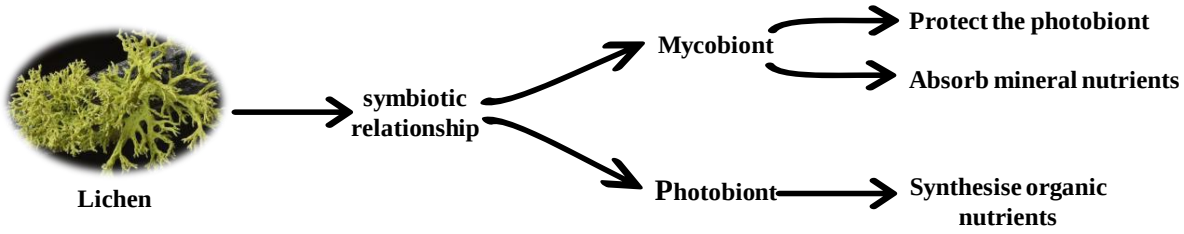

Figure 1- Symbiotic relationship of lichens and different roles of the two partners.

Lichen morphology is extremely diverse; these organisms come in a fantastic array of colors and can vary in size from very small individuals to large structures. They can be found growing in almost all parts of the terrestrial world, can colonize ice-free polar areas, tropical rainforests, desert areas or bare rock surfaces for example [17]. Lichens are often found in high humidity areas [18]. Despite this large range of environmental adaptations, most lichens are sensitive to changes of their preferred ecological conditions and can hardly grow in non-native habitats [19].

In lichen associations, both partners have benefits (**Figure 1**). The mycobiont has two principal roles in the lichen symbiosis: to protect the photobiont from exposure to intense sunlight and desiccation and to absorb mineral nutrients from the under lying surface or from minute traces of atmospheric contaminants. The photobiont also has two roles: to synthesize organic nutrients from carbon dioxide and, in the case of cyanobacteria, to produce ammonium [18]. The lichenization process is a very successful nutritional strategy adopted by more than 20 % of fungal species [20]. Consequently, through the lichen partnership, the photobionts are protected and able to grow in conditions in which they could not grow alone, benefiting from the highly efficient uptake of mineral nutrients by the lichen fungi. In turn, the fungi obtain sugars and in some cases organic

nitrogen from the photosynthetic partner, enabling them to grow in environments deficient in organic nutrients [18].

The symbiotic relationship between mycobiont and photobiont is in some cases so vital that microorganisms cannot survive without this symbiotic relationship. The morphology of lichens is determined by the mycobiont and can be divided in three main groups: crustose, foliose, and fruticose. Crustose lichens look very much similar a thin crust on the substrate. They are markedly two dimensional and firmly attached to the substrate by their entire lower surfaces and cannot easily be removed. Foliose lichens are two-dimensional leafy structures; they grow in a more-or-less sheet-like form, but often with a lobed appearance. They are not attached by their entire lower surfaces to their substrates. Indeed, some foliose lichens are just centrally attached to their substrates with the rest loose, so making it possible to see both the lower and upper surfaces very easily. Fruticose lichens are erect or pendulous and markedly three-dimensional. They grow preferentially in wet and/or humid climates and are organisms that stand out from their substrate and are characterized as hair-like, shrubby, moss-like, or pendulous [17].

Lichen grow in extreme environmental and these specifics adverse conditions allows the production of many metabolites that can provide good protection against various negative physical and biological influences. These protective substances that allows lichens to survive in these extreme environments are also unique, but little understood. Metabolites synthesised by lichens were divided into two groups: primary and secondary [18].

The primary metabolites consist of proteins, amino acids, carotenoids, polysaccharides, and vitamins. These compounds are non-specific and are produced by fungi and some by algae. Some of the primary metabolites also may occur in free-living fungi, algae, and higher plants. All these compounds are synthesized by both the microalgae and the fungus that make up the lichen except the vitamins. Vitamins were identified as metabolic products which biosynthesize microalgae, while the fungi are poor sources of these compounds [18].

The majority of organic compounds found in lichens are secondary metabolites [18]. Numerous lichens are known to produce unique secondary metabolites and have considerable biological activities. Secondary metabolites are organic compounds that are not directly involved in the growth, development and reproduction processes of organisms [21]. The lichen secondary

metabolites represent between 0.1 and 10% of the dry weight of the thallus and in some cases even 30%. These compounds are usually hydrophobic and can usually be isolated from the lichens by organic solvents [18,21]. More than 800 secondary metabolites are known from lichen, most are unique to these organisms and only a small minority occurs in other organisms [18].

The lichen compounds may be classified into the following groups: **(1)** aliphatic lichen substances (including acids, zeorin compounds, polyhydric alcohols); **(2)** aromatic lichen substances (including pulvic acid derivatives, depsides, depsidones, quinones, xanthone derivatives, diphenyleneoxide derivatives, nitrogen containing compounds, triterpenes, tetronic acids); and **(3)** carbohydrates (polysaccharides) [21]. Lichen-derived bioactive compounds have a lot of potential in many areas and with a wide range of applications like for medical use in antibiotics, antitumor and antimutagenic, antiviral and enzyme inhibitor [19].

2.2 Microalgae

Microalgae are an extremely diverse group of microscopic organisms, representing one of the oldest forms of life on Earth. Recently, these organisms have begun to be used as source of important bioproducts. These microorganisms are defined as primitive plants (thallophytes)-not presenting roots, stems and leaves- comprising unicellular plants (Chlorophyta), bacteria (Cyanobacteria), diatoms (Chromalveolata) and protists (Chromista) [1].

Microalgae are largely a diverse group of microorganisms comprising eukaryotic photoautotrophic protists and prokaryotic cyanobacteria [22]. Microalgae are generally a spherical microscopic cell and their cellular structure is extremely similar to the cellular structure of a plant cell [23]. Morphologically, microalgae are constituted by a set of organelles that perform the different metabolic functions necessary for cell survival.

Unlike the reproductive structures of plants, most algae reproduce asexually or by cell separation [22] (**Figure 2**). In this manner, four daughter cells having their own cell wall are formed inside the cell wall of the mother cell. After maturation of these newly formed cells, the mother cell wall ruptures, allowing the liberation of the daughter cells and the remaining debris of the mother cell will be consumed as feed by the newly formed daughter cells [23] . Since they do not need to generate elaborate support and reproductive structures, they can devote more of their energy into trapping and converting light energy and CO_2 into biomass [22].

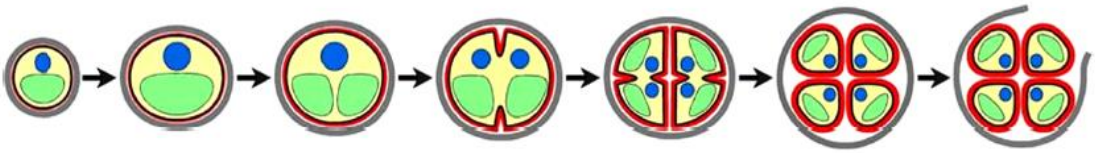

Figure 2- Scheme of the reproductive process of a microalgae [from 10].

The microalgae can be classified as autotrophs or heterotrophs, according to their nutritional requirements. (**Figure 3**). If they grow under autotrophic schemes, they use inorganic compounds as a source of carbon. Autotrophs can be photoautotrophic, using light as a source of energy, or chemoautotrophic, oxidizing inorganic compounds in order to obtain energy. If they grow under heterotrophic schemes, they use organic compounds for growth. Heterotrophs use organic compounds as a source of carbon; these can be photoheterotroph, using light as a source of energy, or chemoheterotrophs by oxidizing organic compounds for energy. Some photosynthetic microalgae can grow under mixotrophic schemes, combining heterotrophy and autotrophy by photosynthesis [24]. This capacity gives to the microalgae the ability to grow and survive under extreme conditions of pH, temperature or light intensity [25].

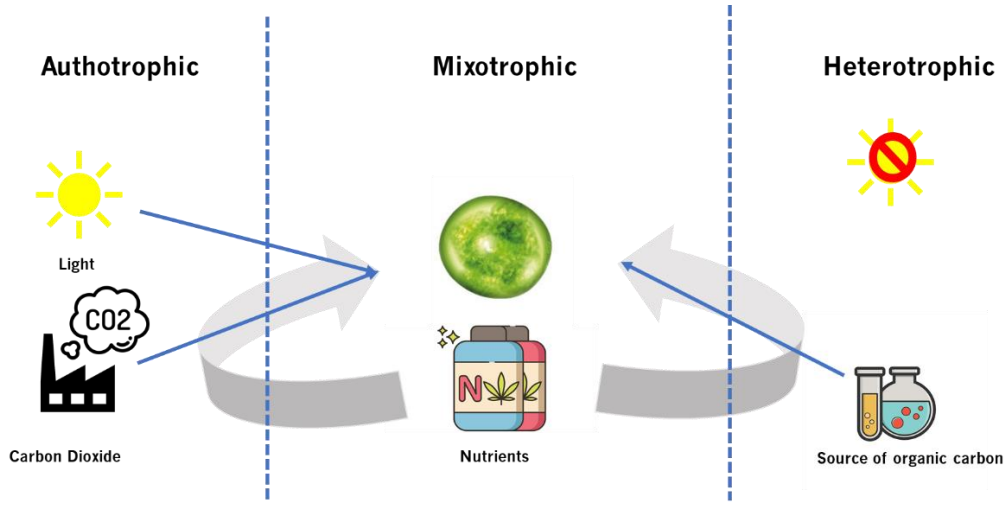

Figure 3- Nutritional reequipments of microalgae. Microalgae can be classified as autotrophic (carbon dioxide as carbon source), heterotrophic (organic compounds as a carbon source) or mixotrophic (combining heterotrophy and autotrophy) according their nutritional requirements.

The microalgae are a group of microorganisms that remain greatly unexplored since several million of species are estimated to existed [26]. As the predominant component of the marine and freshwater plankton, microalgae are primarily responsible for the 40-50 % of total global photosynthetic primary production. While the contribution by pelagic, coastal, and freshwater communities is relatively well understood, the significance of terrestrial microalgae is not known

with any degree of precision, but they may be important on a local or regional scale because of their predominance on extensive areas of exposed land surface in arid and semi-arid steppes and deserts [27].

Despite the importance of microalgae in the ecosystem, the recent growing interest in these microorganisms is due to their high biotechnological potentialities in several areas. Depending on the type of microalgae used, numerous bioactive molecules (**Figure 4**) such as pigments, antioxidants, proteins, fatty acids, carbohydrates and vitamins can be obtained for commercial purposes in different industrial segments, which include pharmaceuticals, cosmetics, nutraceuticals, functional foods, antifouling agents and biofuels [1,28].

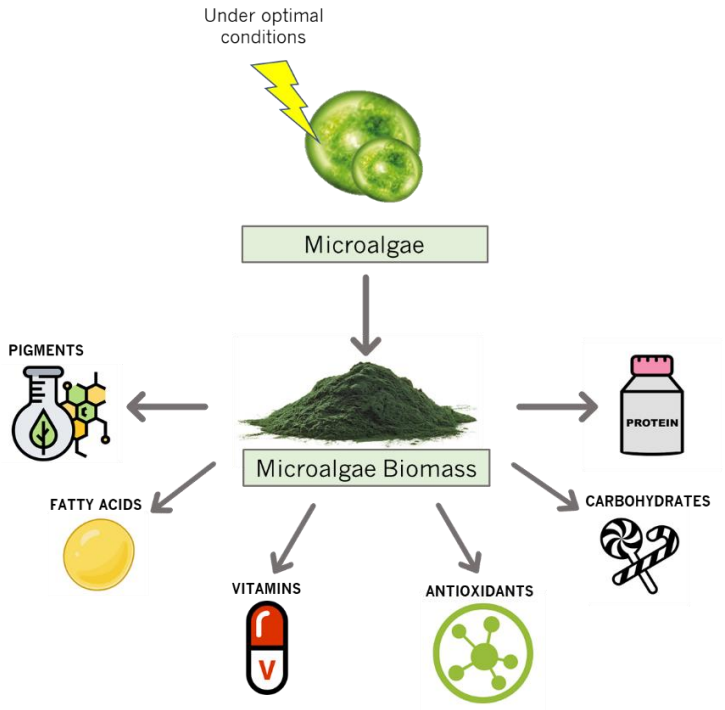

Figure 4- Microalgae metabolites of interest. Microalgae can be a source of a numerous metabolites such as pigments, antioxidants, proteins, fatty acids, carbohydrates, and vitamins used in several industrial segments.

2.2.1 Microalgae bioprocessing

In order to maximize the production of potential bioproducts obtained through the cultivation of microalgae, it is important to know their ideal growth conditions. There are many types of microalgae cultivation techniques depending on; i) the investment cost; ii) the desired products; iii) the source of nutrients; and iv) CO_2 capture [29].

After selecting a suitable microalgae strain to obtain the product of interest, all the process details from biomass production to product extraction and purification must be considered to get a viable commercialization strategy. As a result, the choice of an adequate cultivation method, whether by choosing an open or closed systems, must be intimately related to the product of interest and its application [30]. To reduce microalgae production costs and therefore stimulate its generalized commercialization, a bioeconomy concept should be implemented, for example by combining algal growth (in open or closed systems) with CO_2 fixation (i.e. utilize the CO_2 in stack gases from thermal power plants to synthesize a variety of valuable substances like starch or oil [31]) and/or wastewater treatment processes, supressing nutritional needs of microorganisms [1]. This will not only contribute to control issues involving greenhouse gases emissions (namely CO_2) and contaminants removal from wastewater, but also generate economic value utilizing wastes or by-products from other industrial processes [1,30]. Thus, besides ensuring environmental sustainability, such production scheme is also suitable to produce high added-value bioactive compounds [1].

Microalgae need the carbon source (carbon dioxide for example) and light within the photosynthetically active radiation (PAR) range to obtain energy by photosynthesis. The wavelength of the PAR ranges from 400 to 700 nm, which is equal to visible light. Illumination is the most important factor influencing biomass composition, growth rate, and product formation [31,32].

As the interest for microalgae increases, continuous production systems are attracting attention once, these systems are the most suitable way to achieve successful large scale production of those photosynthetic microorganisms mainly due to higher volumetric productivities, constant product quality, reduction of space requirement, decrease of labour costs, lower investment and operational costs and decrease of “unprofitable” periods [33]. Another important fact is that the microalgal biomass composition can be influenced via different cultivation conditions in order to achieve better outputs, e.g., of reserve materials (starch and oil) [31].

The batch culture is the most common method for cultivation of microalgae cells. In a batch culture a limited amount of culture medium is used, and the microalgae inoculum is placed in a reactor and incubated in a growth-friendly environment. The agitation is an important factor to ensure nutrient and gaseous exchange at the cell–water interface. Batch culture is widely used for

commercial cultivation of algae for its ease of operation and simple culture system. Since the process was widely standardized there is low requirement for complete sterilization. For mass algal culture production, a portion of the culture could be retained as inoculum for the next culture batch. The different growth phases, which may occur in a batch culture, reflect changes in the biomass and in its environment [34].

The first of these phases is the lag phase which is characterized by an adaptation of microorganisms to the culture in which there is a reduced growth of the biomass. The lag phase is followed by exponential one, which is characterized by an abrupt growth of the biomass present in the culture. This growth is a result of the availability of light and nutrients and the perfect adaptation of microalgae to the reaction medium. In turn, the stationary phase is characterized by a stagnation of biomass growth resulting from the accumulation of products from cellular metabolism as well as the depletion of some essential nutrients. In this phase also occurs the production and consequent accumulation of reserve products as is the case of starch, among others. The last phase is the death phase where there is a decrease in biomass resulting from the depletion of essential nutrients and the large amounts of products resulting from cell metabolism causing the culture to decay [35].

2.2.2 Microalgae and its applications

Depending on the type of microalgae used and the growth conditions, numerous bioactive molecules can be obtained for commercial purposes in different industrial segments (**Figure 5**).

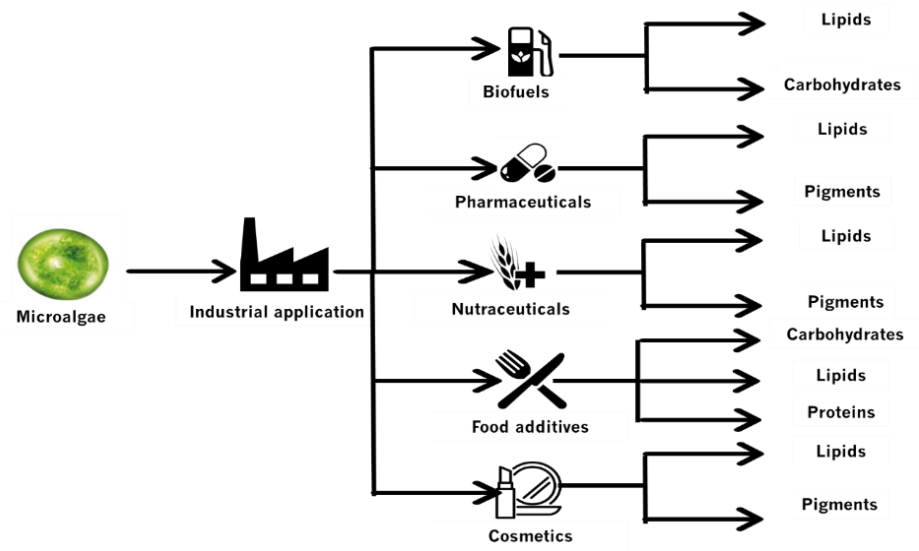

Figure 5- Potentials application areas of metabolites from microalgae. Microalgae can be directly used by humans as food supplements, can be used as a different source of biofuels and even can be used as sources of different bioproducts applied in industrial branches such as the pharmaceuticals or the cosmetics.

By virtue of their larger size and accessibility, seaweeds have attracted more attention than microalgae as source of natural products, including identification of novel compounds for pharmaceutical development [27]. In the last years, due to the considerable quantity of molecules obtained from microalgae with potential application in the pharmaceutical industry, these microorganisms have gained much more relevance. Microalgae may be potential sources of antiviral sulfolipids (e.g. sulfoquinosovyl diglyceride) [27] and carotenoids, such as astaxanthin and β-carotene. Astaxanthin is a high-value carotenoid produced by microalgae [36] that can act as an immune system enhancer and hormone precursor, as well as, having anti-inflammatory properties that might have a positive effect on human health, while β-carotene is well known for its effective control over cholesterol, as well as its anti-carcinogenic and anti-heart disease activities [1].

Components of microalgae are frequently used in cosmetics as thickening agents, sun protectors, hair care products, anti-ageing products, water-binding agents and antioxidants. The use of some microalgal species is already established in the skin care market, being the main ones *Arthrospira* and *Chlorella.* Certain microalgae also produce organic metabolites, such as sporopollenin, scytonemin and mycosporine-like amino acids, to protect themselves from ultraviolet radiation (UV) while allowing visible radiation involved in photosynthesis to pass through. Their capacity to protect themselves from UV radiation is very important because these microorganisms are predominantly found in aquatic environments where they are exposed to high levels of UV

radiation. The compounds responsible for this function are frequently used in the cosmetics industries due to their natural capability to absorb armful radiations [37]. Other compounds, like the carotenoid astaxanthin have potential applications in cosmetics as well. Astaxanthin is a potent antioxidant and has possible roles in human health such as UV-light protection, immune system enhancer, hormone precursor, source of pro-vitamin A and an anti-inflammatory agent. It is also a strong coloring agent that is used for coloring muscles in fish. The microalgae *H. pluvialis* is a rich natural source of astaxanthin and can produce 1–8 % astaxanthin dry weight. A lot of compounds used in the cosmetics industry can be used in other industries thanks to their untapped potential in other applications [38].

The age of cheap petroleum is ending. According to many analysts, at the present staggering rates of consumption, the world fossil oil reserves will be exhausted in less than 40 years [39]. The necessity to find new alternatives to fossil fuels is very important and today the potential value of microbial and, particularly, microalgal photosynthesis to produce biofuels is widely recognized [39]. The accumulation of large amounts of high quality lipids and carbohydrates, places microalgae as a very promising potential candidate to become the basis of the production of a great variety of renewable fuels like biodiesel, biomethanol, biomethane, bioethanol, biohydrogen, biobutanol and biohydrocarbon [1,24,40]. They have much higher growth rates and productivity when compared to conventional forestry, agricultural crops, and other aquatic plants, requiring much less land area than other biodiesel feedstocks of agricultural origin – i.e. up to 49 or 132 times less when compared to rapeseed or soybean crops, for a 30 % (w/w) of oil content in algae biomass. Therefore, the competition for arable soil with other crops, in particular for human consumption, is greatly reduced [40]. Microalgae biodiesel contains no sulfur and performs as well as petroleum diesel, while reducing emissions of particulate matter, CO_2, hydrocarbons and SO_x. However, emissions of NO_x may be higher in some engine types [40]. There are several ways to convert microalgal biomass to energy sources, which can be classified into biochemical conversion, chemical reaction, direct combustion and thermochemical conversion. Thus microalgae can provide feedstock for a renewable liquid fuels [37].

Microalgae are a rich source of carbohydrates, proteins, enzymes and fibers. Besides these, many vitamins and minerals like vitamin A, C, B1, B2, B6, niacin, iodine, potassium, iron, magnesium and calcium are abundantly found in microalgae [37]. This characteristic makes

microalgae an excellent food additive. The starting point on microalgae used by humans occurred about 2000 years ago, when the Chinese started using them as a food source [1]. Asian countries, like China or South Korea, widely use microalgae in food [41,42]. In spite of the significant efforts to introduce microalgae into the human diet linking them to healthy food, in the western world, the strict food regulations have shown to be restrictive to this mind-set shift [1]. Consequently, microalgae cultures have been mostly used as feed additives in aquaculture food chain. The survival, growth, development, productivity, and fertility of animals reflect their health. Feed quality is the most important exogenous factor influencing animal health, especially in connection with intensive breeding conditions and the recent trend to avoid "chemicals" like antibiotics. Animals whose diet is based on microalgae show an improvement in these factors [1,26,38]. Even do there are certain fatty acids that our organism is not able to synthesize – the essential fatty acids. These have a vital importance, contributing to the health of human beings and therefore must be acquired throughout a person's diet [43]. One group of those fatty acids is the polyunsaturated fatty acids (PUFAs) which consumption has been associated with good health indicators. Fish and fish oil are currently the main sources of PUFAs but their application as a food additive is limited due to the possible accumulation of toxins, fish odor, unpleasant taste, poor oxidative stability, presence of mixed fatty acids which turn them unsuitable for vegetarian diets [38]. Microalgae are a primary source of PUFAs and can sustain whole food chains suppling other organisms, such as higher plants and animals that lack the presence of enzymes to synthesize PUFAs. Microalgal PUFA also have many other applications such as additives for infant milk formula [38].

Microalgae have developed chemical strategies to communicate, defend, and adapt to their environment. Hence, they are able to produce a large diversity of metabolites including those being potentially toxic or having biological activities [44]. Therefore, more attention has been given to microalgal intracellular and extracellular metabolites, namely toxins produced by several strains of cyanobacteria [1,45]. Marine cyanobacteria have been shown to produce a diverse array of biologically significant natural products with activity in models for anticancer, neuromodulatory and anti-inflammatory drug discovery, and other areas [46]. These toxins and other metabolites present a wide range of biological activities including antialgal, antibacterial, antifungal and antiviral activity which are certainly useful from the pharmaceutical point of view [1].

The worldwide nutraceutical market has been increasing due to current population needs and health trends. Nutraceuticals are food or parts of food that have an important role in normal physiological functions in order to maintain human beings healthier [47]. Microalgae are an important source of a lot of compounds that can be classified as nutraceuticals. Compounds such as PUFAs and their derivatives, some lipids obtained from microalgae, clionasterol, lutein or C-phycocyanim are examples of nutraceuticals obtained from microalgae [1,11].

2.3 Cell disruption and extraction methodologies

The capacity to generate compounds of interest from microalgae itself, is not a factor that contributes to the valorisation of microalgae biomass. It becomes necessary to access these compounds so that the whole process can be viable. Most of the products resulting from the metabolism of microalgae are intracellular, which is a disadvantage. Traditionally, in order to overcome the physical barriers offered by the cell structures (wall and membrane) and obtain the bioproducts of commercial interest, the microalgae is subjected to different conventional extraction methods such as homogenization, heating, osmotic shock or exposure to organic solvent [1]. These methods often compromise cell wall and cell membrane resulting on the releases of the bioproducts. These methodologies have the purpose of maximizing the collection of one specific product only, even if it represents destruction or damage and subsequent loss of all the other high-added value metabolites that may be present [1,48]. These traditional extraction techniques have some drawbacks as follow: low eco-friendly character since due to the use of polluting raw materials, such as organic solvents, that subsequently must be treated in a way that does not affect the ecosystem; long extraction or treatment times; low selectivity and/or low extraction yields; and exposure of the extracts to excessive heat, light, and oxygen [48,49]. Furthermore, many of extracted fractions must be purified before being applied in the different industrial branches since they are contaminated with organic solvents used in the extraction process. These additional treatments are also time consuming and entail costs, which at industrial scale are impracticable. In the **Table 1** are present a set of cell disruption methodologies used in microalgae in a general way. Cell rupture methods consists of the complete or partial destruction of the cell wall to release intracellular compounds. This methodologies requires the breakdown of cell wall compounds, for the destruction of contact resistance [50].

Table 1- Different cell disruption techniques that could be applied in microalgae biomass to obtain bioactive molecules for commercial purposes.

Cell Disruption	Principles	References
Acid treatment	Hydrothermal acid treatment used to extract biocompounds (eg: sulfuric acid).	[51]
Alkaline treatment	Extraction under alkaline conditions using for example sodium hydroxide.	[52]
Bead Milling	The sample is placed in a chamber together with glass beads and subjected to rotational speed for a certain period of time.	[53]
Electrotechnologies	The exposure of cells to an external Electric Fields can alter the structure of their membrane since it causes a transmembrane charge exchange. Consequently, membrane loses its barrier function and becomes permeable, a phenomenon often referred to as electroporation or electropermeabilization	[1]
Enzymatic Lysis	Use of enzymes that will cause microalgae cell disruption. Long incubation periods are usually required and enzymes such as cellulase are used.	[53]
Manual Grinding	Mechanical breaking methodology in which the sample is ground with a pestle. To enhance the extraction rate, compounds such as liquid nitrogen or quartz sand may be used.	[53]
Ultra-sonication	The samples are subjected to ultrasound in order to cause its heating and subsequent extraction. To avoid denaturation of the extracted compounds, the treatments consist of repetitions of short-term treatments that occur in an ice bath.	[53]
Microwaves	Use of a microwave oven at a high temperature (about 100 °C and 2,450 MHz) for a certain period to promote the cell disruption.	[53]
Osmotic shock	Osmotic shock is physiologic dysfunction caused by a sudden change in the solute concentration around a cell, which causes a rapid change in the movement of water across its cell membrane	[54]
High pressure homogeniser	High pressure homogenisation is a purely mechanical process, which is evoked by forcing a fluidic product through a narrow gap (the homogenizing nozzle) at high pressure.	[55]

In addition to cell disruption methodologies, there are also extraction methodologies that consist of using a certain organic solvent that has an affinity for a given bioproduct. Organic solvents such as diethyl formamide, methanol, ethanol and acetone can be used for example for chlorophyll

extraction. These solvents penetrate the cell membrane to dissolve lipoproteins of chloroplast along with the lipids into the extract phase. This type of methodology is more efficient when before the extraction occurs a pre-treatment (e.g.; acid treatment, thermal or electrothermal treatment or beading meals) [50,56].

2.4 Ohmic Heating

Currently, OH is in the restricted range of technologies considered to be emerging in food processing. Also referred to as Joule heating or electroheating, it is a process in which the electric current passes through a (semi-) conductive material allowing heat dissipation. The resistance that the product offers to the passage of the electric current allows the internal generation of energy, dissipated in the form of heat, that makes this technology not dependent on phenomena of conduction of heat transfer from an external source [12,57]. Many of actual and potential applications for OH, includes unit operations such as blanching, evaporation, dehydration, fermentation, extraction, sterilization, pasteurization and heating of foods to serving temperature, including in the military field or long-duration space missions [12]. Additionally, research data suggests that the applied electric field under OH can causes electroporation of cell membranes independently of heat dissipation [57].

The beginning of this technology goes back to the 20th century and, at an early stage, due to lack of knowledge of fundamental and technological principles, as well as high operating costs, its development and application become limited. Currently, an OH system can easily be integrated into existing processing lines, consisting of hollow pipe that in one part of its path has a pair of electrodes placed in opposite positions or in integrated rings, which has the function of applying the electric field in a uniform way to the food that its placed or passes between them (**Figure 6**) [12].

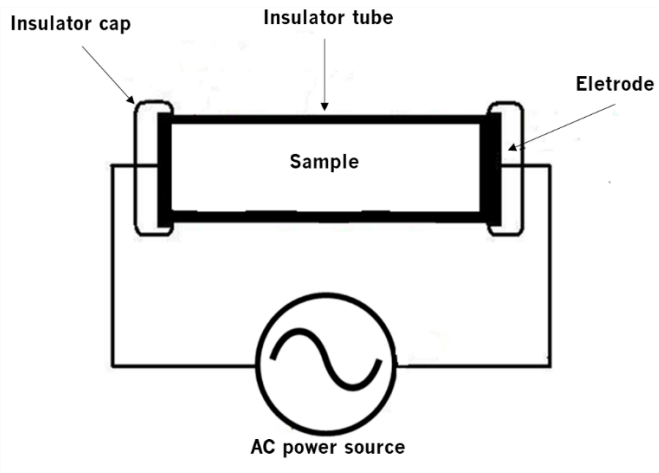

Figure 6 - Schematic representation of the OH system.

There are several advantages of this technology, and the one that has aroused much more interest from the point of view of industrial use is to reduce the exposure of the food product to heat thus reducing the time required to obtain the target temperature of a particular heat treatment. In addition, the OH also has other advantages, as follows [12]:

- Absence of hot surfaces, with reduction of the over processing of the product;
- High heating efficiency both in terms of fastness (HTST principle) and in terms of product temperature uniformity in liquid and solid states; minimizing the loss of organoleptic and nutritional properties;
- Ideal for the processing of viscous products, since it allows a uniform heating not being limited to the phenomena of conduction and convection;
- High energy efficiency (over 95%);
- Is considered environmentally-friendly technology;
- Low mechanical stress induced in the food, ideal for sensitive foods (e.g. pieces of fruit); whose integrity is intended to be preserved;
- Simple base process with low maintenance costs.

The main disadvantages associated to this technology are the lack of information/research on many industrial processes, which makes difficult its validation, and the assurance of the initial investment. The lack of data for technology validation can also raise several food safety issues.

During the last decades, and as a result of the important technological advances and consequent increase of fundamental research related to the electrical processing, several questions

have been posed on the effects of some electrical variables, such as frequency and intensity of the electric field. This systematic research has been contributing to technology validation and new applications regarding food biotechnology. Some studies prove the existence of non-thermal effects associated with OH and the inherent presence of moderate electric fields in the order of 1 to 1000 V/cm, also known as MEF. Specifically, the application of MEF can induce effects at the cellular structures, promoting for example the inactivation of contaminating microorganisms at room temperature, due to the eventual formation of pores in cell membranes - a phenomenon also known as electroporation. Other potentialities of the electroporation phenomenon promoted by the application of MEFs are the permeabilization and rupture of plant tissues in biotechnological processes. Combination of thermal and electrical effects offer potential to facilitate the extraction of cellular compounds of interest and promote the diffusion of solutes, this is now another reason of interest for this technology [12].

2.5 Extraction of bioactive compounds - OH as an extraction methodology

Based on these facts, there is an opportunity to create an alternative to the existing extraction methodologies. OH offer potential advantages regarding conventional extraction technologies: is more environmentally friendly methodology that does not require purification steps and presents low application costs; can be easily applied at industrial scale once equipment at industrial scale is already developed; and that above all it can highly efficient in the thermal extraction of the desired bioproducts. This lead to growing interest on the effects of EFs (Electrical Fields) processing in biological systems attending the need for more efficient and cost-effective technologies [1]. The presence of an electrical field may result in different outcomes and practical applications. In the case PEF, inherent electrical properties of sample,strength of the external field, pulse shape, pulse duration, number of pulses applied, and specific treatment energy are important parameters to be controlled [58]. The exposure of biological cells to electric field can alter the structure of the cell membrane and promote electroporation that enables extraction of bioproducts [58]. PEF processing is being also extensively investigated as an innovative nonthermal technique to increase mass transfer across cellular membranes due to electroporation effects [59]. PEF involve the application of electrical pulses usually at high voltages (kV range) and short durations (micro or nano-seconds) to a product placed between two electrodes [1]. The use of high intensity voltages causes an increase in the permeability of cellular membrane, promoting the phenomenon of

electroporation. This increase in permeability may be temporary which the cell recovers to original state, to a permanent permeabilization or even, in extreme cases, cell lysis [1,60]. Applying PEF in a reversible way increases the mass transfer across the cell membrane without impeding the cell's physiological state [60]. Considering these facts, it is therefore possible to think of a cyclical extraction approach where biocompounds can be extracted sequentially and over time from the same microalgae sample [60]. Compared with the established extraction methodologies, the treatment with PEF showed lower yields, but considering the possibility of cyclical extraction, these lower yields can be increased.

The effectiveness of the EF is the result of the field strength applied and the cell radius, meaning the smaller the cell radius is, the higher the external field needs to be applied in order to achieve the same effect [1]. Another consequence of exposure to EF is heat generation in the medium through the Joule effect. In this process, commonly referred as OH, where heat is produced directly within the material itself, as the electric current passes through the semi-conductive material. Heating occurs due to friction and molecular agitation provoked by the motion of charged molecules within the material [1].

In recent years, the use of MEF, that is, electric fields of intensity ranging from 1 V/cm to 1 kV/cm, associated with OH, has aroused interest in the scientific community about their applicability in industrial processes as a method of extracting cellular bioproducts [1,58,61]. OH presents high heating rates with a precise temperature control allowing mild processing, which helps to preserve nutritional, functional and structural properties. Heat is generated inside the material to be heated (Joule effect), the heating process does not depend on heat transfer between phases and interfaces, allowing uniform heating and an extremely rapid heating rate [61]. For a more clearer understanding it is important to highlight that MEF and OH are very often used in an interchangeably way, but usually MEF is associated to non-thermal or mild thermal processing effects where electric effects can be proven, while OH refers to thermal processes where thermal and electrical effects occurs simultaneously.

The electroporation effects of MEF and their use in extraction of compounds of interest from cells is well recognized. Either alone or conjugated with mild thermal effects, the potential for MEF extraction has been significantly reviewed [8,14,62], especially in vegetable tissues. According to

some experimental approaches [9] MEF was used for the extraction of lipids and carotenoids from microalgae. Results have shown that MEF can improve the carotenoid extraction, while lipid is only influenced by the solvent [1,9]..

Within OH, there are three possible extraction approaches: 1) application of MEF for long periods of time together with a controllable increase/maintenance of temperature; 2) application of MEF of increasing intensity maintaining temperature as low as possible; 3) or combining MEF with high-temperature for a short-time (HTST) period in an attempt to combine thermal and electric effects at their full potential. These different approaches will be responsible for different disturbances in the level of the microalgae cell membranes, therefore different extraction profiles are expected. In this work, we intended to explore some these treatments strategies, in particular the one where high temperature and electrical field effects are combined in attempt to seek synergistic effects.

3.AIMS

Microalgae present themselves as a class of microorganisms with high capacity to generate diversified bioproducts with potential of application in several branches of the industry. Despite this aptitude, the great challenge lies in obtention of these bioproducts, since these, as a rule, are intracellular and are therefore inaccessible. The existing methodologies present several shortcomings due to the low extraction efficiency, in the inability to extract compounds from different classes, or even the fact that they are not environmentally friendly methodologies. In this context, the need to find a new methodologies or strategies that can respond positively to the existing challenges has arisen. OH through the application of electric fields in a closed circuit can be a potential solution for sample pre-treatment for further enhanced or selective extraction, but there stills a lack of information regarding this possibility.

The objective of this work is to perform a multi-exploratory evaluation of OH as methodology for the pre-treatment of microalgae envisioning enhanced extraction of in order to conclude about their feasibility and possible application in research studies but also at possible industrial applications.

4.MATERIALS AND METHODS

4.1 Microorganism, culture and experimental conditions

In this approach it will be used microalgae isolated from a folicular lichen, labelled as LFR1. (species under identification) was found in rocks of Castro de São Lourenço, Esposende, a city of the north region of Portugal. These microalgae live in organisms capable of surviving on extreme environments and with deprivation of nutrients and because of that they are considered to be an enriched source of bioactive compounds.

Batch culture experiments were carried out in 5000 mL Erlenmeyer in autotrophic conditions with constant light supply (86,54 µmol) and air stream. The growth medium consists in macro and micronutrients important for microalgae growth and maintenance (**Table 2**). After 15 days of growth, the microalgae were submitted to extraction treatments.

Table 2- Growth medium constituents and their concentrations.

Reagent	Concentration (g/L)
Urea	1.100
Monopotassium phosphate	0.238
Magnesium sulfate heptahydrate	0.204
EDTA NaFe	0.040
Calcium chloride dihydrate	0.116
Boric acid	0.416
Copper (II) sulfate pentahydrate	0.473
Manganese (II) chloride tetrahydrate	1.647
Cobalt (II) sulfate heptahydrate	0.308
Zinc sulfate heptahydrate	1.339
Ammonium molybdate tetrahydrate	0.086
Ammonium metavanadate	0.007

4.2 Thermal extraction methods

Microalgae samples were centrifuged for 10 minutes at 3000 G (Centurion, model CR7000R (2/3L), India) to be concentrated to a concentration of 10 g/L. In each test, an aliquot of 10 mL of the microalgae solution were used. The treatment reactor (**Figure 7**) consisted on a cylindrical

glass tube of (30 cm total length and an inner diameter of 2.3 cm) made of a tempered glass (pirex 3 mm thick), with a cooling / heating recirculation jacket and equipped with a thermocouple type-K (temperature precision of ±1 °C; Omega Engineering, Inc., Stamford, CT, USA), connected to a data logger (USB-9161, National Instruments Corporation, Austin, TX, USA) for online acquisition. A magnetic stirrer was included in the reactor to allow a good homogenization of the culture under the different treatments. Several pre-treatments combining temperature and electric fields were evaluated as follow.

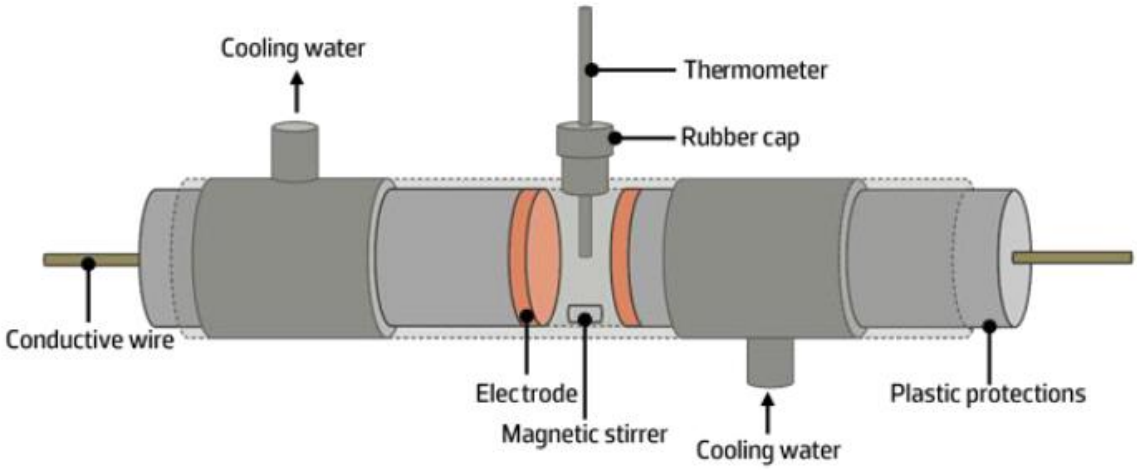

Figure 7- Schematic representation of the ohmic heating system using during the practical assays.

4.2.1 Ohmic Heating (OH)

The OH was carried out for 10 minutes in a temperature range of 50-70°C. The choice of these treatment conditions were based on preliminary work (results not shown) [63] and on reported evidence that electroporation and subsequent extraction at temperatures above 60°C can decrease the extraction rate [13]. The OH system consisted in i) a function generator that generates an electrical wave to be applied (Agilent 33220A, Penang, Malaysia); ii) an electrical signal amplifier (Peavey CS3000, Meridian, MS, USA) which will amplify the electrical signal previously produced; iii) a jacketed glass reactor (described above), with two stainless steel electrodes isolated at each edge with Teflon; v) and a portable multimeter for electric current and voltage acquisition (ScopeMeter® 125/S, Fluke, Everett, WA, USA). In this range of treatments electric fields of 10 V/cm to 100 V/cm were applied and electrical frequency was kept constant at 50 Hz.

4.2.2 Ohmic Heating Pulses (OHP)

In this method was applied the principle of HTST processing using OH [12]. Based on this principle, a set of temperatures were tested from 60°C (temperature selected based on the facts mentioned in the previous parameters [13]) to 100°C with treatment times varying from 7 to 20 s. In each treatment, different number of thermal pulses were applied (from 1 to 5) In this this range of treatments electric fields of approximately 100 V/cm to 230 V/cm were allowed to be applied and electrical frequency was kept constant at 50 Hz.

4.2.3 Conventional Heating (COV)

These treatments were carried out for 10 minutes in a temperature range of 50-70°C. simulating thermal history OH treatments described in section 2.3.1. COV was performed through indirect heating using recirculation jacket connected to a temperature-controlled water bath. **Figure 8** shows an example of the thermal profiles of different pre-treatments.

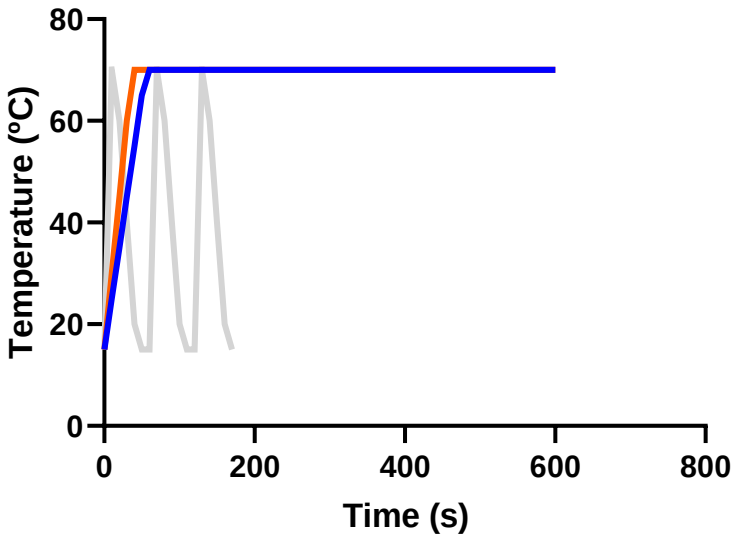

Figure 8- Temperature variation profile over time during the extraction test where ● corresponds to COV, ● corresponds to OH, ● corresponds to OHP.

4.3 Total extraction

To evaluate the effectiveness of pre-treatments on the aqueous extraction, referenced physical and chemical disruption methods were employed to according with metabolite or molecule of interest.

4.3.1 Bead milling technique

The bead milling equipment was used as disruption technique for the extraction of maximum organic matter. This method is described as having a high disruption efficiency greater than 98% [64]. The total volume used was 2 mL with 42% of glass beads (Sigma Aldrich, EUA) (0,149–0,250 nm) and 58% of microalgae biomass. The treatment time was 5 minutes in a vortex mixer Clifton Cyclone CM-1 (Nickel Electro Ltd., UK.)

4.3.2 Solvent extraction (methanol)

Methanol was used for the total extraction of the three main pigments – chlorophyll a and b and carotenoids [65] This extraction methodology is responsible for an almost total efficiency of the pigments. Aliquots of 1mL microalgae biomass samples were centrifuged for 5 min at 10000 g in microcentrifuge (Mikro 120, AndreasHettich GmbH & Co.KG, Tuttlingen, Germany) and the resulting supernatant discarded, this process was repeat two times with distilled water. After the last centrifugation, 1 ml of methanol was added to the pellet and then incubated for 30 minutes at 45 °C. According to the following equation we estimated the total concentration of carotenoids, chlorophyll a and b in microalgae biomass [66]:

$$\text{Chlorophyll a} = 16.72\ A_{664} - 9.16\ A_{652}$$

$$\text{Chlorophyll b} = 34.09\ A_{652} - 15.28\ A_{665}$$

$$\text{Carotenoids} = \frac{1000\ A\ 470 - 1.63\ Ca\ - 104.96\ Cb}{221}$$

4.3.3 Alkaline hydrolysis

Total amount of protein was determined using alkaline hydrolysis with NaOH. It is described that the use of alkaline conditions contributes to an increase in protein extraction yield [64]. This extraction methodology is responsible for an almost total recovery of the proteins. At 500 µL of microalgae biomass was added 500 µL of 1M NaOH and this mixture was incubated for 10 minutes at 100 °C and kept for further analysis.

4.3.4 Anthrone Method

Carbohydrates were measured by anthrone method [67]. This method is considered as one of the most convenient colorimetric method for determination of total sugar concentration. The

microalgae sample (1 mL) was centrifuged for 5 minutes at 10000 g in microcentrifuge (Mikro 120, AndreasHettich GmbH & Co.KG, Tuttlingen, Germany) and the supernatant discarded. The pellet was hydrolysed with 1 ml of HCl in a bath at 100°C for 2 hours. To 200 µL of the hydrolysate were added 400 µL of sulphuric acid (74 %). After mixing, 800 µl of Anthrone solution was added. Subsequently, the mixed was placed to the water bath at 100 °C for 15 min. After incubation time, samples cooled down to the room temperature and measured optical density at 578 nm [68,69].

4.3.5 SPV Method

For the quantification of lipids by the sulfophosphatevaniline (SPV) method, 100 µL of supernatant were collected from the extractions performed. To the collected supernatant, 2 ml of sulfuric acid (97 %) was added, and the samples were then placed in a bath at 100 °C for 10 minutes and then the samples were placed on ice for 5 minutes on ice. About 5 ml of the phosphate-vanillin solution was added to each sample and placed for 15 minutes in a bath at 37° C with a stirring of 200 rpm. Finally, absorbance was read at 530 nm and related to the calibration curve (0-70 µg) [70]. The phosphate-vanillin solution was prepared by diluting vanillin in absolute ethanol (60 g/L). This solution was then dissolved in 1:10 distilled water. Finally, this solution was dissolved (1:5) in phosphoric acid [70].

4.4 Chemical characterization

Untreated (negative control) and treated microalgae suspensions using classing disruption methods (positive control, described in section 4.3) and COV, OH and OHP methods (described in section 4.2) were centrifuged and the supernatant collected for characterization. regarding organic content, pigments (chlorophylls and carotenoids), proteins, lipids and carbohydrates as follows:

4.4.1 Protein content

Widely used Lowry estimation is acceptable alternative to determination of proteins. The sensitivity of this method is constant for various proteins. Method is based on Biuret reaction, and sensitive down to 0.01 mg of protein/mL. Immediately before determination of proteins complex-forming reagent (Reagent I) was prepared. **Reagent I** consist of three stock solution a that solution A, B and solution C in the ratio 10:1:1, in this sequence.

Solution A: 2.5 g Na2CO3 in 50 ml distilled water

Solution B: 0.1 g NaK Tartrate in 5 ml distilled water

Solution C: 0.05 g CuSO4.5H2O in 5 ml distilled water

Reagent II consists of 2N Folin-Phenol reactants with distilled water, in the proportion 1:2, respectively. Stock solution is prepared from standard protein, bovine serum albumin (BSA), with concentration 1 mg/mL and diluted this solution to the desired concentration (0-100mg/L).

Hydrolysis was performed added 500 μL of sample, 500 μL of 1 M NaOH, in water bath at 100 °C for 10 min. After incubation, samples were centrifuged. Subsequently, 500 μL of supernatant was removed and added to the Eppendorf with 1.25 mL of Lowry reagent (Reagent I) and incubate in the dark at room temperature for 10 min. After incubation time, add 250 μL of Folin reagent and incubate in dark for 30 min [71,72].

4.4.2 Determination of pigments

In order to evaluate the effects of OH, COV and OHP treatments on the extraction of the chlorophyll a, chlorophyll b and carotenoids, aliquots of 1 mL of treated samples were taken and centrifuged for 5 min at 10000 g. After that, the supernatant was discarded, and 1 ml of ethanol was added to the pellet. After 30 minutes, the solution was centrifuged for 5 min at 10000 g in microcentrifuge (Mikro 120, AndreasHettich GmbH & Co.KG, Tuttlingen, Germany) and the supernatant was measurement at 470 nm, 648 nm and 664 nm. According to the following equation we estimated the concentration of carotenoids, chlorophyll a and b [66].

$$\text{Chlorophyll a} = 13.36\ A_{664} - 5.19\ A_{648}$$

$$\text{Chlorophyll b} = 27.43\ A_{648} - 8.12\ A_{664}$$

$$\text{Carotenoids} = \frac{1000\ A\ 470 - 2.13\ Ca - 97.64\ Cb}{209}$$

4.4.3 IOMR measurement

After OH and conventional thermal treatments, samples of 500 μL were taken and then centrifuged at 11290 g in microcentrifuge (Mikro 120, AndreasHettich GmbH & Co.KG, Tuttlingen, Germany) for 10 min. The supernatant was collected and its absorbance was measured at a

wavelength of 254 nm in microplate absorbance reader. This procedure allowed detecting the IOMR from the cells at different conditions. The extracellular organic matter present in original sample (before starting disruption process) was also determined applying the same method [73,74]. To facilitate the interpretation of results, an IOMR factor was calculated for each sample tested according to the following equation:

$$\text{IOMR factor} = \frac{Abs_t - Abs_0}{Abs_0}$$

where Abs_t refers to the absorbance measured at 254 nm after disruption treatment during a certain period of time (t, min) and Abs_0 is the absorbance measured at 254 nm of the original sample (before treatment).

4.4.4 Determination of Lipids

The determination of lipids was carried out by the SPV method [70] (described in section 4.3.5). After the electrical treatments, 1 mL of microalgae suspension was centrifuged at 10000 g in microcentrifuge (Mikro 120, AndreasHettich GmbH & Co.KG, Tuttlingen, Germany) for 5 minutes and the supernatant was discarded. 1 mL of ethanol was added to the pellet and after 30 minutes 100 µL of the extract was analyzed by SPV methods.

Regarding calibration curve, we used canola stock solution with concentration 100 mg/mL and diluted this solution to the desired concentration (0-70µg). These solutions were used for preparing the standard curve [70].

4.4.5 Determination of carbohydrates

The determination of carbohydrates was carried out by the method of anthrone [67] (described in section 4.3.4). The only change to the protocol was the replacement of hydrolysis with HCl by the tested OH, OHP and COV treatments.

Regarding calibration curve, it was used D-glucose stock solution with concentration 250 mg/L. This stock solutions was diluted to concentrations in the range between 250 and 0 mg/L for the preparation of a standard curve [68].

4.5 Antioxidant activity

Antioxidant activity was measured by three assays - ABTS, DPPH and FRAP - for each of these methods a specific calibration curve was also made.

4.5.1 ABTS

The ABTS radical scavenging assay was carried out preparing a solution of 7 mM ABTS and 2.45 mM potassium persulphate in distilled water. The reaction mixture was prepared join the previous solutions in the dark for 16 h at room temperature. After the necessary time, 250 µl of the solution were diluted in 22 mL of absolute ethanol. 50 µl of sample was mixed with 1 mL of diluted ABTS solution. After 3 minutes incubation at room temperature, the absorbance was measured at 734 nm [75,76].

The ABTS radical scavenging activity was analyzed considering the next equation.

$$\textbf{ABTS radical scavenging activity (\%)}\left[\frac{(Ac-As)}{Ac}\right] \times 100$$

where A_c refers to the absorbance at 734 nm of the blank and A_S refers to the absorbance at 734 nm of the different samples analyzed.

Regarding calibration curve, we used trolox stock solution in ethanol solution (10%) with concentration 50 $mg{\cdot}L^{-1}$ and diluted this solution to the desired concentration (0-50 mg/mL). These solutions were used for preparing the standard curve.

4.5.2 DPPH

The DPPH radical scavenging assay was carried out preparing a 0.1mM DPPH in ethanol solution (50 %). 150 µl of the previous solution was added to the 50 µl of sample and after 30 minutes in the dark at room temperature the absorbance was measured at 515 nm [76].

The DPPH radical scavenging activity was analyzed considering the next equation:

$$\textbf{DPPH radical scavenging activity (\%)}\left[\frac{(Ac-As)}{Ac}\right] \times 100$$

where A_c refers to the absorbance at 734 nm of the blank and A_S refers to the absorbance at 734 nm of the different samples analyzed.

Regarding calibration curve, we used BHT stock solution in ethanol solution with concentration 2 mg/mL and diluted this solution to the desired concentration (0-1,2 mg/mL). These solutions were used for preparing the standard curve.

4.5.3 FRAP

The FRAP assay was carried out preparing the FRAP reagent. The FRAP reagent was constituted by 10 mL of 300 mM sodium acetate buffer with pH 3.6, 1 mL of 10 mM TPTZ in hydrochloric acid (40mM) and 1 mL of 20 mM $FeCl_3$. To complete the preparation of the FRAP reagent, it was heated to 37 °C for 20 minutes. 20 µl of sample was mixed with 150 µl of FRAP and the absorbance was measured at 593 nm [75].

Regarding calibration curve, we used $FeSO_4.7H_2O$ stock solution in distilled water with concentration 2 mg/mL and diluted this solution to the desired concentration (0-1000µM). These solutions were used for preparing the standard curve.

4.6 Fluorescence analysis

In order to confirm the presence of chlorophylls and proteins in the samples resulting from the thermal extraction methods, fluorescence at respective excitation and emission wavelengths was performed. The presence of proteins was made through the evaluation of fluorescence spectra from 300 to 400 nm after excitation at 290 nm (tryptophan excitation), which allowed to confirm the presence or absence of the proteins with tryptophan residues [77]. The presence of chlorophylls was made through the evaluation of fluorescence spectra from 400 to 450 nm and spectra emission from 500 to 800 nm [78].

4.7 Extraction rate

Extraction rate (in percentage) and impact of treatment on microalgae suspensions was determined by the ratio between concentration of metabolites determined after pre-treatments – i.e., COV, OH and OHP (in accordance with methods described in section 4.4) - and the concentration determined for the same metabolite after applying methods for total extraction (positive control) described in section 4.3.

4.8 Flow cytometry

The cellular integrity was measured by flow cytometry, through a *Cell analyzer Sony EC800* (Sony Biotechnology Inc., USA). The cytometry analysis was done for every experimental condition in triplicate. In each run, a volume of 200 μL was used by the flow cytometer for analysis of cells in the sample. A specific protocol was designed in the cytometer software to evaluate the cell rupture of the culture used. This protocol can identify the different cell populations present in the culture and the disrupted cells of those populations. Several parameters were analyzed with this protocol: the cell complexity (SS), the cell size (FS) and the cell disruption.

Each cell that enters the cytometer is read as a single event, and the light that passes through the cell reaches the filter that corresponds to the emission zone of chlorophylls, the filter is FL3. The FL3 signal is used to plot a graph capable of show the cells disruption, the graph was plot with the axes FL3-Lin and FL3-Peak-Lin, given by the software, an example of this type of graphs are shown in **Supplementary Information 1** and **2**. The cells disruption efficiency was measured by drawing gates manually in these graphs, given four different quadrants. These quadrants are upper-right (UR), upper-left (UL), lower-right (LR) and lower-left (LL). In the UR were plot the healthy microalgae cell population, with their normal metabolism working. In the LL quadrant were the cells that lost their integrity after the treatment those are the disrupted cells. And in the other two quadrants is essentially cellular debris [74,79].

The cytometer software can also give the cell percentage on each run. With the use of a control sample before the treatment it is possible to establish the total cell percentage of cells in the culture. By analyzing of the different conditions of treatment, it is possible obtain the cellular disruption percentage.

4.9 Statistical analysis

Statistical analysis was carried out using GraphPad Prism 8 (GraphPad Software, La Jolla, USA). Two-way analysis of variance (ANOVA) with Tukey *post-hoc* test was applied to analyse all data. Data is represented as mean ± standard error of the mean (SD). Statistical significance was set for $p<0.05$ and is indicated by different letters according to their significance (a,b,c,d). Principal component analysis (PCA) and K-cluster means was performed using PAST – Paleontological Statistics Software Package for Education and Data Analysis, version 3.26. were performed [80].

Combination of these tools allowed to highlight similarities and correlations between a set of preliminary results and extraction methods. Ellipses were established within each highlighted group with 95% of confidence interval.

5. RESULTS AND DISCUSSION

5.1 Impact of treatments on release of intracellular components

Preliminary assessment of the impact of thermal treatments (COV, OH and OHP) were evaluated through IOMR factor, which is an indicator of cell disruption efficiency [74]. **Figure 9** compares a extraction rate based on ratio between IOMR factor of the different treatments with that obtained after total cell disruption with bead milling method as described in section 4.3.1 OH and COV (**Figure 9a**) promoted a notable increase in the release of organic matter (of approximately 40 % of the total) with increasing temperature ($p < 0.05$), but there were no significant differences ($p > 0.05$) between OH and COV performed at the same temperature. This indicates, that at this level the presence MEF at ≈ 12 V/cm did not favour additional release effect of organic matter, and that this effect were exclusively of thermal nature. **Figure 9b** shows the effect of OHP performed at maximum registered temperature ranging from 70 and 95 °C, at MEF of 133 V/cm and with varying number of applied heating pulses (from 1 to 5). In this case, release of organic matter attained the same levels previously observed for OH and COV (i.e. 40 %) which suggest that OHP with only a few seconds of treatment can contribute to the same level of destabilization or permeabilization of microalgae cell structure previously observed. This can be attributed to the presence of high electric field applied in synergy with thermal effects. OHP with application of 5 pulses was significantly different when compared to the treatments with 1 or 3 thermal pulses. Despite the increase observed, the viability of the extracted compounds is not safeguarded. It is important to take in consideration that the use of a high number of thermal pulses, and consequent increase of thermal load, can also result in degradation of the extracted metabolites [74].

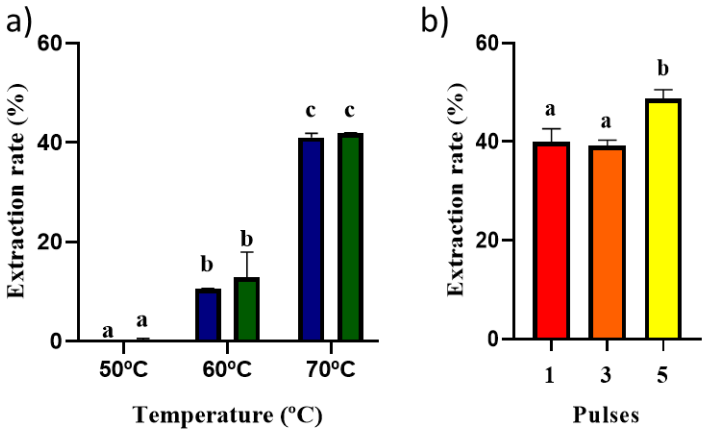

Figure 9- The results of IOMR obtained in the different extraction conditions tested over the practical experiments. **a)** Comparison between COV represented by ● and OH represented by ● of the IOMR. **b)** Comparison between the different number of pulses applied in the OHP treatment under 133 V/cm electric field where 1 pulse (T≈100°C) represented by ●, 3 pulses(T≈70°C) represented by ● and 5 pulses (T≈70°C) represented by ●. According to Tukey's test, the assays presenting the same letter do not differ significantly (α=0.05).

5.2 PCA

Preliminary assessment (section 5.1.) allowed to establish a set of thermal treatments with potential impact on microalgae cell disruption/permeabilization and release of compounds of interest. PCA and k-means cluster analysis (KCA) were performed as way to highlight patterns about the impact of different treatment strategies and identify clusters of interest for further validation. **Table 3** and **Supplementary Tables 1-6** (see supplementary section) resume treatment conditions and concentrations of extracted metabolites, respectively. PCA and KCA analysis (**Figure 10**) was based on the results from the release of protein and pigments contents (i.e., chlorophyll a and b, and carotenoids), which were considered a measure of cell structure permeabilization; permeabilization of cellular wall and membranes of microalgae allows for easier recovery of the intracellular molecules such as pigments and proteins [3–5]. The two principal components were responsible for 95.9 % of the total data variance showing that dimension reduction can be performed while maintaining relevant information of data set. The component 1 (79.7 % of variance) represents the horizontal axis on which the variance of the data is greater. The greater dispersion of data along component 1 is driven by differences on the contents of pigments promoted by the different treatments applied. While component 2 (16.6 %) explains different profiles regarding release of organic compounds, such as proteins.

Table 3- Samples resulting from the different treatments (UNT, COV, OH and OH) and respective treatment conditions in which these tests took place.

Sample	Treatment	Temperature / ºC	Electric field / V/cm	Treatment time /s	Heating cycles	Cluster
2	UNT	-	-	-	-	3
3	UNT	-	-	-	-	3
4	UNT	-	-	-	-	3
99	UNT	-	-	-	-	1
100	UNT	-	-	-	-	1
101	UNT	-	-	-	-	1
114	UNT	-	-	-	-	1
115	UNT	-	-	-	-	1
116	UNT	-	-	-	-	1
123	UNT	-	-	-	-	1
124	UNT	-	-	-	-	1
125	UNT	-	-	-	-	1
8	COV	50	-	600	1	1
9	COV	50	-	600	1	1
10	COV	50	-	600	1	1
11	COV	60	-	600	1	7
12	COV	60	-	600	1	7
13	COV	60	-	600	1	7
14	COV	70	-	600	1	7
15	COV	70	-	600	1	7
16	COV	70	-	600	1	4
32	COV	70	-	600	1	4
33	COV	70	-	600	1	4
34	COV	70	-	600	1	4
105	COV	70	-	600	1	4
106	COV	70	-	600	1	4
107	COV	70	-	600	1	4
20	OH	50	12	600	1	1
21	OH	50	12	600	1	1
22	OH	50	12	600	1	1
23	OH	60	12	600	1	7
24	OH	60	12	600	1	2
25	OH	60	12	600	1	7
26	OH	70	12	600	1	4
27	OH	70	12	600	1	4
28	OH	70	12	600	1	4
35	OH	70	35	600	1	4
36	OH	70	35	600	1	5
37	OH	70	35	600	1	6
108	OH	70	36	600	1	6
109	OH	70	36	600	1	6
110	OH	70	36	600	1	6
38	OHP	70	133	30	3	6
39	OHP	70	133	30	3	5
40	OHP	70	133	30	3	6
44	OHP	95	133	12	1	5
45	OHP	70	133	12	1	2
46	OHP	60	133	12	1	1

47	OHP	75	133	21	3	3
48	OHP	75	133	21	3	3
49	OHP	75	133	21	3	3
50	OHP	80	133	30	5	4
51	OHP	75	133	30	5	4
52	OHP	80	133	30	5	6
111	OHP	100	133	18	1	5
112	OHP	100	133	18	1	5
113	OHP	100	133	18	1	5
120	OHP	90	216	6	1	6
121	OHP	90	216	6	1	5
122	OHP	90	216	6	1	5
129	OHP	90	216	39	1	6
130	OHP	90	216	37	1	6
131	OHP	90	216	32	1	6

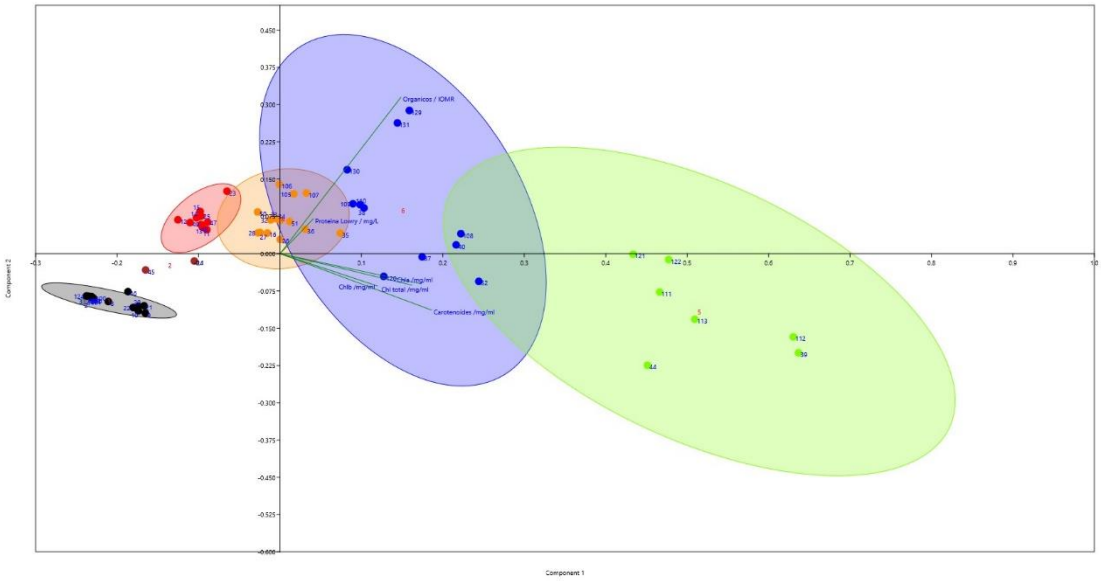

Figure 10- Exploratory analysis of the data obtained regarding the extraction of biocompounds from microalgae biomass.

From PCA and KCA it is clear that OHP strategy allows a great potential to extract both pigments and proteins rather than continuous and prolonged thermal treatments which seem to favour only protein extraction. Chlorophyll and carotenoids are better extracted when OHP with an average temperature, treatment time and electric field of 92.1 °C, 156.7 V/cm and 15.4 s, respectively (cluster 5; Figure 10 green colour); in this set of sample concentration of chlorophyll a, b and carotenoids was of approximately 135.0 mg/100g, 38.8 mg/100g and 42.6 mg/100g, respectively. While the release of protein and organic compounds seem better extracted when

electric field is increased up to 217 V/cm or punctually when OH at 70 °C for 10 min are applied (cluster 6; Figure 10, blue colour). For example, OH and COV treatments at 70 °C gave higher concentration of protein ranging from 24.7 to 25.1 mg/100 g, which is well compared with 22.4±0.08 mg/100 g obtained with OHP treatments at 100 °C, but significantly less chlorophyll a, b and carotenoids (e.g. 135.0 mg/100g, 38.8 mg/100g and 42.6 mg/100g). Thermal treatments (COV and OH) at temperatures of 60 °C for 10 min (cluster 4 and 7; Figure 10, orange and red colours, respectively) also attain level differentiation regarding untreated samples or treated samples at 50 °C for 10 min (cluster 1 and 3; Figure 10 black colour). Depending on the heating pulses (3 to 5) OHP treatments performed at 70 °C can also attain the same level of extraction observed for the continuous treatments (COV or OH) but in a lesser time. Another evidence on PCA is that the release of organic compounds and proteins are well correlated with each other. This means that 40 % of extraction regarding organic compounds release is sufficient to allow recovery significant amounts of intracellular components such as proteins and chlorophylls. Overall, this analysis evidences that destabilization of microalgae cell structure can be attainted at temperature above 50 °C, being this effect more significative with OHP at temperatures ranging from 90 to 100 °C performed in a short-time (less than 30 s) and at electric field of 133 V/cm and 217 V/cm. Luengo et al,[5] using pulsed electric fields (PEFs) at different intensities (10–25 kV/cm) and in combination with temperatures ranging from using 10–40 °C), demonstrated that an increase in temperature during the extraction can contributes to an increase in the sensitivity of the microalgae and consequently to an irreversible electroporation. In this work, it can be observed that permeabilization effects can also be attained when HTST treatments are performed under MEF effects. Based on these exploratory analysis OHP at temperatures of 100 °C (1 heating pulse) showed to be the most promising one on cell permeabilization and thus were applied at different MEF intensities to further study effects on extraction of pigments, lipids, carbohydrates, as well as to address the extract bioactivities.

5.3 Pigments

5.3.1 Impact of MEF in the extraction of pigments

Figure 11 (a to i) resumes in a more detailed way the influence COV, OH and OHP treatments on extraction of pigments. The results in **Figure 11 a1, a2,** and **a3** demonstrate that in resemblance to the organic matter, also the extraction of pigments occurs at higher temperatures, but in this case at 70 °C there is a statistically significant difference ($p < 0.05$) between the COV and OH; for chlorophyll a, b and carotenoids OH extraction was of 16.3 %, 16.4 % and 16.7 %, respectively, of the total and about 2x times higher than the one observed for COV treatment. This is a clear indication that, at equal extraction temperatures and under identical thermal histories, the presence of MEF and its associated electrical frequency (50 Hz) positively influence the extraction of pigments. Sensoy et al [8] suggest that the extraction of specific metabolites with pigments is positively affected by treatments in which the temperature (25 to 85 °C) and the electric field (0 to 125 V/cm) work together, which is in accordance with the results in here presented. A possible explanation for this phenomenon only occurring at a temperature of 70 °C is related to the fact that the microalgae under study has a double layer cell wall. Jaeschke et al [9] demonstrated in their study that carotenoid extraction was positively affected by the increase in the electric field in MEF treatments. Regarding the effect of applying successive thermal pulses during OHP treatments at 100 °C, the best extraction rate was obtained when 1 pulse was applied (**Figure 11 b1, b2,** and **b3**). In this case extraction rate for all pigments increased up 40 % of the total, with concentration for chlorophyll a, b and carotenoids of 135.0 mg/100g, 38.8 mg/100g and 42.6 mg/100g. However, when 3 and 5 heating cycles were applied extraction rate decreased to levels below 10 % evidencing that thermal load associated to the application of more than one heating cycle at high temperature (≈ 100 °C) promotes degradation of pigments. Even under different thermal and electrical conditions, these results can be corroborated based on previous findings by Luengo et al [5]. According to latter author, in addition to parameters such as the electric field and treatment time, the temperature was also a critical parameter in pigment extraction. In order to understood in more detail, the effect of the MEF at higher temperature range, the extraction of pigments was performed under OHP at 217 V/cm. OHP treatments at 133 and 217 V/cm were compared with COV and OH at 70 °C for a clearer understanding (see **Figure 11 c1, c2** and **c3**). Different outcomes were observed, depending on the intensity of the MEF: chlorophyll a presented higher extraction rate at 217 V/cm ($p < 0.05$); oppositely chlorophyll b was more extracted under 133 V/cm ($p < 0.05$); while carotenoids didn't presented significant differences among the different MEF ($p > 0.05$). These results may indicate that MEF has promoted

different selectivity mechanisms or degradation levels regarding chlorophyll extraction that need to be further investigated. Comparing the best performances obtained in the different treatments tested (**Figure 11 c1 c2** and **c3**), the treatment that presented the best performance was the OHP.

The obtained results are in accordance with the expected range reported in the literature for other electrotechnologies such as PEFs and MEFs. Leonhard et al [81] observed a pigment extraction rate of 3.25 mg/g DW that corresponds to a yield of 14 %. In this work we obtained a pigments extraction rate of 1.35 mg/g DW that corresponds to yield of 37 %. Despite the lower concentration, our results demonstrated an increase in the yield of the extraction with less aggressive treatment conditions. Our results when compared with MEFs display similar extraction rates. Moreover, Jaeschke et al [9] in his study indicated an extraction rate of pigments of 0.71 mg/g DW using an electrical field of 12 V/cm and 1.21 mg/g DW using an electrical field of 23.9 V/cm, demonstrating that the increase of electrical fields results in increased extraction of pigments which is accordance with our results.

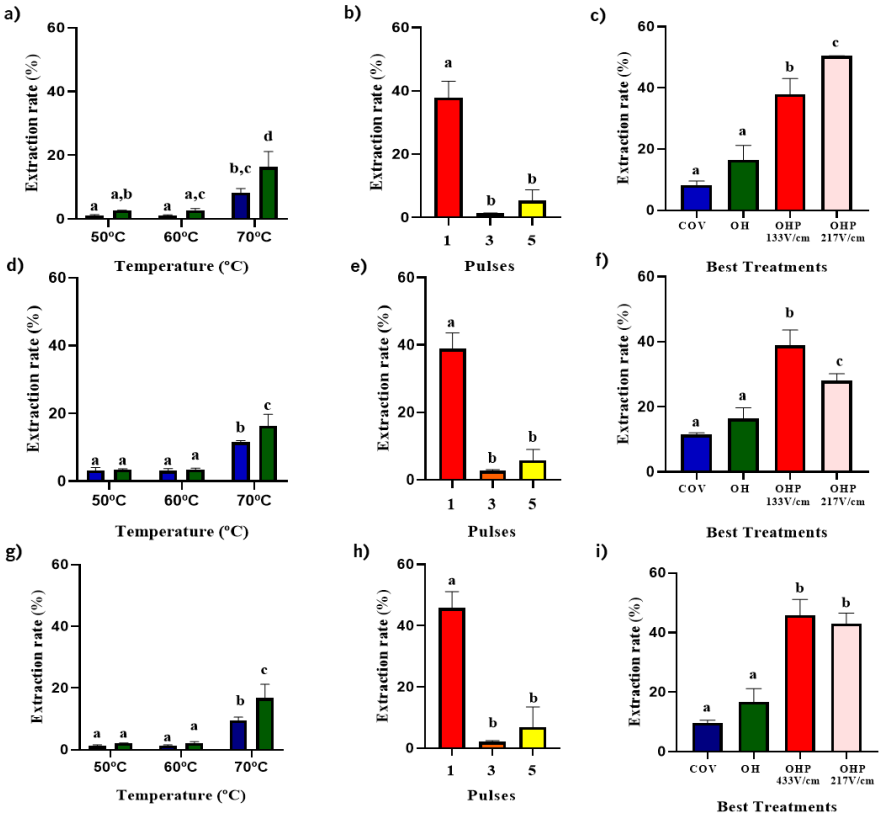

Figure 11- The results of pigment extraction obtained in the different extraction conditions tested over the practical experiments**. a)** Comparison between COV represented by ● and OH (35 V/cm) represented by ● of the chlorophyll a extracted **b)** Comparison between the different number of pulses applied in OHP treatment under 133 V/cm electric

field where 1 pulse (T≈100°C) represented by ●, 3 pulses(T≈70°C) represented by ● and 5 pulses (T≈70°C) represented by ● of the chlorophyll a extracted **c)** Comparison of calculated extraction yields between the best conditions for each type of treatments where COV represented by ●, OH (35 V/cm) represented by ● , OHP treatment under 133 V/cm (T≈100°C) represented by ● and OHP treatment under 217 V/cm (T≈100°C) represented by ● of the chlorophyll a extracted **d)** Comparison between COV represented by ● and OH (35 V/cm) represented by ● of the chlorophyll b extracted **e)** Comparison between the different number of pulses applied in the OHP treatment under 133 V/cm electric field where 1 pulse(T≈100°C) represented by ●, 3 pulses(T≈70°C) represented by ● and 5 pulses (T≈70°C) represented by ● of the chlorophyll b extracted **f)** Comparison of calculated extraction yields between the best conditions for each type of treatments where COV represented by ●, OH (35v/cm) represented by ●, OHP treatment under 133 V/cm (T≈100°C) represented by ● and OHP treatment under 217 V/cm (T≈100°C) represented by ● of the chlorophyll b extracted **g)** Comparison between COV represented by ● and OH (35 V/cm) represented by ● of the carotenoids extracted **h)** Comparison between the different number of pulses applied in the OHP under 133 V/cm electric field where 1 pulse(T≈100°C) represented by ●, 3 pulses (T≈70°C) represented by ● and 5 pulses(T≈100°C) represented by ● of the carotenoids extracted i**)** Comparison of calculated extraction yields between the best conditions for each type of treatments where COV represented by ●, OH (35V/cm) represented by ● , OHP treatment under 133 V/cm (T≈100°C) represented by ● and ohmic OHP under 217 V/cm (T≈100°C) represented by ● of the carotenoids extracted. According to Tukey's test, the assays presenting the same letter do not differ significantly (α=0.05).

5.3.2 Fluorescence analysis of the extracted chlorophyll´s

Fluorescence analysis was performed as a way to qualitatively confirm the results obtained in the previous sections. **Figure 12** shows and example of fluorescence spectra evidencing that all performed treatments (COV, OH and OHP) presented a similar design, regardless of the intensity. Considering the results presented, the observed maximum intensity at ranging from 675 to 685 nm points out to the presence of chlorophyll a [82]. Other indications that confirm the presence of chlorophyll a are related to the decrease in the area of fluorescence from 685 nm to 710 nm where a shoulder was found. This shoulder precedes a new peak between 720-740 nm, due to vibrational satellite bands of the main chlorophyll a bands [82]. A more detailed observation indicated the presence of a shoulder or higher fluorescence intensity at 660 nm (**Figure 12b**) for the treated samples. The presence of this shoulder was an evidence for the presence of chlorophyll b in the samples analyzed [82]. Extracts resulting from treatments OH, COV and OHP also were characterized by differences on the intensity of the vibrational band at 660 nm. Based on these observations the impact of treatments on structural properties of molecules extracted should not be overlooked and be object of further research. It is important to point out that, independently of the treatment applied the use of solvent already produce released of chlorophyll thus justifying the intentisity band for untreated sample. This solvent effect is much more pronounced within treated samples thus corraborating previous results.

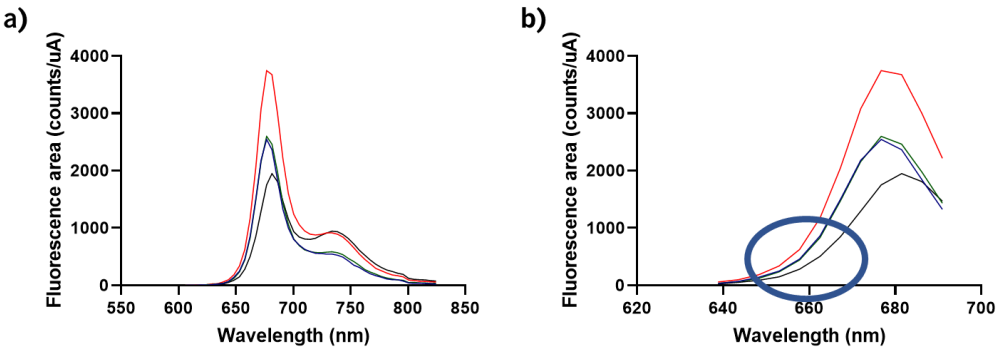

Figure 12- Fluorimetric analysis of chlorophylls obtained in the different extraction conditions **a)** Spectra of the chlorophyll a in the different treatments performed where COV represented by ●, OH represented by ●, OHP treatment represented by ● and the UNT microalgae represented by ● from the pigment extraction performed over the practical experiments **b)** Shoulder in the chlorophyll spectrum that indicates the presence of chlorophyll b in the samples resulting from the treatments performed where COV represented by ●, OH represented by ●, OHP treatment represented by ● and the UNT microalgae represented by ● from the pigment extraction performed over the practical experiments.

5.4 Protein

5.4.1 Impact of the MEF in the extraction of proteins

Regarding extraction of proteins, results from **Figure 13** show that the OH presents a better performance against COV in the extraction of proteins at temperature below 70 °C; OH presented an extraction rate of approximately 3 % (50 °C) and 6 % (60 °C), while COV extraction rate was significantly lower ($p < 0.05$), presenting the values 1.5 and 2.4 %, for 50 and 60 °C, respectively. At temperatures of 70 °C both OH and COV resulted in a similar extraction ($p > 0.05$) of approximately 6.5 %. This unveils that MEF even at low intensity (≈ 12 V/cm) can resulted in an additional effect that is superimposed by the temperature increase. These results also suggest that through of OH it is possible extract the same amount of protein with lower temperature requirements when compared with COV. This phenomenon can be very advantageous since proteins are molecules that are very sensitive to temperature and thus lose some of their biological value. Considering that electrotechnologies can only extract about 30 % of the total protein content of the cell [4] that corresponds to the free total available protein, we conclude that in the best tested condition, the extraction yielded up to 21.67 % of these proteins. This study is in accordance with the literature, where electrotechnologies have lower protein extraction yields when compared to other mechanical methods such as bead milling or high pressure homogenization [4,7]. The great advantage of electrotechnologies is related to the mode of operation, once they can cause cell electroporation or some level of permeabilization contrasting to the mechanical methods

mentioned that cause the total disintegration of the cell [7]. The electroporation phenomenon is directly correlated with the possibility of performing extraction cyclical proteins that corresponds the possibility to extract protein multiple times from a sample over the time demonstrated by Buchmann et al[4]. Similar to that observed in pigment extraction, OHP strategy also resulted in higher protein extraction rates (**Figure 13**). OHP treatments where three and five pulses were applied resulted in less extraction rate probably due to thermal degradation of the molecules. Our results are supported by previous findings where Carullo et all [3] demonstrated that eletrotechnologies based on the HTST principle were not suitable for the extraction of high molecular weight proteins.

The results present in the **Figure 13c** also demonstratea that the increase in the MEF (from 133 to 217 V/cm) contributes to a significant increase ($p < 0.05$) in the rate of protein extraction from aproximatelly 5 to 8 %. The results obtained under this treatment conditions are in agreement with the reported in some previous studies. Buchmann et al [4] in their study demonstrated that the increase of the electric field had an positive effect on the extraction rate of protein. Considering that of the total protein content only about 30% is available for electrical fields extraction [4], the overall yield raises to 26,17% within this condition. Comparing the best performances obtained in the different treatments tested (**Figure 13c**), the treatment that presented the best performance was the OHP at 217 V/cm. Regarding the protein extraction, slightly less extraction was observed when compared to other biocompounds under study, such as the pigments. There are two possible explanations for this event: one of these explanations is related to the high temperature reached during the OHP (100°C) that can denature the extracted proteins; another explanation is related to the reduced treatment time that may not be enough for having an appropriate protein extraction.

In this work, we obtained a protein extraction of 0.23 mg/g DW, which corresponded at approximately of 8 % of total protein. Carullo et al [3] demonstrated a protein extraction of 1.65 mg/g DW which corresponded at approximately of 5.2 % of total protein, for *Chlorela vulgaris* using PEF and high pressure homogenization . Our results show that OHP offer potential to attain same levels of extraction with less energy input.

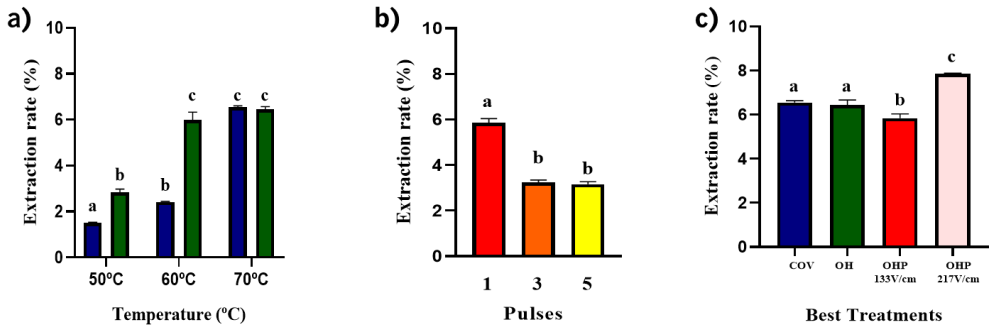

Figure 13- The results of protein extraction obtained in the different extraction conditions tested over the practical experiments. **a)** Comparison between COV represented by ● and OH (35 V/cm) represented by ● of the protein extracted **b)** Comparison between the different number of pulses applied in the OHP treatment under 133 V/cm electric field where 1 pulse (T≈100°C) represented by ●, 3 pulses (T≈70°C) represented by ● and 5 pulses (T≈70°C) represented by ● of the protein extracted **c)** Comparison of calculated extraction yields between the best conditions for each type of treatments where COV represented by ●, OH (35 V/cm) represented by ●, OHP treatment under 133 V/cm represented by ● and OHP treatment under 217 V/cm are represented by ● of the protein extracted. According to Tukey's test, the assays presenting the same letter do not differ significantly (α=0.05).

5.4.2 Protein extraction under alkaline conditions

Based on the previous results regarding protein extraction, a new approach was tested. Considering the fact that the use of alkaline conditions contributes to an increase in protein extraction yield [64], a quantity of 5 mM NaOH was added to the most promising treatment. The results obtained are shown in the **Figure 14**.

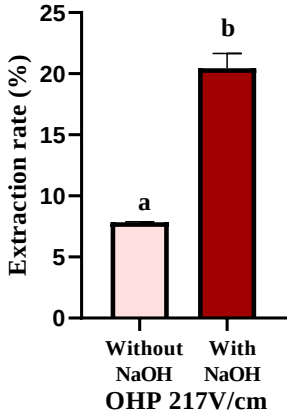

Figure 14- The results of protein extraction obtained under a 217 V/cm electric field (T≈100°C) with and without the addition of NaOH where the assay without NaOH represented by ● and the assay with NaOH (5Mm) represented by ● . According to t-test, the assays presenting the same letter do not differ significantly (α=0.05).

These results suggest the addition of a small amount of NaOH has been shown sufficient to enhance protein extraction – i.e., protein concentration increased from 23.5 mg/100 g to 29.4 mg/100g. The combination of this solvent with the application of OHP on the microalgae biomass

proved to be the most effective methodology for extracting protein. Considering that of the total protein content only about 30 % is available for electrical fields extraction [4], the overall yield raises to 68.17 % giving an indication of higher disintegration of cellular structures. In order to understood in more detail, the effect of MEF in the extraction of proteins was performed an OHP (1 pulse) under an electric field (217 V/cm). This treatment was performed at a temperature below 37°C under low conductivity where the sample was subjected to 5 pulses. It was verified that there is a statistically significant difference between the negative control and the treatment under the influence of the electric field, showing that the influence of the electric field was responsible for the verified extraction (**Supplementary information 9**). These results also open perspectives of using OHP in alkaline environment for best extraction performances.

5.4.3 Fluorescence analysis of the extracted proteins

Fluorescence analysis of the protein extracts was performed in order to confirm the results obtained in the previous sections and as way monitor structural changes regarding local structure and dynamics of tryptophan groups [77] (**Figure 15**). The λ_{max} of native proteins containing tryptophan is contained between 308 and 355 nm [83]. Regarding the obtained spectra, the λ_{max} observed in the different treatment performed (COV, OH, OHP and untreated) were within the expected range confirming intensity peak around 340 nm, which is typical of tryptophan residues.

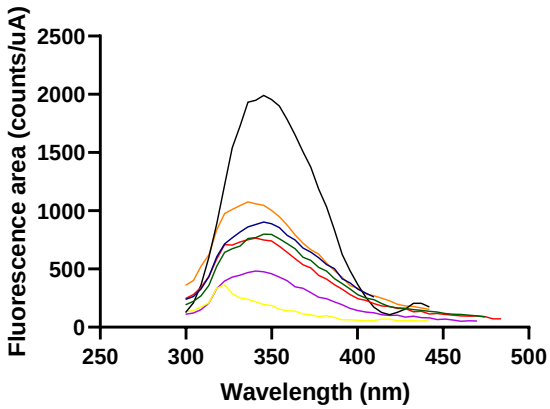

Figure 15- Spectra of the tryptophan fluorescence wavelength in the different treatments performed where where COV represented by ●, OH represented by ●, OHP treatment under 217 V/cm electrical field represented by ●, OHP treatment under 133 V/cm electrical field represented by ●, OHP treatment under 217 V/cm electrical field with 5mM of NaOH represented by ●, the negative control represented by ● and the untreated microalgae represented by ● from the protein extraction performed over the practical experiments.

Comparing the λ_{max} observed in the positive control (total disruption) with the treated samples (COV, OH and OHP) it was clear the occurrence of a blue shift. **Table 4** shows the wavelength observed at maximum intensity fluorescence. The blue shift is when the maximum fluorescence shifting decrease in wavelength with a corresponding increase in frequency [84]. The λ_{max} can vary significantly depending on where the fluorophore is located and polarity of the environment [77]; a blue shift is usually an indication that the tryptophan (fluorophore) is in an extremely nonpolar environment inside the protein sphere [83,84]. The occurrence of the blue shift gives an indication that the tryptophan ring was negative charged near the benzene end or positively charges near the pyrrole end [77] and that integrity of the protein is more preserved. On the other hand, some studies points to the emission of a red shift when the proteins are unfolded [83,85]. Based on these facts and considering the results from **Table 4** different outcomes regarding positioning of tryptophan residues can be obtained depending on the thermal treatment applied. These events should be thoroughly investigated using more advanced characterization methods. Samples that presented higher levels of protein concentration were subjected do circular dichroism analysis but neither secondary or tertiary structure was detected.

Table 4- Maximum wavelength (mean ± standard deviation) obtained for the different treatments in the fluorometric analysis of proteins.

Treatment	λ_{max} (nm)
Positive control (total disruption)	347.67±1,78
UNT	322.00±0,00
COV	345.00±0,00
OH	343.33±2,22
OHP	340.83±1,38
OHP*	337.33±1,78

* with NaOH 5 mM

5.5 Most promising treatments

The objective of this study was not the optimization of the extraction of biomolecules, but rather to prove that the application of OH depending on thermal and MEF conditions can enhance the extraction of biomolecules with treatments much more shorter in time than traditional thermal strategies. The influence of MEF (non-thermal events) was clearly verified in the OH extraction of pigments at a temperature of 70°C and in the OH extraction of proteins at a temperature of 50 °C

and 60 °C. Regarding the OHP extraction, the results demonstrate that with higher temperatures and high electric field intensity it is possible to obtained a significantly higher extraction rate being this effect even more pronounced in combination with alkaline conditions.

Considering the results presented in this work, treatments performed at 100°C as the maximum temperature, at 217 V/cm (1 heating pulse of 7 seconds) resulted in higher extraction of pigments and proteins , thus showing one of the two events: **1)** OHP following the principle of HTST extraction presents a high heating efficiency and uniformity during the process - the application of this principle (HTST) allowed a better preservation of the compounds of interest and this better preservation may be responsible for the higher extraction rates verified. **2)** OHP treatments in less than 10 s under the influence of MEF ranging from 133 to 217 V/cm allowed higher extraction than continuous OH at 70 °C for 10 min at MEF of 12 V/cm.

Based on these facts and obtained results, a series of complementary analyses such as lipid analysis, carbohydrates and the antioxidant activity of the extracts resulting from most promising treatment conditions were evaluated. OHP treatments were the ones that presented the best results regarding cell permeabilization and metabolite extraction **(Table 5)**, and consequently those that were analyzed in more detail.

Table 5- Summary table of the best performing treatments for the extraction of different metabolites.

Metabolites		**Better performance treatment**
Protein		OHP 217V/cm with NaOH
Pigments	chlorophyll a	OHP 217V/cm
	chlorophyll b	OHP 133 V/cm
	carotenoids	OHP 133 V/cm and OHP 217 V/cm

5.5.1 Lipids

Regarding the lipid extraction, the results in the **Figure 16** showed that OHP at 133 V/cm resulted in an increase in the extraction of lipids when compared with treatments performed at 217 V/cm. These results evidence that the use of the lower electrical field during a higher period of time (17 s instead of 7 s) is an important factor in efficiency of lipid extraction.

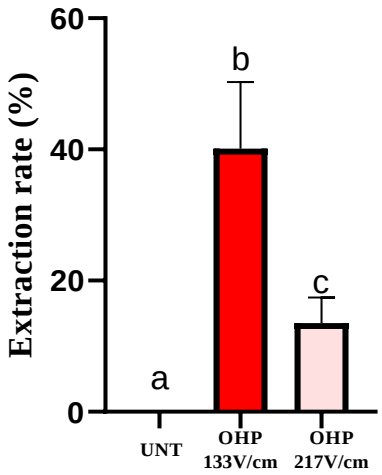

Figure 16- The results of lipid extraction obtained in the different extraction conditions tested over the practical experiments where Unthread samples represented by ●, OHP treatment under 133 V/cm represented by ● and OHP treatment under 217 V/cm represented by ●. According to t-test, the assays presenting the same letter do not differ significantly (α=0.05).

Considering that the extraction of lipids in microalgae biomass is usually performed essentially through the use of solvents [86,87]. The use of these solvents represents environmental and safety issues. Based on this information, new and green approaches to enhance lipids extraction from microalgae biomass are attracting attention. Some previous studies [86,87] about the using of electrotechnologies (PEFs) for lipid extraction points to enhanced lipid recovery but solvent extraction remained the rate-limiting step and the results obtained in this study were concordant with these evidences. Indeed, this practical work demonstrated that OHP can be used as tool to soften cellular microalgae structure thus helping lipid extraction when ethanol (food grade) In this practical work OHP at 133 V/cm enhanced ethanol effect and about 40 % of the total content of lipids were extracted. This is a preliminary evidence that OHP can be optimized by combining the influence of electrical and thermal parameters with green solvents composition.

Results obtained in this work are in accordance with ones observed in other studies that use electrotecnhologies for the lipid extraction. Herein, we obtained an extraction of 2067 µg/L when using OHP 133 V/cm and ethanol as a solvent (incubation period of 30 minutes), whereas Zbiden et al[87] in their work extracted 2500 µg/L of lipids using PEF and ethyl acetate-methanol as a solvent (incubation period of 2 h). Thus, in this work OHP allowed the use of a solvent that is food grade and a lower incubation period which consist in a great advantage.

5.5.2 Carbohydrates

The carbohydrates extraction was also positively affected by the application of MEF in the microalgae biomass. Although no significant differences were observed ($p > 0.05$) between samples suggesting that the different MEF conditions did not promote any additional effects on release of carbohydrates **(Figure 17).**

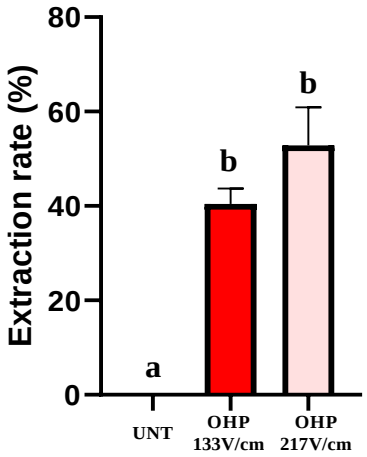

Figure 17- The results of carbohydrates extraction obtained in the different extraction conditions tested over the practical experiments where Unthread samples represented by ●, OHP treatment under 133 V/cm represented by ● and OHP treatment under 217 V/cm represented by ●. According to t-test, the assays presenting the same letter do not differ significantly (α=0.05).

Carullo et all [3] suggest in their work that the application of PEFs in microalgae biomass was responsible for extract 36 % of total content of carbohydrates. The approach used in this practical work (OHP) was responsible for extract 46 % of total content of carbohydrates. These results suggest that the using of OHP contribute to attain expected extraction rates of carbohydrates using a less extreme electrical conditions. Being a good indication, of course that care should be taken once different microalgae species are being compared.

5.5.3 Bioactivities

The microalgae biomass present himself as promising alternative source of antioxidants compounds [75]. Based on this fact, the antioxidant activity of the best extraction conditions obtained in this practical work was measured using three different methods – i.e., ABTS, DPPH and FRAP. Radical scavenging activities and reducing power were analyzed to determine the antioxidant activity of the extracts [76] and results obtained are present in the **Figure 18**.

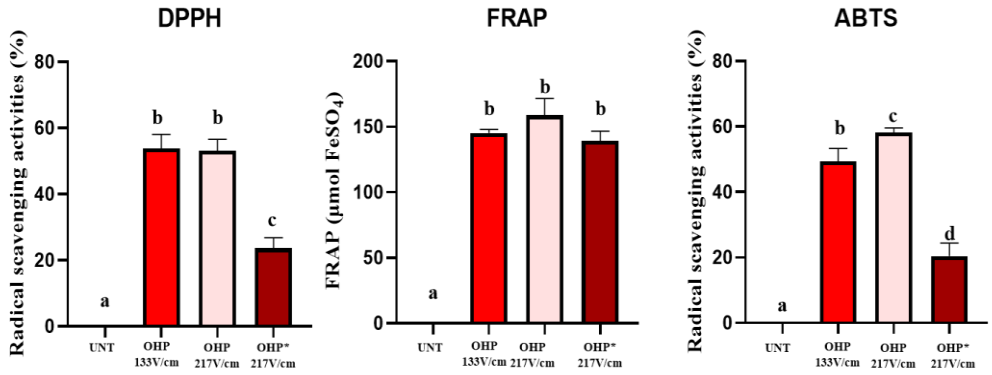

Figure 18- The results of bioactivities obtained in the different extraction conditions tested over the practical experiments where UNT samples represented by ●, OHP treatment under 133 V/cm represented by ● and OHP treatment under 217 V/cm represented by ● and OHP* treatment under 217 V/cm electrical field with 5mM of NaOH represented by ●. According to t-test, the assays presenting the same letter do not differ significantly (α=0.05).

The antioxidant capacity of the microalgae results from two different sources such as carotenoids and phenolic compounds. Carotenoids contribute for the quenching reactive oxygen species (ROS). The carotenoids are considered the major contribution to the antioxidant capacity of microalgae. The phenolic compounds such as the flavonoids participate in some antioxidant mechanisms. For example, the flavonoids can inhibit the lipid oxidation. In this practical work the measure of the antioxidant capacity was analyzed considering the contribution of the two different source the carotenoids and phenolic compounds [75,76]. Considering the results obtained (**Figure 18**) OHP at 133 V/cm or 217 V/cm without NaOH resulted in the extraction of more antioxidant compounds evidencing higher radical scavenging activity. These results point to the ability to extract the same amount of antioxidant compounds using either 217 V/cm or using 133 V/cm; however regarding ABTS, higher scavenging activity at a significant level was observed for the OHP at 217 V/cm ($p < 0.05$). It is important to point out that OHP in alkaline conditions resulted in lesser bioactivity ($p < 0.05$) regarding DPPH and ABTS when compared with other treatments. This means that the use of NaOH may not compensate or its concentration should be adjusted to attain extracts with an interesting level of bioactivity. This aspect should be further investigated.

Similarity to the observed in the carbohydrates extraction, the extraction of antioxidant compounds can be performed from two different approaches: the application of 1 pulse of an upper electric field for a shorter period of time (OHP 217 V/cm) or the application of 1 pulse of a lower electrical field for a longer period of time (OHP 133 V/cm). That was a good indication that allow choice between two different options.

4.6 Flow cytometer

Flow cytometer analyzis was performed in order to evaluate and confirm the cell disruption and viability after most promising electrical treatments (**Figure 19).** In terms of cellular content (number of counts), the treatment of 217 V/cm presented similar values to the ones observed in the untreated sample ($p > 0.05$), unlike the other treatments where there was a significant decrease ($p < 0.05$) to approximately half of that observed in the control. However, samples resulting from all electrical treatments presented only 40 % of viable cells.

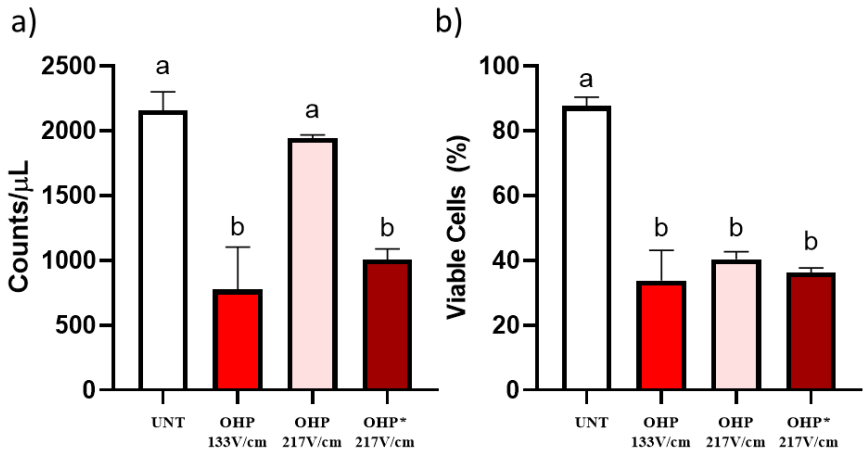

Figure 19– a) Quantification of the absolute counts by flow cytometry after applying the different electrical treatments conditions where UNT samples represented by ●, OHP treatment under 133 V/cm represented by ● and OHP treatment under 217 V/cm represented by ● and OHP* treatment under 217 V/cm electrical field with 5mM of NaOH represented by ●.**b)** Quantification of the percentage of viable cells by flow cytometry after applying the different electrical treatments conditions where UNT samples represented by ●, OHP treatment under 133 V/cm represented by ● and OHP treatment under 217 V/cm represented by ● and OHP* treatment under 217 V/cm electrical field with 5mM of NaOH represented by ● . According to t-test, the assays presenting the same letter do not differ significantly (α=0.05).

Considering the results obtained (**Figure 19 a**) the treatment condition OHP at 217 V/cm despite resulting in a number of cells per µL identical to the ones observed at the control level, (**Figure 19 b**) presents a higher level of unviable cells. These results suggest that under an electric field of 217 V/cm the microalgae cells in terms of morphology or apparent integrity, are less affected, but in practice they are as not viable as those of other treatments. This may result from the fact that treatment time is shortened at this electric field. Moreover, addition of NaOH to OHP 217 V/cm treatment contributed to a decrease of approximately 50 % of number of cells when compared to the OHP 217 V/cm without the addition of NaOH, which indicates that the addition of NaOH is responsible by itself for the observed decrease.

Data from the two different approaches tested in this practical work indicate that the application of OHP at 217 V/cm or the application at 133 V/cm are both responsible for an equal percentage of viable cells but may imply different levels of permeabilization or disruption that can have different outcomes in the protection of extracted metabolites, thus corroborating previous results.

5 FINAL REMARKS

As final remarks of this work, it is possible to conclude that the application of MEF in microalgae biomass contributes for an increase of the extraction rate of pigments. This evidence was observed when comparing the assays COV and OH at the same temperature (i.e. 70 °C) and heating kinetics. This increase was accompanied by the maintenance of the integrity of the pigments confirmed by the fluorescence analyzis. Of the two electric field based methodologies (OH or OHP) the one that presented the most promising results was the OHP demonstrating that the HTST principle can be applied for the extraction of biocompounds particular for microalgae species that present resistant cell walls. Within the OHP methodology there is also an indication that the use of high electric fields (i.e. from 133 to 217 V/cm) can contributes to an increase in the rate of extraction of proteins and pigments (chlorophyll a) while maintaining the same levels of bioactivity. Similar to what was previously observed, this increase was accompanied by the maintenance of the integrity of the pigments and proteins confirmed by the fluorometric analysis. Overall, the application of OH has demonstrated a great applicability in the extraction of different biocompounds. The optimization of certain factors studied, such as treatment time and use of NaOH, can also contribute to an enhanced extraction of specific biocompounds such as lipids and proteins, respectively.

6. FUTURE PRESPECTIVES

In this thesis new insights were opened regarding the application of OH as an extraction methodology. As future work it would be interesting to perform a more precise quantification of the extracted biocompounds by using different techniques, such as high performance liquid chromatography (HPLC), fast protein liquid chromatography (FPLC), gas chromatography (GC) to identify and quantify the pigments, lipids and carbohydrates. This works has shown that extraction of proteins can be highly potentiated by applying OHP in alkaline conditions. This condition could be more explored in order to maximize the extraction rate of proteins.

Moreover, the OH technology could be optimized to perform the electrical treatments using different types of solvents. These solvents could have a positive effect on the extraction of some biocompounds such as nonpolar compounds that do not have as much affinity for the aqueous part used in the tests. After electrical treatments different types of solvents could be applied to increase the extraction rate of this biocompounds, for example ethyl acetate has been described as a solvent that contributes to increase the extraction of lipids in other electrical technologies.

In consideration of the results obtained and considering the positive effect that the application of the electrical field has on the extraction of different biocompounds, other design parameters such as waveform, current density and frequency should be tested in order to understand their effect on the extraction rate of biocompounds.

In addition, it would be interesting to study the extraction rate of different biocompounds over the microalgae growth cycle. This study would allow us to infer which phase of the cell cycle would be more suitable for the extraction of a certain biocompounds.

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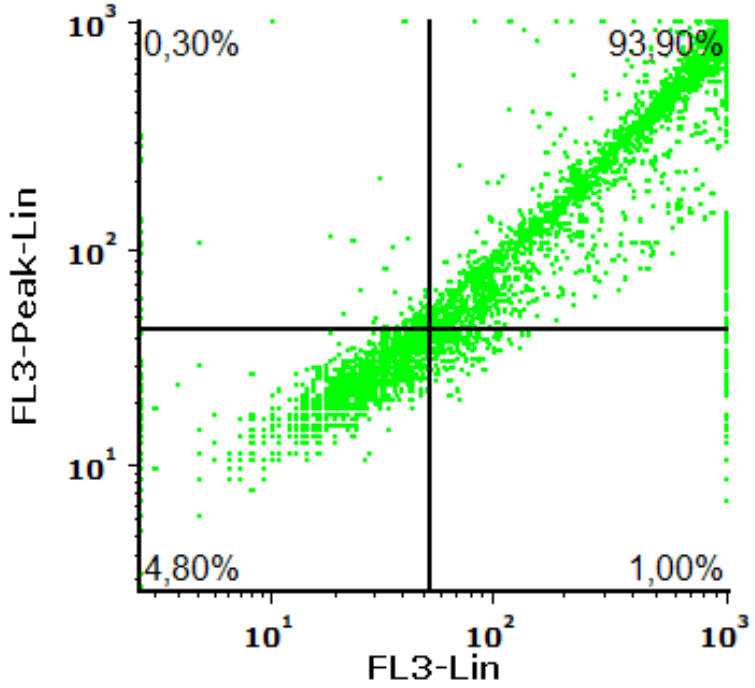
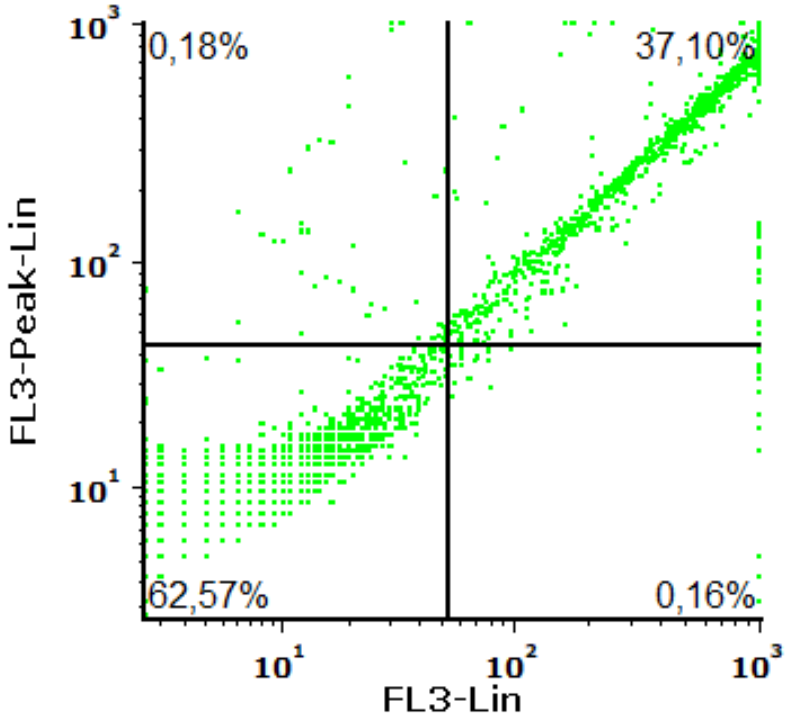
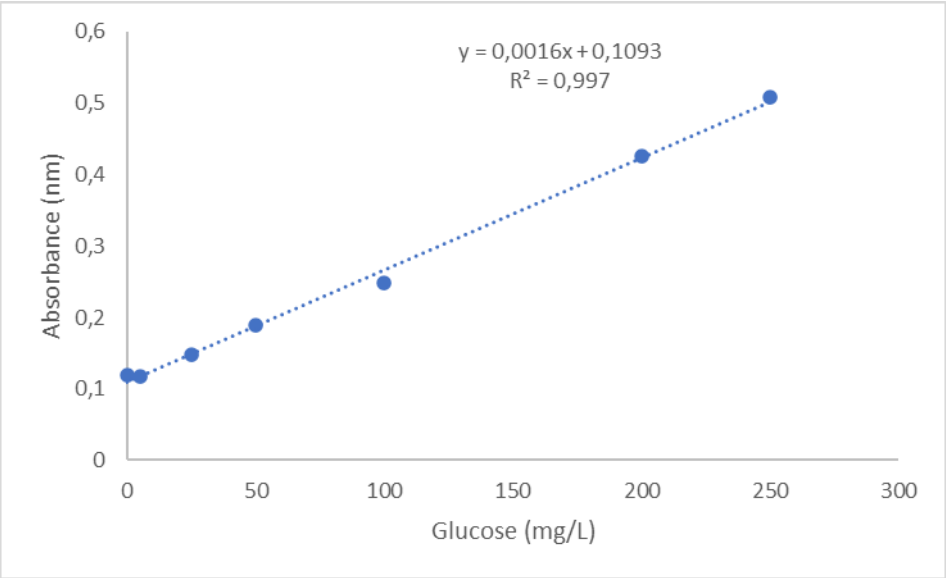
8. SUPPLEMENTARY INFORMATION



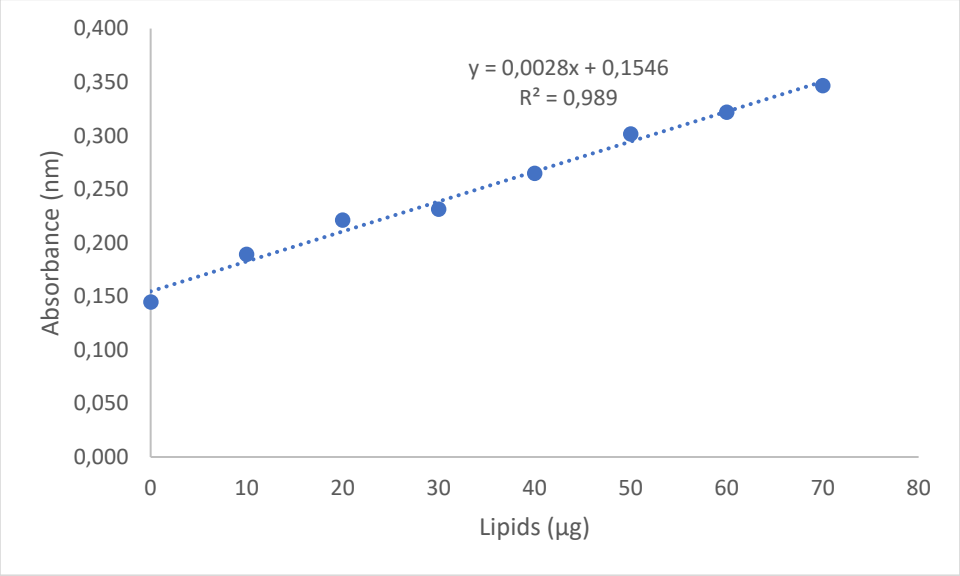
Supplementary Information 1- Graph generated by flow cytometer of a control sample of microalgae with the defined protocol.



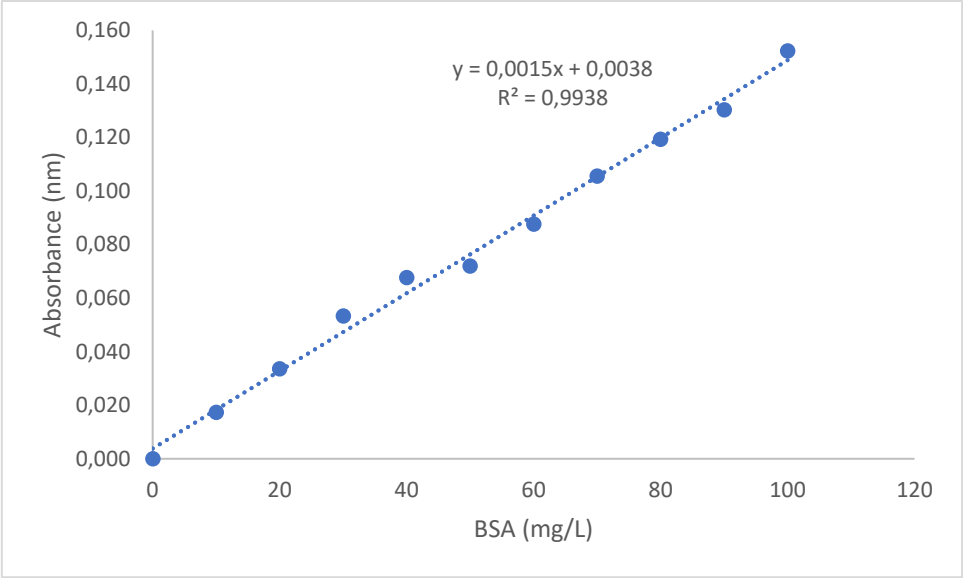
Supplementary Information 2- Graph generated by flow cytometer of a treatedsample of microalgae with the defined protocol**.**



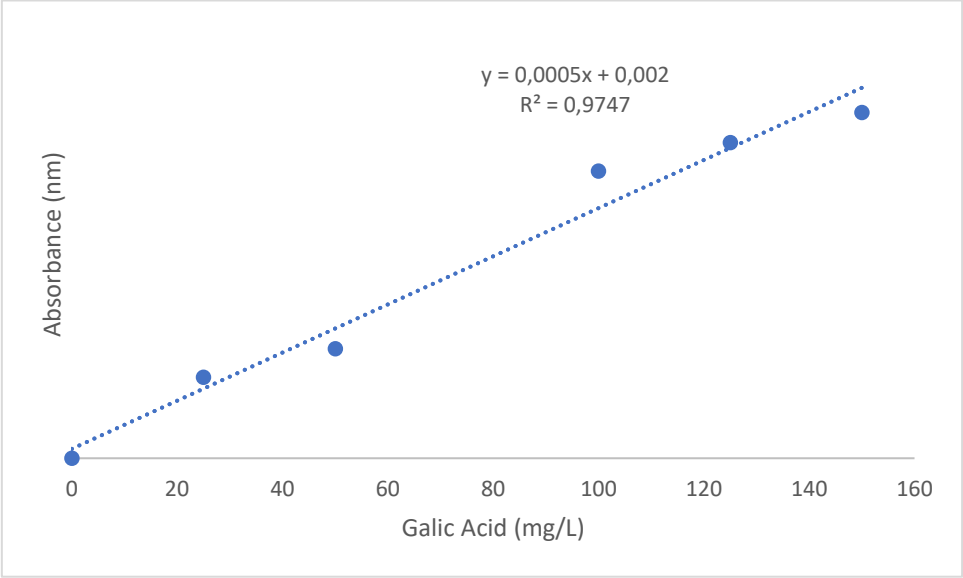
Supplementary Information 3- Carbohydrates calibration curve using different concentrations of a glucose stock solution of 250 mg/L.



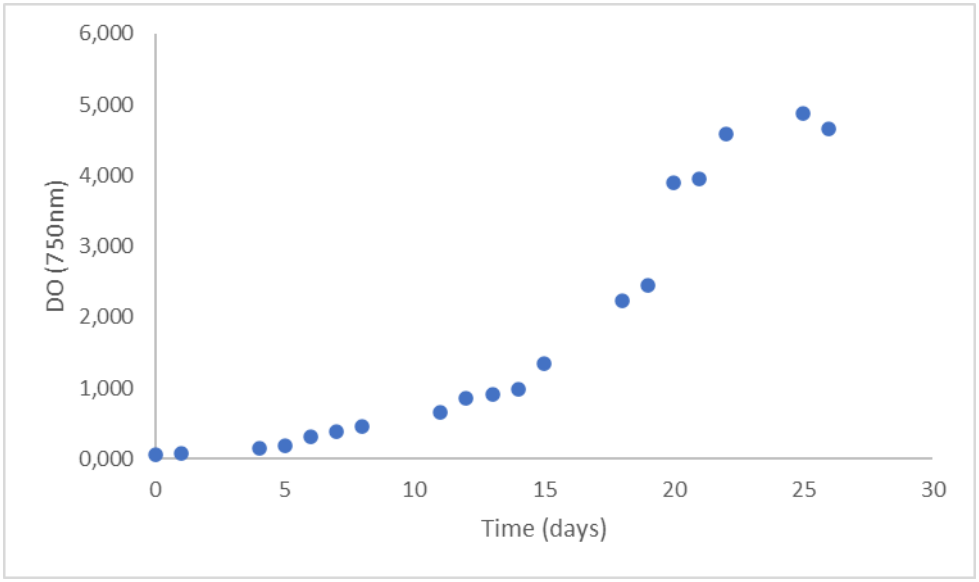
Supplementary Information 4- Lipid calibration curve using different concentrations of a canola oil stock solution of 100 mg/mL.



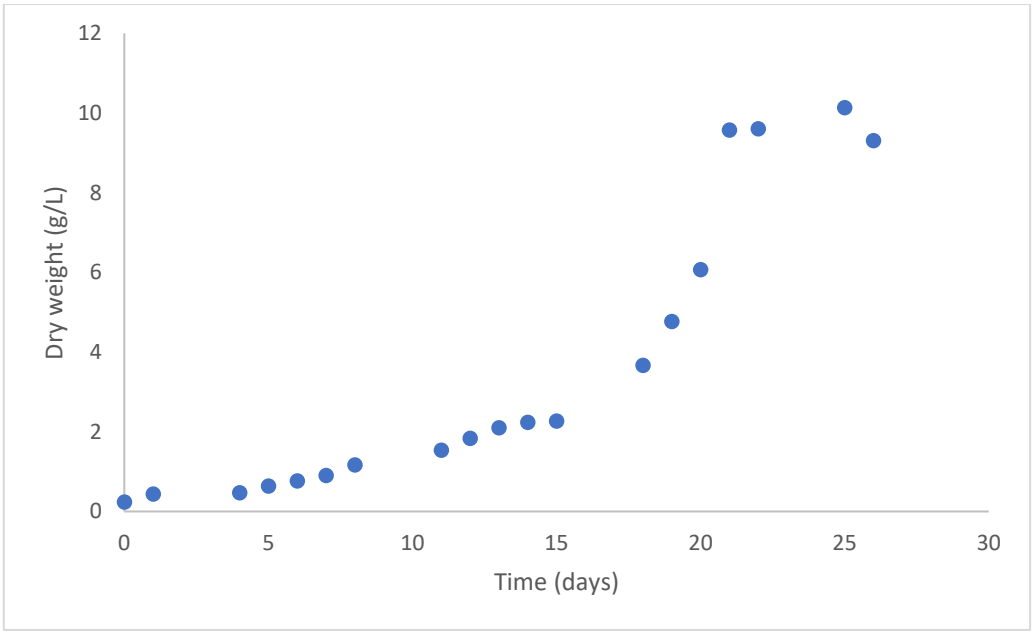
Supplementary Information 5- Proteins calibration curve using different concentrations of a BSA stock solution of 100 mg/L.



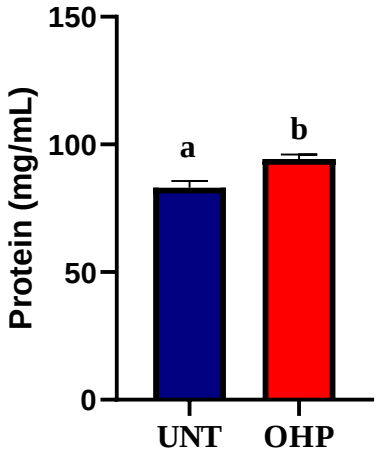
Supplementary Information 6- Antioxidant activity calibration curve (FRAP) using different concentrations of a Iron (II) sulfate stock solution of 200 mg/L.



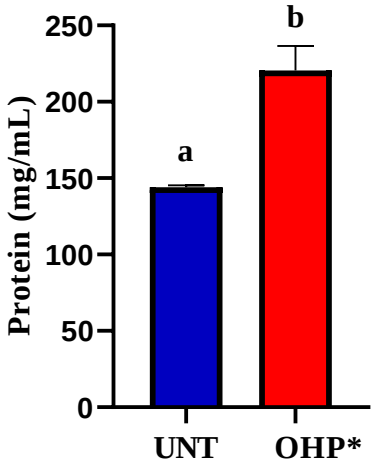
Supplementary Information 7- Growth curve of the microalgae used during the work carried out. The wavelenght used was 750 nm.



Supplementary Information 8- Evolution of microalgae dry weight over time.



Supplementary Information 9- Electrical capacity test to extract without the associated thermal effect.Comparison between the UNT represented by ● and OHP (133 V/cm) represented by ● performed under 37 °C. According to t-test, the assays presenting the same letter do not differ significantly (α=0.05).



Supplementary Information 10- Electrical capacity test to extract without the associated thermal effect (with NaOH 5 mM). Comparison between the UNT represented by ● and OHP (133 V/cm) represented by ● performed under 37 °C.In each assay was used NaOH (5mM). According to t-test, the assays presenting the same letter do not differ significantly (α=0.05).

Supplementary Table 1- Extraction rate and concentrarion of the extraction protein obtained with COV, OH, OHP (133 and 217 V/cm) and Untreated methods.

Treatments		Extraction rate (%)	Mean	SD	Concentration (mg/100g biomass)	Mean	SD	Concentration control (mg/100g biomass)	Mean	SD
COV	**50ºC**	1.58	1.49	0.06	9.28	8.75	0.38	612.83	586.16	24.94
		1.44			8.41			592.83		
		1.46			8.55			552.83		
	60ºC	2.40	2.42	0.03	14.01	14.19	0.21	612.83	586.16	24.94
		2.47			14.48			592.83		
		2.40			14.08			552.83		
	70ºC	6.59	6.55	0.07	25.21	25.06	0.27	416.83	382.60	24.50
		6.61			25.28			360.83		
		6.45			24.68			370.16		
OH	**50ºC**	2.84	2.84	0.19	10.48	10.48	0.71	375.49	368.38	5.03
		3.08			11.35			364.83		
		2.61			9.61			364.83		
	60ºC	6.30	5.98	0.50	23.21	22.01	1.84	375.49	368.38	5.03
		5.27			19.41			364.83		
		6.36			23.41			364.83		
	70ºC	6.42	6.45	0.18	24.55	24.66	0.68	416.83	382.60	24.50
		6.24			23.88			360.83		
		6.68			25.55			370.16		
OHP 133V/cm	**1 pulse**	5.63	5.85	0.16	21.55	22.37	0.60	416.83	382.60	24.50
		6.00			22.95			360.83		
		5.91			22.61			370.16		
	3 pulses	3.35	3.24	0.09	17.08	16.55	0.44	495.49	510.60	17.73
		3.14			16.01			500.83		
		3.24			16.55			535.49		
	5 pulses	3.14	3.16	0.09	16.01	16.12	0.47	495.49	510.60	17.73
		3.06			15.61			500.83		
		3.28			16.75			535.49		
OHP 217V/cm	**1 pulse**	7.84	7.85	0.03	23.55	23.57	0.08	296.16	300.16	2.88
		7.82			23.48			302.83		
		7.89			23.68			301.49		
	1 pulse NaOH (5mM)	20.54	20.45	0.99	29.55	29.41	1.42	142.96	143.85	6.35
		21.61			31.08			152.03		
		19.20			27.61			136.56		
Untreated	**NaOH 5mM**	7.66	7.75	0.10	11.01	11.15	0.14	142.96	143.85	6.35
		7.89			11.35			152.03		
		7.70			11.80			136.56		

Supplementary Table 2- Extraction rate and concentrarion of the extraction chlorophyll a obtained with COV. OH. OHP (133 and 217 V/cm) and Untreated methods.

Treatments		Extraction rate (%)	Mean	SD	Concentration (mg/100g biomass)	Mean	SD	Concentration control (mg/100g biomass)	Mean	SD
COV	**50ºC**	0.66	1.04	0.27	5.66	8.95	2.34	889.19	868.32	14.90
		1.27			10.96			855.36		
		1.18			10.24			860.41		
	60ºC	1.28	1.06	0.23	11.11	9.21	1.95	889.19	868.32	14.90
		0.75			6.52			855.36		
		1.16			9.99			860.41		
	70ºC	8.59	8.26	1.07	29.67	28.55	3.65	406.34	348.78	40.73
		6.82			23.62			318.38		
		9.37			32.35			321.60		
OH	**50ºC**	2.71	2.68	0.09	14.87	14.76	0.53	425.17	553.16	91.71
		2.78			15.35			635.31		
		2.56			14.06			598.99		
	60ºC	2.62	2.78	0.42	14.39	15.30	2.36	425.17	553.16	91.71
		3.36			18.53			635.31		
		2.36			12.97			598.99		
	70ºC	21.94	16.28	4.01	75.87	56.28	13.86	406.34	348.78	40.73
		13.3			45.97			318.38		
		13.59			46.99			321.60		
OHP 133V/cm	**1 pulse**	33.16	37.94	4.18	118.00	135.03	14.89	406.34	348.78	40.73
		43.35			154.26			318.38		
		37.31			132.82			321.60		
	3 pulses	1.44	1.38	0.04	13.09	12.58	0.37	868.83	911.61	42.14
		1.36			12.42			968.94		
		1.34			12.23			897.06		
	5 pulses	2.19	5.30	2.86	19.93	48.14	25.96	868.83	911.61	42.14
		4.61			41.89			968.94		
		9.09			82.60			897.06		
OHP 217V/cm	**1 pulse**	50.53	50.46	0.06	101.63	101.85	0.19	103.95	201.82	72.76
		50.45			101.83			223.23		
		50.39			102.10			278.28		

Supplementary Table 3- Extraction rate and concentrarion of the extraction chlorophyll b obtained with COV, OH, OHP (133 and 217 V/cm) and Untreated methods.

Treatments		Extraction rate (%)	Mean	SD	Concentration (mg/100g biomass)	Mean	SD	Concentration control (mg/100g biomass)	Mean	SD
COV	**50ºC**	2.26	3.24	0.70	8.72	12.44	2.68	391.50	389.58	5.37
		3.88			14.95			394.98		
		3.57			13.65			382.26		
	60ºC	3.56	3.10	0.51	13.97	12.21	1.98	391.50	389.58	5.37
		2.39			9.44			394.98		
		3.36			13.22			382.26		
	70ºC	11.60	11.53	0.41	11.61	11.59	0.46	116.40	105.40	8.63
		11.00			11.02			95.32		
		12.00			12.15			104.46		
OH	**50ºC**	3.35	3.46	0.18	8.55	8.77	0.46	296.63	258.84	32.93
		3.71			9.41			216.38		
		3.31			8.35			263.51		
	60ºC	3.35	3.50	0.32	8.52	8.88	0.81	296.63	258.84	32.93
		3.95			9.99			216.38		
		3.20			8.11			263.51		
	70ºC	20.20	16.37	2.74	20.23	16.37	2.76	116.40	105.40	8.63
		14.00			13.97			95.32		
		14.90			14.92			104.46		
OHP 133V/cm	**1 pulse**	35.10	38.87	3.88	35.05	38.84	3.87	116.40	105.40	8.63
		44.20			44.15			95.32		
		37.30			37.30			104.46		
	3 pulses	2.95	2.74	0.22	8.71	8.10	0.64	289.74	301.06	10.83
		2.84			8.37			315.66		
		2.44			7.22			297.79		
	5 pulses	3.62	5.85	2.57	10.74	17.27	7.57	289.74	301.06	10.83
		4.47			13.19			315.66		
		9.45			27.88			297.79		
OHP 217V/cm	**1 pulse**	26.46	28.10	1.68	31.04	34.95	3.17	162.23	127.56	35.39
		27.43			34.99			78.97		
		30.41			38.81			141.49		

Supplementary Table 4- Extraction rate and concentrarion of the extraction carotenoids obtained with COV, OH, OHP (133 and 217 V/cm) and Untreated methods.

Treatments		Extraction rate (%)	Mean	SD	Concentration (mg/100g biomass)	Mean	SD	Concentration control (mg/100g biomass)	Mean	SD
COV	**50ºC**	0.79	1.22	0.31	2.37	3.70	0.96	308.50	303.17	3.79
		1.52			4.62			300.00		
		1.36			4.10			301.00		
	60ºC	1.49	1.26	0.26	4.45	3.78	0.77	308.50	303.17	3.79
		0.89			2.70			300.00		
		1.39			4.19			301.00		
	70ºC	9.68	9.57	0.84	9.02	8.90	0.74	109.84	94.17	11.24
		8.49			7.94			84.04		
		10.54			9.75			88.64		
OH	**50ºC**	2.13	2.10	0.11	4.85	4.81	0.23	225.60	231.27	4.43
		2.22			5.06			231.80		
		1.96			4.51			236.40		
	60ºC	2.09	2.22	0.32	4.79	5.07	0.73	225.60	231.27	4.43
		2.65			6.07			231.80		
		1.91			4.35			236.40		
	70ºC	21.94	16.70	3.71	20.35	15.52	3.42	109.84	94.17	11.24
		13.87			12.92			84.04		
		14.30			13.29			88.64		
OHP 133V/cm	**1 pulse**	41.40	45.77	4.42	38.47	42.56	4.13	109.84	94.17	11.24
		51.83			48.22			84.04		
		44.09			40.99			88.64		
	3 pulses	2.42	2.14	0.32	6.34	5.58	0.86	255.40	261.13	4.05
		2.31			6.02			264.00		
		1.69			4.37			264.00		
	5 pulses	3.88	6.86	5.45	10.13	17.83	14.17	255.40	261.13	4.05
		2.19			5.67			264.00		
		14.50			37.70			264.00		
OHP 217V/cm	**1 pulse**	40.92	43.06	2.87	29.05	34.71	4.50	75.97	85.10	15.65
		41.13			35.00			72.20		
		47.12			40.07			107.12		

Supplementary Table 5- Extraction rate and concentrarion of the extraction carbohydrates obtained with COV, OH, OHP (133 and 217 V/cm) and Untreated methods.

Treatments		Extraction rate (%)	Mean	SD	Concentration (mg/100g biomass)	Mean	SD	Concentration control (mg/100g biomass)	Mean	SD
OHP	**1 pulse 133V/cm**	36.82	40.41	2.69	44.73	49.09	3.27	116.98	121.48	3.19
		43.30			52.61			123.92		
		41.09			49.92			123.54		
	1 pulse 217V/cm	54.98	52.84	6.58	66.79	64.19	8.00	116.98	121.48	3.19
		59.61			72.42			123.92		
		43.92			53.36			123.54		
Untreated		0	0	0	0	0	0	116.98	121.48	3.19
								123.92		
								123.54		

Supplementary Table 6- Extraction rate and concentrarion of the extraction lipids obtained with COV, OH, OHP (133 and 217 V/cm) and Untreated methods.

Treatments		Extraction rate (%)	Mean	SD	Concentration (µg/L)	Mean	SD	Concentration control (µg/L)	Mean	SD
OHP	**1 pulse 133V/cm**	38.51	40.13	8.29	1983.33	2066.67	426.91	4590.48	5150.00	395.91
		30.88			1590.48			5411.90		
		50.99			2626.19			5447.62		
	1 pulse 217V/cm	17.71	13.55	3.15	911.90	697.62	162.36	4590.48	5150.00	395.91
		12.85			661.90			5411.90		
		10.08			519.05			5447.62		
Untreated		0	0	0	0	0	0	4590.48	5150.00	395.91
								5411.90		
								5447.62		