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Microalgae Biorefinery Routes and Unit Operation Pre-Project Design, Selection and Sizing

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Dedicated to my beloved parents...

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Resumo

Nos últimos anos, as microalgas têm sido amplamente estudadas como um recurso natural para várias aplicações economicamente relevantes e atualmente existem várias opções para a recuperação e processamento da biomassa, a fim de obter metabolitos intracelulares. No presente trabalho, são apresentados os resultados a escala laboratorial de duas propostas de biorrefinaria de *Nannochloropsis* sp. para a produção de lípidos e pigmentos de alto valor comercial, e ainda proteínas e carboidratos. Relativamente ao método de ruptura das células, foi feita uma revisão da literatura dos métodos existentes, concluído-se que o moinho de esferas é a tecnologia mais adequada para biorrefinarias de microalgas em larga escala.

Como os solventes derivados do petróleo como o diclorometano, tolueno ou hexano são tóxicos e não renováveis, foram testadas extrações inovadoras e sustentáveis usando como solventes o D-limoneno, o azeite e o etanol para extrair lipídos e pigmentos de alto valor comercial. Diferentes membranas de filtração foram testadas com o intuito de recuperar os solventes: etanol e D-limoneno. Em relação, aos coeficientes de rejeição dos pigmentos e dos ácidos gordos, foram obtidos resultados promissores, demonstrando ser possível fracionar biomoléculas com alto valor com um processo verde e limpo.

Posteriormente, os resultados laboratoriais foram extrapolados para cálculos de balanços de massa, com o intuito de selecionar a melhor proposta de biorrefinaria, através de uma análise económica. A biorrefinaria proposta em que se utiliza azeite como solvente, mostrou ser o processo mais promissor para futuros estudos, visto que, aliado ao CAPEX estimado ser mais baixo, o extracto de azeite rico em ácidos gordos EPA poderá ser um produto final altamente lucrativo e com altos benefícios para a saúde humana.

Palavras-chave: Biorefinaria de *Nannochloropsis* sp.; ácidos gordos EPA; D-limoneno; azeite; etanol.

Abstract

Microalgae, or microscopic algae, have been studied as a natural marine resource for a number of economically relevant applications and several options exist for recovering and processing the biomass to obtain intracellular metabolites. This work presents the laboratory results from two proposed *Nannochloropsis* sp. microalgae biorefineries for the production of oil, high-value pigments, proteins and carbohydrates. Concerning the microalgal cell disruption processes, an overview of the technologies available was performed and bead milling was found to be potentially suitable for large scale microalgae biorefineries.

Since petroleum-derived solvents such as dichloromethane, toluene or hexane are toxic and non-renewable, innovative and sustainable extractions using D-limonene, olive oil and ethanol were tested to extract valuable lipids and pigments. Different membranes were also tested aiming the solvent recovery of ethanol and D-limonene. Regarding rejections of pigments and fatty acids, high values were achieved using organic solvent resistant membranes, proving to be possible to fractionate valuable biomolecules with a green and clean process.

Afterwards, the laboratory results were extrapolated to mass balance calculations to select the biorefinery route by conducting a simplified economic analysis through equipment sizing and the cost estimation of the major equipments. The biorefinery route using olive oil as solvent was found to be the most promising process to study because, combined with the lower estimated CAPEX, the olive oil extract might be a highly profitable product rich in EPA fatty acid, as a product with significant health benefits for the human population.

Keywords: *Nannochloropsis* sp. biorefinery; EPA fatty acid; D-limonene; olive oil; ethanol.

Contents

List of Tables

List of Figures

List of Abbreviations

TAG – Triacylglicerides

NL – Neutral lipids

GL – Glycolipids

PL – Phospholipids

EPA - Eicosapentaenoic acid

SFA – Saturated Fatty Acid

MUFA – Monounsaturated Fatty Acid

PUFA – Polyunsaturated Fatty Acid

PEF – Pulsed Electric Field

HPH – High Pressure Homogenization

MWCO – Molecular Weight Cut-Off

CF – Concentration Factor

FAME – Fatty Acid Metyl Ester

OPEX – Operational Expendidure

CAPEX – Capital Expendidure

V – Volume

J_v – Permeability Flux

$\bar{J_v}$ – Average Permeability Flux

J_{avg} – Average flux rate

A – Area

P – Pressure

t – Time

$\mathbf{C_i}$ or $\mathbf{[compound]_i}$ – Concentration of compound i

m – mass

Q – Volumetric flow rate

F – Flow rate

$\mathbf{U_i}$ – Velocity

Σ – Area of a gravity settler

ρ – Density

d – Diameter

μ – Viscosity

r – Radius

τ – Residence time

Chapter 1

Introdution

1.1. Background

It is known that fossil fuels are considered the main global source of energy, however they are unsustainable and non-renewable. In the last century, due to exponential increase of world population and energy consumption, the use of new resources became a need [1]. Moreover, the level of carbon dioxide (CO_2) present in the atmosphere caused by fuel combustion from industrial and transportation sectors achieved the highest number ever, hence it is necessary to introduce other approaches for CO_2 sequestration for instance such as agriculture, reforestation and photosynthetic microorganisms [2].

In the area of photosynthetic microorganisms, microalgae have been widely studied and presented as one of the great alternatives for the production of biofuels, aiming at replacing the use of fossil fuel and serving as the main raw material for the mitigation of greenhouse gases. This is due to microalgae photosynthetic efficiency in bio-conversion of carbon dioxide (CO_2), high biomass productivity, high lipid accumulation and its valuable non-combustible co-products [3].

Although microalgae have high potential to produce biofuels and replace fossil in power generation, challenges to produce at large scale need to be considered. One of the great challenges of this process is the low market price of fossil fuels which have a negative effect on the demand for biofuels [4].

The viability of a microalgae-based biofuel industry seems not to be possible and directing the potential of microalgae to biofuel alone seems to be no longer plausible. These microorganisms are like microscopic factories producing all sorts of valuable compounds, not just lipids dedicated to the biodiesel industry. They are composed of lipids but also valuable proteins, pigments and

carbohydrates [5]. As the interest for microalgal compounds grows, so does the need for greener and renewable strategies for energy. From this arises the concept of biorefinery associated with microalgae and the main theme of this thesis.

Like in petroleum refinery processes, the biomass feedstocks are constituted by elements as carbon, hydrogen, nitrogen and oxygen but in different proportions. Therefore, the principles of petroleum refinery can be used for biomass processing to find a potential way to obtain marketable products and energy in different industries like pharmaceutics, biofuels, chemicals, food/feed and cosmetics. In addition, a complete biorefinery aiming at viable and profitable exploitation of the biomass components allocating them in different markets may lead to several market combinations [6].

1.2. Framework and Goals

The main goal of this work is to develop biorefinery routes of the microalgae *Nannochloropsis* sp. in order to obtain a economically viable project for production of commercial products. To achieve this goal, this microalgae strain will be fully characterized to know the proportions of the most valuable products to be extracted from the cell and identify expected economic bottlenecks and opportunities. To make the best decisions in any entrepreneurial projects, it is imperative to take into account the separation of each fraction with minimum cost (equipment and process) and also minimum environmental footprint. The solution must be compatible with industrial realities.

To evaluate the viability of the proposed biorefineries, various possible processes were tested at laboratory scale. Afterwards, the results were extrapolated to mass balances calculations to select a biorefinery route by doing an economic analysis through the balance sheets and equipment sizing.

Chapter 2

State of the Art

Biorefinery: General Concept and Challenges

2.1. A biorefinery focuses on the designing of a sustainable process that converts biomass (including waste) into a spectrum of marketable products and energy, maximising the value of the biomass and minimising waste [7][8]. This definition is analogous to today's integrated petroleum refinery and petrochemical industry that produces multitude of fuels and organic chemicals from petroleum.

Due to the diversity of raw materials and processing technologies, three grades of biorefinery can be distinguished [9][10]:

- A “Phase I” biorefinery: processes a single raw material in a simple and fixed transformation process, yielding one main product;
- A “Phase II”: biorefinery also processes a single raw material, but is able to produce various end-products due to the flexible transformation technology in response to the market;
- A “Phase III”: biorefinery uses many types of raw materials and production technologies which allows the production of various industrial products.

In any case, there are constraints to be managed towards the development of a biorefinery. In biomass utilization, one issue to consider is the cost of the respective supply chain and the technology to convert biomass into useful forms of energy. Therefore, several attempts have been made to optimize the simulations of a specific biomass supply chain to reduce the costs and achieve efficient logistics operations. In this type of refinery, biological issues such as harvesting/collection of biomass, high transportation costs (biomass is bulky and difficult to transport), storage, seasonal availability and others must be managed [11][12].

Also, regarding the market viability of an integrated biorefinery, it should also optimize the use of biomass to create products that match the demands and requirements of the market [10].

Microalgae Biorefineries

2.2.1. General Approach

Microalgae are photosynthetic microorganisms and can live either in marine or freshwater environments and can include bacteria and unicellular plants. These microorganisms are unicellular differing from macroalgae and may exist individually or form cell chains [13].

2.2.

Over other biomass feedstocks, the major advantages of microalgae reside in their metabolic flexibility (by varying culture conditions), which offers the possibility of modification of their biochemical pathways (e.g., towards protein, carbohydrate or oil synthesis) and cellular composition[14]. Due to biomass compositions differences, it is essential the assessment of commercial relevance of the microalgae strain as a raw material for producing high-value products [15], see some examples in Table 2.1**.**

Table 2.1. Commercially valuable biomolecules from microalgae (adapted) [16].

Algal Components		Applications	Species
Lipids	Docosahexaenoic acid (DHA)	Nutraceutical suplements	*Crypthecodinium, Schizochytrium*
	Eicosapentaenoic acid (EPA)	Nutraceutical suplements	*Nannochloropsis, Phaeodactylum, Nitzschia*
	γ-Linoleic acid (GLA)	Infant formula	Spirulina, *Chlorella*
	Arachidonic acid (AA)	Infant formula	*Porphyridium, Lobosphaera*
	Triacylglyceride (TAG)	Energy source for diesel vehicles	*Nannochloropsis, Chlorella, Botryococcus*
Pigments	Astaxanthin	Natural food color, antioxidant	*Haematococcus*
	β-carotene	Natural food color, antioxidant	Spirulina, *Dunaliella*
	C-phycocyanin	Natural food color, antioxidant	Spirulina
Carbohydrates	Polysaccharide	Food Thickener	*Chlorella, Dunaliella*
Protein	Whole cells, delipidated biomass or extracted protein	Animal and fish feed	Spirulina

In spite of the promising microalgae biorefinery for biofuel production, the process is technically feasible, but it has not yet reached an economical viable level. Economics are currently the main barrier to produce microalgae on a large scale for the exploitation of lower value compounds such as biofuels, since industries are still in R&D phase[17]. Apart from potential feedstock for biofuel production, microalgae plays an important role in environmental pollution control, human

health, animal and aqua nutrition, cosmetic industry, pharmaceutical filed and as a source for bioactive compounds, biomedical components and high value pigments [4][18].

As microalgae biorefinery perspectives, its success will only be achieved if all the biochemical components of biomass are exploited through an optimized downstream procedure composed of different streams for diverse commercial end-products purposes[19]. According to Figure 2.1, there are in total five steps to achieve the end-products: culture production, harvesting, disintegration, fractionation and purification. However, for this report the focus will be made only on the last three steps.

Figure 2.1. Overall general process for microalgae production and biorefinery

Nevertheless, this new concept has been recognized as an opportunity to build a future economy based on renewable sources. Although society started to finance Research and Development (R&D) activities for biorefineries implementation, this concept is still in pilot or small-scale demonstration phases, thus far from commercialisation [20][21].

2.2.2. *Nannochloropsis* sp. Biorefinery

The microalgae strain used for the development of the present work was *Nannochloropsis* sp. from Eustigmatophyceae lineage which can be found in brackish and ocean waters. The cells are small (2-5 um in diameter), spherical to slightly ovoid and possess a strong cell wall composed by fibrillar and amorphous components [22].

The nutritional composition of this microalgae is highly influenced by the residence time in the photobioreactor, for example for short residence times, richer in protein is the biomass harvested; for high residence times higher polyunsaturated fatty acids (PUFA) omega-3 concentrations [23]. Yet, on average, the biomass contain 20% (w/w) available carbohydrates, 50% crude protein of which 20% are soluble, 20% total lipids, 3% pigments and 7% of minerals [24].

The composition of pigments in this strain is characterized by chlorophyll a (lack of chlorophyll b or chlorophyll c), violaxanthin and vaucheriaxanthin as the major pigment contents, Table 2.2. [25]. Found in minor quantities were zeaxanthin and several chlorophyll a derivatives [26].

Table 2.2. Pigments profile of *Nannochloropsis* sp. Obtained from [25]

	Pigments/Total pigments [%]	**Pigments/Biomass [%]**
Chlorophyll a	54	1.7
Violaxanthin	22	0.7
Vaucheriaxanthin	15	0.5
Canthaxanthin	3	0.1
Astaxanthin	3	0.08
Zeaxanthin	1	0.02
Antheraxanthin	2	0.05
Total	100	3.1

Marine microalgae are the primary producers of ω-3 PUFAs, therefore, due to the limitation of global market of fish oils, they have been strongly studied and are considered as promising alternative for PUFA production. *Nannochloropsis* sp. has a particular interest because it can accumulate high value oil (from 20-45 % of dry weight) containing omega-3 fatty acids, specifically eicosapentaenoic acid (EPA) [27].

Despite being an excellent source of EPA, there are some factors such as availability of phosphorous, nitrogen or silicon, temperature, salinity, high level, light/dark cycle and the growing phase that have effects on the fatty acids profile [28]. Aiming this fatty acid accumulation, there are cultivation conditions to manage since the deprivation of a key nutrient induces accumulation of triacylgrlyceride (TAG) and decreases other lipid classes containing EPA. The nutrient deprivation causes the induction of the *de novo* fatty acid synthesis, aside from the rearrangement of some polar lipids to form neutral lipids [22][29].

It is also crucial the knowledge of the portion of fatty acids between lipid classes to develop extraction methods capable to reach high yields of omega-3 fatty acids [30].

This microalgae gender has the advantage of being one of the few species recognized and accepted as human food due to its nutritional value. Thus, a *Nannochloropsis* sp. biorefinery is highly interesting where a greener bioprocess accomplishing zero-waste, multiple-products concept can be applied [22].

2.2.3. Microalgae Cell Disruption Step

The first biorefinery step focused in the present work is the disintegration/disruption of the cell. Considering the cell wall structure, the location of the products and the small size of microalgae, this step represent a significant challenge for inexpensive extraction of the commercial products [31].

Thus, the microalgae-based biorefinement process requires efficient methods to recover intracellular biomolecules. Due to the microalgae cell structure diversity and rigidity, cell disruption is the most critical step in the process in terms of costs, energy-intensity and/or environmental implications when organic solvents are needed. Therefore, robustness, energy-efficiency, minimization of environmental impact and product quality are the process requirements for this downstream step [32].

To achieve the maximization of the value of the materials obtained from refining microalgae, fast and precise disruption needs to be used. In an industrial setting, an appropriate cell disruption technology is selected based on strength of cell walls, scalability, risk of sub-cellular destruction of important products, the costs of the process and the safety concerns, i.e. non-contamination of products since most of them are for food grade purpose [33].

Several disruption methods are available and they can be broadly divided into two big categories based on their working mechanism: mechanical and non-mechanical disruption [34].

2.2.3.1. Mechanical Disruption

This category, as its name implies, uses only mechanical processes, putting aside the chemical and enzymatic processes. These methods are considered harsh and non-selective and they are based on:

1 Cells are subjected to shearing by liquid flow;
2 Pressure differences between inside and outside the cell causing its explosion;
3 Collision forces by impact of beads or paddles
4 Combination of the previous forces [35].

This driving force has the advantage to be continuous, with no addition of matter into the system, avoiding further contaminations in the final product which simplifies downstream processing and scalability to industrial level [35][36].

Nevertheless, if excessive force is used, it will generate detrimental heat that can ruin the desired proteins and pigments, hence require more sophisticated equipment and higher energy inputs for processing [35][37]. In the following topics several techniques were presented such as bead milling, high pressure homogenization, pulse electric field, ultrasonification and microwave.

2.2.3.1.1. Bead Milling

A bead mill is a homogenizer originally designed for size reduction of paint or lacquer particles. This equipment is composed by a grinding chamber with a stirrer (concentric or eccentric disks or rings) that spread kinetic energy to small beads resulting in multiple collisions. The operation can occur under batch (recirculation) or continuous (single pass though milling chamber) conditions depending on the biomass concentration [34][38].

Using this technology requires a high number of operation parameters, which should not be considered a disadvantage since it opens up possibilities for optimizing performance for different products. Parameters such as feed rate of the suspension, agitator speed, cell density, bead

diameter, bead density, bead filling (% of the grinding chamber volume) can be controlled and optimized during the operation [39].

However, the optimal interaction between the different operating parameters seems to vary towards morphology of the microorganism, which makes them complex and difficult to predict [40].

As stated before, one of the most important factors that can be controlled during the process is the correct ratio of the size of the grinding balls to the size of the particles. If the beads are too small, they will not reduce the size of the particles ("destroy" the particle. In addition, a set of beads could start to act as a filter and accumulate product and agglomerates in the chamber. Thus, the mill will block. On the other hand, if the beads are very large, the probability of the product not colliding with the beads increases drastically, see Figure 2.2. In the previous cases, the grinding yield will be low [41]. Also, previous researches found out that the beads filling up to 85% of grinding chamber volume have a positive effect on the process [34].

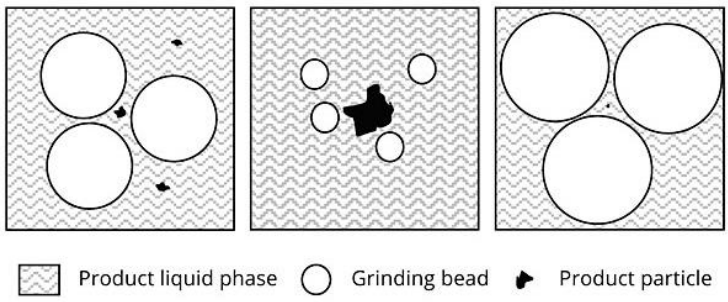

Figure 2.2. A: Correct particle size/bead size ratio; B: Beads are too small case; C:Beads are too large case [40].

Regarding impacts and friction between the elements inside the chamber, production of heat is an intrinsic problem in agitated mills related to denaturation of proteins and degradations of pigments. Although a cooling jacket integrated in the chamber is generally sufficient to handle the generated heat, it is necessary to optimize the demand of cooling energy to achieve a favourable process [42].

2.2.3.1.2. High Pressure Homogenization (HPH)

The driving force of this technique, as the name suggest, is high pressure (shear forces) caused by the accelerated fluid jet on the stationary valve surface and the hydrodynamic cavitation from the pressure drop induced shear stress, see Figure 2.3 [34].

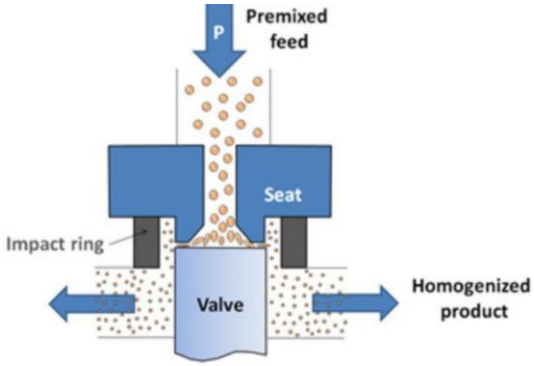

Figure 2.3. A typical HPH equipment [43].

Nowadays, high pressure homogenizers are unit operation widely used in pharmaceutical, chemical, and food industries, for example to recover recombinant proteins Escherichia coli and Saccharomyces cerevisiae [44]. Thus, this technique started to be widely studied for algae processing proposes and found out that is feasible for process-scale algal cell disruption [34][45][46][47].

The major controllable parameter for HRH is the pressure applied on the medium and the subsequent pressure drop across the orifice, valve seats and impact ring. Other operating parameters which affect the disruption efficiency are the process temperature, number of passes, the valve and orifice design and the medium flow rate [48].

The main drawback of using HPH, together with bead mill technique, is energy demand and the possible denaturation of the metabolites [34][47].

2.2.3.1.3. Pulsed Electric Field (PEF)

In this technique, electric pulses are applied to the cells, either in suspension or tissue, causing the electroporation of the cell membrane, increasing its permeability and allowing larger molecules that otherwise cannot cross the membrane, such as molecules of drugs, DNA or even biomolecules, enter or exit the cell. If the amplitude of the pulse is suitable, the electric field and the induced transmembrane voltage associated are high enough to cause permeabilization of the cell membrane [49].

PEF has an excellent potential as a preliminary step of aqueous extraction of algae components. Besides, PEF technique allows selective extraction of some pure biomolecules [50].

Some of these critical factors to keep in mind are: the field strength, treatment time, treatment temperature, pulse shape, strain of microalgae, growth stage of microorganism, and characteristics of the treatment substrate [50][51].

In spite of being easily scaled-up and combined with different biomass treatment methods, the biomass to treat must be electrically low conductive, which represents a limitation for microalgae biorefineries [52].

2.2.3.1.4. Ultrasonification

The general principle of this technique is based in two mechanisms: cavitation and subsequent acoustic streaming. Cavitation is the production of microbubbles consequence of the applied ultrasound that eventually become unstable and implode violently, sending shock waves that disrupt surrounding cells [53]. In the food industry, ultrasounds are preliminarily used as a control system using their high frequency, but recently they became more attractive given to high-power (or low-frequency) ultrasound as an innovative and alternative process, as the process simplifies the handling and processing conditions [54].

However, in case of high cell concentrations, power is diminished even more quickly due to the increased viscosity. Also, the subsequent heat generation can denature proteins and degrade pigments. Very reactive hydroxyl radicals can be formed by thermolysis of water and interact with most biomolecules. These factors potentially are critical barriers to practical, scaled-up based microalgal biorefinery applications [34][55].

2.2.3.1.5. Microwave

Microwave treatment is a technique that includes heating locally a material causing evaporation of the medium water exposed, which generates higher pressure in the bubbles and the rupture of cells favouring the release of the desired intracellular contents [34][56].

Nevertheless, the efficiency of this microwave treatment in the presence of non-polar solvents or target compounds is poor. It also depends on their dielectric, electrical and magnetic properties as well as on composition and shape. This disadvantage makes this method unattractive to microalgae biorefineries because most of the extracted lipids (triglyceride) from microalgae were non-polar [57][58].

2.2.3.2. Non-mechanical disruption

Compared to mechanical cell disruption, the non-mechanical cell disruption techniques consume less energy. Generally, this method is more gentle and specific but is difficult to scale up to industrial levels [59]. This section, is divided by several examples of non-mechanical methods such as chemical treatments, enzymatic treatments and osmotic shock.

2.2.3.2.1. Enzymatic Treatments

Enzymatic degradation of the microalgal cell walls prior to further processes has the potential to increase extraction yields by reducing solvent and energy inputs, improving accessibility of cell wall polymers to microorganisms, enzymes or reagents involved in downstream algal biomass processing [60].

Highly selective disruption allowing the extraction of targeted bioproducts, mild reaction conditions, such as neutral pH and incubation temperatures between 25°C and 50°C, and the absence of energy intensive drying steps are the major advantages of this process. Based on composition and cell wall structure, the best enzyme or cocktail of enzymes are selected [61].

The key barrier for this widespread plant-scale application is the cost of purified enzyme production or commercial enzymatic cocktails and also long process times, thus a low production capacity compared to mechanical disruption [34][62][63].

2.2.3.2.2. Chemical Treatments

A wide variety of chemical compounds can cause microalgae cell disruption such as antibiotics, chelating agents, chaotropes, detergents, solvents, acids and alkali [34]. This possible treatment relies on the selective interactions of a chemical with the cell wall [64].

Surfactants are a chemical option to disrupt the cell as they could easily bind with microalga membranes that have a negative charge and depend on the affinities of other components: i.e., the hydrophilic–lipophilic interplay, which may cause cell disruption. Despite being cost-effective and energy-efficient, a change in cell-wall structure will affect the efficacy of cell disruption. The composition of the cell membrane not only changes with microalgae species, but also varies with physiological state for a single strain [55][65][66].

Acid/Alkaline addiction leads to hydrolysis of the cell and they might be promising in treating various biomass feedstocks, however it can cause degradation of some biomolecules and also the material selection, safety issues, and wastewater treatment are essential [34][67][68].

Using reasonable concentrations of detergents will compromise the integrity of cell membranes, penetrating between the membrane bilayers at concentrations sufficient to form mixed micelles with isolated phospholipids and membrane proteins while chelating agents bind the cations that cross-bridge adjacent cell membrane molecules [34][69].

Nevertheless, the use of chemicals harms the subsequent downstream process acting as a contaminant and must be used in low concentrations, which might impact process yields [70].

2.2.4. Extration Step

2.2.4.1. General Principles

According to the literature, the definition of an extraction operation says that “two phases come into contact with the objective of transferring a solute or particle from one phase to the other” [73].

Regarding single-stage processes, one feed stream contacts one extraction solvent stream, and the mixture divides into equilibrium extract and raffinate phases. When the equilibrium between the phases is reached, the distribution of the target solute is defined by the partition coefficient as

$$Partition\ coefficient = \frac{[solute]_{extract}}{[solute]_{raffinate}} \quad \textbf{(2.1)}$$

Considering this equation, it is desirable to have high value as possible, enabling low volumes of extraction solvent. Partition coefficients near unity would require large volumes and many serial extractions for full recovery.

The partition coefficient can depend on many parameters, such as the size of the molecule being extracted, pH, types of solvent, temperature, and concentration and molecular weight of polymers (or salt) in the phases [73]. Therefore, the selection of proper solvent and development of efficient extraction technique for biomolecules extraction is necessary. Despite solvents such as methanol, acetone, ethyl acetate, hexane, chloroform and various ionic liquids ([P(CH2OH)4][Cl], [BMIM][HSO4], [EMIM][DBP]) can be used for lipid and carotenoid extraction from microalgae biomass, most of the processes being demonstrated are using these solvents only at a lab-scale. Aiming industrial scale microalgae biorefineries, an efficient, cost effective and environmentally friendly extraction technology has to be developed to extract demanded products [74]. The application of the final extracts (e.g. for food products) is also relevant in the solvent selection process.

On the other hand, physiological properties of the desirable metabolites in the cell can impact on the efficacy of the solvent [75].

2.2.4.2. Extractable Compounds

2.2.4.2.1. Lipids

Some microalgae species are considered oleaginous because they can accumulate large amounts of lipids. Reports about this subject present lipid contents about 20% to 50% of dry biomass [76][77].

Microalgal lipids can be divided into two groups according to their structures: nonpolar neutral lipids (NLs) (acylglycerols, sterols, free fatty acids, waxes, and steryl esters) and polar lipids (phosphoglycerides, glycosylglycerides, and sphingolipids) [77]. When grown under ideal conditions and sufficient nutrients, microalgae can synthesize lipids including small amounts of glycerides or free fatty acids and large amounts of polar lipids, such as glycolipids (GL) and phospholipids (PL) [78].

Neutral Lipids

Neutral lipids are non-polar compounds and they include acylglycerols and free fatty acids. Acylglycerols consist of fatty acids ester-bonded to a glycerol backbone and are categorized according to their number of fatty acids: triacylglycerols (TG), diacylglycerols (DG), monoacylglycerols (MG) while free fatty acids are fatty acids merely bonded to a hydrogen atom [79], see Figure 2.4

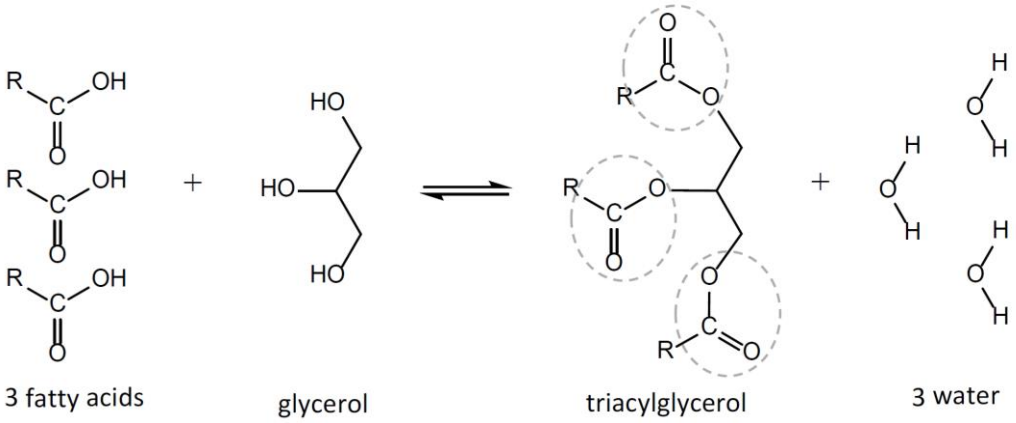

Figure 2.4. The main constituent of fats and oils is triacylglycerol, which in turn is formed by three fatty acids condensed with glycerol [80].

This type of lipids is used primarily as a storage form of carbon and energy and they are storage within the cell in specialized compartments in the cytoplasm called lipid particles (LPs), lipid droplets or oil bodies. Recent studies suggest that, in algae, the TAG biosynthesis pathway may play a more active role in the stress response, in addition to functioning as carbon and energy storage under environmental stress conditions [81][82][83]. Therefore, microalgae became attractive as feedstock for biofuel production by transesterification into fatty acid methyl esters (FAMEs)[24].

In microalgae, the unsaponifiable lipophilic compounds are present in the form of sterols, phenolic compounds, hydrocarbons and waxes.

As the biodiesel production is based on transesterification of vegetable oils and fats through the addition of methanol (or other alcohols) and a catalyst [84], the presence of unsaponifiable compounds in the production of biofuels can lead to engine coking carbon depositing. Therefore, in this context, they are considered as waste.

Nevertheless, in other industries such as food and cosmetic, they are a potential source of added-value compounds since they exhibited antioxidant properties causing the delay of deterioration of food and cosmeceutical products [19].

Waxes and sterols commonly contribute to the extracellular surface layers and they may act as energy stores especially in some organisms from cold water habitats [85].

Polar Lipids

The polar lipids present in algal cells are phospholipids and glycolipids. They are important components of membranes, which confer cellular characteristics of extreme relevance, being present in the outer cell membrane and also in the membranes associated with internal organelles, particularly the chloroplasts and the endoplasmic reticulum [25].

Highly unsaturated lipids occur more frequently in polar lipid fractions with high value polyunsaturated fatty acids (PUFAs) [86]. However, in downstream processes the extraction of this lipid

fraction may present some difficulties related to their behaviour when using solvents because they are amphiphilic molecules, i.e. consist of a hydrophobic and a hydrophilic portion [87]. Moreover, having a higher viscosity can lead to reduced transesterification velocity and FAME conversion. Also, they present higher degree of unsaturation hence lower oxidation stability [88].

2.2.4.2.2. Proteins

Proteins are macromolecules composed by chains of different amino acids and hence the nutritional quality of a protein is determined basically by the content, proportion and availability of its amino acids[89].Although microalgae have emerged as a promising alternative to fishmeal and soybean protein sources (typically 25–40% of the dry weight), there are several difficulties in mass production due to the cell wall barrier of these microorganisms [90][91][92]. According to comprehensive analyses and nutritional studies, algal proteins are considered high quality and comparable to conventional vegetable proteins (rich in essential amino acids). Therefore, they constitute a high value product to process from microalgae biomass to obtain enriched flours, concentrates or isolates for various food and feed industries[93][94].

Regarding extraction operations, the properties of these macromolecules may become a problem because they can act as natural emulsifiers in the process. They are constituted by hydrophilic and hydrophobic amino-acid groups forming three-dimensional conformations. If their hydrophobic groups are exposed at the surface, there is a possibility of attracting oil droplets and form protective coatings around them impairing the extractive power of the solvent. On the other hand, this protein coating may provide protection against chemical degradation by acting as a physical barrier, chelating agent, or antioxidant [95].

Integrating a stream to obtain protein based-product in microalgae biorefinery requires several inputs to satisfy all the food industry requirements. For example, if the supply of nutrients to an algae cultivation came from wastewaters, there is a high probability that the final processed protein would be prohibited for nutritional applications [96]. Therefore, the presence of antinutritional compounds such as allergens, toxins, pathogens, heavy metals, and pesticides in the extract with possible hazardous effects on human health should also be monitored and limited by appropriate processing, as in the case of any other food source [97].

Using organic solvents to extract these biomolecules is usually not feasible since proteins are often denatured or degraded, but they were already successfully extracted by means of two immiscible aqueous liquid phases that consist of solutions of two water-soluble but incompatible polymers, or one polymer plus a high concentration of certain salts. [73] Also, the conditions associated to the extraction of proteins may be adverse to obtain economically advantageous protein yields, consequently this adversity can be problematic in terms of preservation of protein quality, both in terms of amino acid profile and digestibility [98].

2.2.4.2.3. Pigments

One of the characteristics that distinguish algae is their colour, which is determined by the presence of certain pigments in their structure. These colourful chemical substances can be divided by three major classes: chlorophylls (a,b and c), carotenoids (carotenes and xanthophylls) and phycobiliproteins [99][100].

Chlorophylls are greenish pigments with a porphyrin ring in their structure. They are usually found in algae, higher plants and cyanobacteria [101]. Moreover, carotenoids are considered their accessory since they increase the light-harvesting properties of algae by passing the light excitation to chlorophylls. In Cyanophyceae and Cryptophyceae, it is possible to find brilliant-coloured and water-soluble antennae-protein pigments named phycobiliproteins which are organized in supramolecular complexes [102].

In biorefinery context, the solubility and degradation products of pigments are important parameters to predict their path in the process. Some factors such as light, oxygen, organic solvents and weak acids results in the formation of numerous degradation products [103]. The degradation product of chlorophyll a is usually pheophytin a and this convertion occur when the magnesium ion bonded to four nitrogen atoms in a ring structure is lost [104].In terms of solubility, chlorophylls and carotenoids are generally hydrophobic molecules, whereas phycobiliproteins are hydrophilic. Hence, carotenoids and chlorophylls can be extracted by using organic solvents such as acetone, methanol or dimethyl sulfoxide (DMSO) [100].

2.2.4.2.4. Carbohydrates

Carbohydrates in microalgae are distributed between polysaccharides, which comprises 80 to 95% of the total carbohydrates and the soluble fractions of simple sugars (mono-,di- and oligosaccharides) [105]. Despite being considered a potential sourcefor bioethanol production as well as in understanding the biosynthetic pathways and carbon allocation, the accumulation in these microorganisms has not been thoroughly studied [106][107].

After the extraction of other marketable products, residual biomass of microalgae rich in carbohydrates remains and can be converted in other products. For bioethanol production, using two types of microorganisms in two separate processes (accumulation of starch by microalgae followed by the anaerobic conversion of starch to ethanol) has associated complications, such as the complexity of the installation and the high number of intermediate stages of the process. Despite this, the residue obtained from the distillation contain valuable compounds, such as minerals, which can be used as agricultural fertilizer ("biofertilizer") [108].

2.3. The Role of Green Chemistry in Bioprocesses and Economical Evaluation

Nowadays, the concept of green chemistry has achieved great importance in various industries, and it can be defined as a ‘‘design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances”[109]. For bioprocesses such as microalgae biorefineries, the principles of green chemistry have to be followed, considering that they effectively provide a framework for designing and/or improving materials, products and systems from an environmental protection perspective [110].

An ideal processing of microalgae to final products would be without the use of solvents, however solvents are almost unavoidable because of their crucial role in solids dissolution, mass transfer and heat, influencing viscosity and the separation and purification steps. Thus, two main strategies for the development of green solvents were proposed: substitution of solvents derived from petroleum by solvents from renewable sources and substitution of hazardous solvents with others that present better environmental, health and safety properties [111].

The step of extracting biomolecules in the microalgae biorefineries is one of the steps in which the choice of a solvent is required. According to the REACH regulation, n-hexane, used most often in extraction phases, was considered toxic and this fossil source is not suitable for extraction in microalgae biorefineries targeting food applications. Therefore, the search for a biodegradable, non-hazardous and greener solvent has become a concern for industrial uses [112]. The following figure (Figure 2.5) relates polarities and studied green solvents that possible meets all the requirements such as supercritical fluid extraction, limonene, pressurized solvents (example: ethanol or ethyl lactate), gas expanded liquids and water [110].

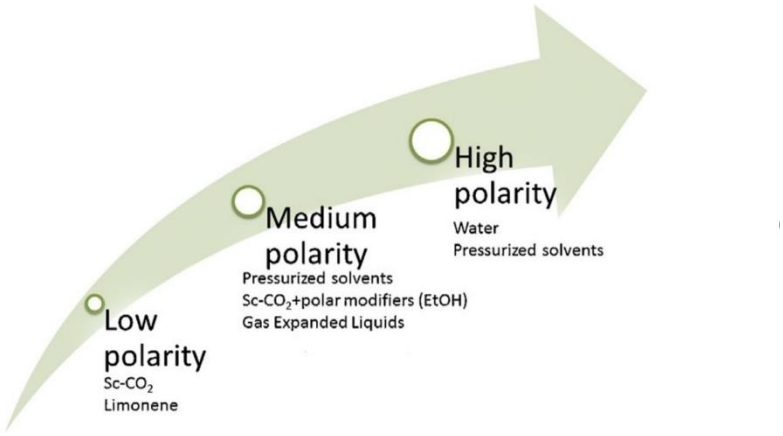

Figure 2.5. Green solvents and environmentally friendly technologies used to extract high added-value products from natural sources [107].

Low-cost unit operations which maximize product output are vital for this process. The recovery of both products and solvent must be studied to prevent excessive costs and environmental

unfriendly wastes. Despite membrane fractionation of microalgae products is still under research, it could be a friendly way of solvent recovery as it is already a successful strategy in the dairy industry [113].

Additionally, to be aware of the best decisions regarding any entrepreneurial segment, it is necessary to study the behavioural tendencies of the market of both consumers and the possible combinations of commercial products. There are many possible products of microalgae biorefinery and consequently, there are also many possible market combinations. In the following figure is presented the value of the fractionated microalgae biomass and the conclusion is that the lowest revenue per unit of biomass comes from biofuel (0.3 €·kg-1) and the application of microalgae for food is more attractive with a three times higher potential value [6]. For the development of this biorefinery pre-project work, the respective commercial of the components will be considered to construct a more profitable project.

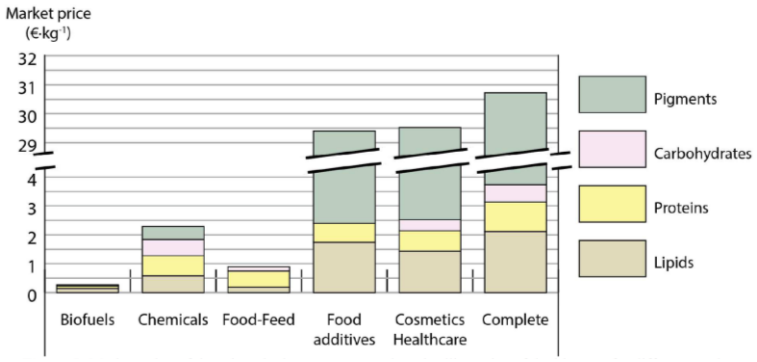

Figure 2.6. Total selling price of microalgae components [6].

On the other hand, the costs are an important part in project design and engineers facing an industrial project that is permanently undergoing technical changes are usually less comfortable with it. Considering the cost consequences of a technical change, engineers typically resort to the principles of engineering economics, emphasizing the dynamics of cash flows and evaluating their net present value to select among the alternatives [114]. For this pre-project costs, only capital expenditures (CAPEX), i.e plant and equipment purchases will be considered to compare the biorefinery paths.

Chapter 3

Materials and Methods

3.1. Reagents

Table 3.1. List of reagents used for the development of this work and their respective manufacturer.

Use	Reagents	Manufacturer
Extraction	(+)-D-Limonene 96%	Acros Organics
	Ethanol absolute anhydrous	Carlo Erba Reagents
	Extra Virgin Olive Oil	Sovena
Fatty Acids Analysis	Petroleum ether	Fisher Chemical
	Methanol	Fisher Chemical
	Acetyl Chloride	Scharlau
	Heptadecanoic acid (C17:0)	Acros Organics
	n-Hexane	Valente e Ribeiro
Pigments Analysis	Acetone	José Manuel Gomes dos Santos, Lda

3.2. Lab-Scale Operation Tests

3.2.1. Cell Disruption

A 100 g/L biomass suspension of *Nannochloropsis* sp. was subjected to cell disruption. This process was performed in the supplier facilities (undisclosed) by bead milling experiments in a horizontal stirred bead mill, see Figure 3.1. Two trials were performed at two different temperatures: 6°C and 25°C. To maintain the feed temperature at required values, a cooling water bath connected to a cooling jacket integrated in the milling chamber was used. The equipment was

fitted with a 0.2 mm dynamic gap separator and filled to 85% with grinding beads. The conditions for the milling of the product were a tip speed of 10 m/s and a product throughput of 13 kg/h. To prevent oxidation, the collecting vessel was Argon blanketed during the entire trial. The number of passes of the biomass in the chamber equipment was also tested (one and two passes). For each trial, samples were taken after one and two passes.

Figure 3.1. Photograph taken during the trials

3.2.2. Extraction

3.2.2.1. Conventional

Dewatered and ruptured biomass was placed in a stirred container (agitated by Agimatic-S-Selecta). Two different solvents were tested and the operational conditions of each trial are shown in the following table. To avoid degradation by light of compounds, aluminium foil was used to cover the container. Samples were taken with intervals of 1h.

Table 3.2. Operational conditions of the conventional extractions.

Solvent	Trial	Duration (h)	Ratio (bio-mass:solvent)	Temperature (ºC)
D-limonene	1	6	1:10	23
	2	4	1:12	23
Ethanol	1	6	1:10	23
	2	3	1:16	23

3.2.2.2. Soxhlet

Dewatered biomass containing some of the targeted lipids was placed inside a cartridge made of thick cellulose paper, which was loaded into the chamber of the Soxhlet extractor. The instrument was placed onto a flask containing ethanol in continuous agitation and was also, equipped with a condenser on the top. The flask was immersed in oil bath heated by a magnetic stirring hotplate (Heidolph MR 3001 K) and the temperature was controlled by contact thermometer (Heidolph EKT 3001) to keep it at 140ºC.

Ethanol roise through a distillation arm and flooded the chamber where the cartridge with the solid were. When the chamber was filled with solvent, it was automatically emptied by a siphon side arm connected to the initial flask. This experiment was carried out for 48h, with an interruption during the night, until the distilled ethanol regained the original appearance (colourless).

3.2.3. Distillation

Distillation for ethanol recovery were tested through lab-scale simple distillation apparatus, see Figure 3.2. A 300 mL extract of ethanol was placed in a 500 mL round-bottomed flask and heated until boiling temperature (~78,5ºC) of ethanol through heating mantle. 285 mL of distilled ethanol were recovered in an Erlenmeyer.

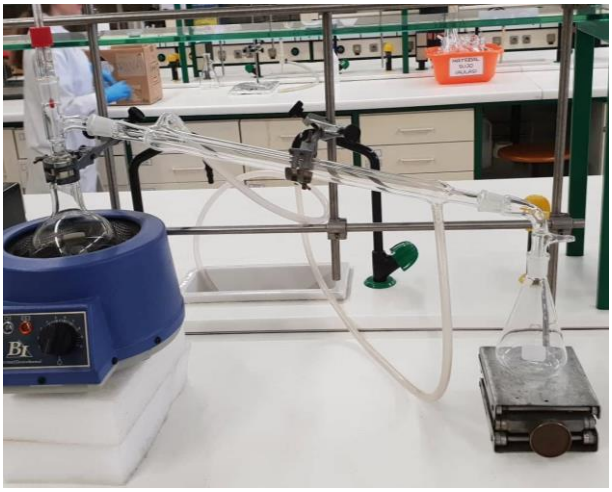

Figure 3.2. Simple distillation assembly.

3.2.4. Membrane Filtration

The membranes used in this study were chosen according to the characteristics of the solvent and the size of the molecules dissolved. The experiments were performed in a stainless steel METcell test cell (supplied by Membrane Extraction Technology, UK) at room temperature. The feed tank has a total volume of 250 cm^3 and the agitation is promoted by a cross head magnetic bar, providing the adequate fluid dynamic conditions. The pressure applied through the membrane (circular sheet with an effective area of 51.4 cm^2) was regulated by a pre-assembled argon unit. The permeate was collected in a recipient, during the course of the experiment and the flux

was monitored by acquisition of the permeate weight, using an electronic balance with an accuracy of 0.1 g. The target concentration factor (CF) were 5. The most relevant conditions of the membranes used are described in the following table (Table 3.2).

Table 3.2. Properties of the selected membranes. *The performance data are approximate and based on tests using different solvents.

Membrane	Manufacturer	Solvent Used	Operation Pressure	MWCO (g/mol)*
Puramem280	Evonik	D-limonene	40 bar	280
030306F	Solsep	D-limonene	35 bar	1000
Puramem S600	Evonik	D-limonene	40 bar	600
Duramem 200	Evonik	Ethanol	40 bar	200
010206	Solsep	Ethanol	15 bar	300
NP030	Microdin Nadir	Ethanol	20 bar	500-1000

For experimental data treatment, the permeability flux (L/m^2.h.bar) and rejection coefficient (%) were calculated through equation (3.1) and (3.2), respectively.

$$J_v = \frac{V}{A\,\Delta P\,t} \tag{3.1}$$

Where A represents the membrane area, ΔP the pressure across membrane (transmembrane pressure), V is the permeate volume and t the time of permeation.

$$R_i = \left(1 - \frac{C_{i,perm}}{C_{i,feed}}\right) \times 100 \tag{3.2}$$

Where $C_{i,perm}$ and $C_{i,feed}$ are respectively the concentration of compound i in the permeate and the feed extract.

The average permeability flux was calculated through equation (3.3).

$$\overline{J_v} = \frac{\int_0^t J_v\,dt}{\int_0^t dt} \tag{3.3}$$

Differences in concentration factor (CF) at the solution–membrane interface as a result of packing density may impact the mass transfer and fouling rate and the applicability of the systems and for that reason the CF (-), was calculated along the experiment by equation (3.4).

$$CF = \frac{m_0}{(m_0 - m_{permeate})} \quad \textbf{(3.4)}$$

Where m_0 is the mass in the feed reservoir at the beginning of the experiment and $m_{permeate}$ is the mass of permeate recovered up to a given instant.

3.2.5. pH shift

To determine the yield of the pH adjustment of the supernatant to obtain proteins, the following experiment was performed. First, centrifugation of biomass processed by bead milling to obtain volumes of supernatant (14–20 ml). These supernatant samples were adjusted to pH 3, 4 and 5 (portable pH meter by Mettler Toledo). This test was carried out with 1.0 M HCl. To weight the amount of precipitate, centrifugation at 4500× g and 10 °C for 15 min (Hermle Z 400 k) was carried out and supernatant was recovered for protein analysis. For elimination of the moisture in the pellet, a vacuum filtration (Millipore XX1104700 by Merck) and weight measurement at 105ºC a moisture analyser was conducted. For proteins analysis, the previous step was bypassed, and the wet pellet was redissolved in distilled water.

3.3. Analytical Methods

3.3.1. Quantification of Pigments

Chlorophylls, pheophytins and carotenoids concentrations were determined by total wavelength spectrophotometric scan (Genesys 10S UV-Vis by Thermo Scientific) of the pigment solution obtained from biomass samples by extraction with bead beating and acetone or directly diluted in case of liquid samples. After measuring the visible absorption spectrum of the pigments solution, each pigment concentration was determined by spectral decomposition: an iterative method that matched the sum of the absorbance spectra of each accounted pigment to the measured spectrum.

3.3.2. Quantification of Proteins

Protein quantification of the extracts was performed using Pierce™ BCA Protein Assay Kit (Thermo Scientific), and the methodology protocol is implemented at A4F Laboratory. The calibration curve and quantification assays were performed according to the manufacturer's instructions (with minor modifications). For the calibration curves, a 2 mg/mL of bovine serum albumin (BSA) solution was prepared with the working buffers (culture medium and 100 mM Tris-HCl buffer). The two BSA solutions were then diluted in order to obtain the series of standard solutions of known concentrations. The concentrations of the standard solutions ranged from 0 (blank) to 500 µg/mL. In a 96-well microplate, 25 µL of each standard solution or unknown sample were mixed with 200 µL of BCA working reagent (prepared according to manufacturer's instructions).

The microplate was shaken during 30s and then incubated for 30 min at 37°C on a microplate reader (SPECTROstar Nano, BMG LABTECH). After the incubation period, the mixtures in the microplate were cooled for 5 min and their absorbance measured at 562 nm (SPECTRO star Nano, BMG LABTECH). All unknown samples or standard solutions were measured in quadruplicate. For the standard solutions, the average absorbance was plotted as a function of BSA concentrations in order to obtain the calibration curves.

3.3.3. Quantification of Fatty Acids

Algal biomass and extracts obtained during this work were strictly weighed (PA114C, Ohaus) and mixed with known concentration of internal standard heptadecanoic acid (C17:0) dissolved in petroleum ether. For transesterification of the lipids, a mixture of methanol and acetyl chloride (proportion of 100:5) was added to the analysis tubes and heated at 90-100ºC for 60 min. The samples were thoroughly mixed during heating.

Then, 2 mL of n-hexane was added to the tubes and mixed through vortex stirrer. To aid in phase separation, 2 mL of distilled water was added to the mixture and after centrifugation (for 3 min at 2,500 x g) the n-hexane layer that contained fatty acids methyl esters was collected and transferred to a glass vial passing through a cotton filter filled with sodium sulphate to prevent moisture and impurities. The gas chromatography (GC) required for the analysis was performed by an external supplier.

Fatty acid identities and contents in the microalgae samples were assigned by analysis of the resulting GC profiles and compared to those of known compounds.

3.3.4. Dry Weight and Salinity

The dry weight was obtained by drying the sample at 105°C in a moisture analyser (MS-70, AND). To measure the salinity of the sample, the dried mass used in the dry weight measurement was removed from the container and resuspended in demineralized water. Afterwards, the salinity value was obtained by reading through a refractometer. (Portable Refractometer ZUZI).

3.3.5. Statistical Analysis

Analytical measurements inherently exhibit variation when repeated many times even if using accurate instruments. Data obtained from repeated measurements are sets of values that do not give an exact result. Statistical methods are then employed to extract the maximum information from these data. Firstly, it is necessary to establish the distribution, generally, by graphical representation. Then this statistical distribution is characterized by a measure of its value as mean, median or mode. Finally, the spread or dispersion of the distribution is determined in terms of the variance or standard deviation.

For the treatment of the experimental data obtained in the present work, the employed measure of the central tendency of data is the sample mean, given by Equation (3.5), and its standard deviation for further discussion of the results, given by Equation (3.6).

$$\bar{x} = \sum_{i=1}^{n} \frac{x_i}{n} \quad \textbf{(3.5)}$$

$$S_x = \sqrt{\left[\sum_{i=1}^{n} \frac{(x_i - \bar{x})^2}{(n-1)}\right]} \quad \textbf{(3.6)}$$

Where n is the total number of measurements, x_i is the ith value of the quantity. [115]

Chapter 4

Results and Discussions

A chemical engineering pre-project is composed by sequential steps to come up with a proposal solution to a problem. Following this approach, this section is divided into steps to achieve the main goal: profitable *Nannochloropsis* sp. biorefinery.

Firstly, the theoretical values of the feedstock compositions obtained through relations between previous studies and experimental values are exposed. Then, through several comparisons between biorefinery studies and according to theoretical parameters of profitability and sustainability, two biorefinery proposes were presented as well as its detailed description. The third section discloses the obtained results of experimental tests related to each biorefinery proposal along with their analysis. The three final sections were performed through the previous experimental results and they are the three steps necessary for an industrial project: mass balances, equipment sizing and economical evaluation. Thus, calculation steps and their results are exposed.

4.1. Characterization of *Nannochloropsis* sp. Biochemical Composition

Process design of an engineering project require a full characterization of the feedstocks. In this section, the constitution of the cells of *Nannochloropsis* genus were analysed through bibliographic research to determine which macromolecules of the biochemical composition might prove to be flowing in the process and to assess their potential as feedstocks for the target industries. The main goal of this section is to obtain a calculation basis for further mass balances and which

will be compared to experimental data. Proteins, carbohydrates, lipids, and pigments were the distinguished macromolecule groups while minerals were considered a minor group.

Although there are several studies about fatty acid profiles, the published results are inconsistent and could be misleading since the findings are likely to be skewed by nitrogen starvation and the contribution of the invading algae contaminants in the culture [116]. Since it was not possible to have access to an acceptable lipid profile in the literature, a calculation of the theoretical composition was achieved from previous analyses of fatty acid composition of the same microalgae strain performed by A4F procedures. By the results, there are 12,7% of fatty acids in the biomass, but the percentage of remaining fractions of lipids is lacking. Following the distribution of fatty acids achieved by Schneider et al.(1995) [117] extrapolations were performed, and it was possible to estimate the fatty acids profile of *Nannochloropsis* biomass.

The fatty acids were grouped based on the class of lipid and degree of saturation for subsequent discussion, see Table 4.1.

Table 4.1. Calculated percentages of individual fatty acids in the neutral lipid (NL), glycolipid (GL), and phospholipid (PL) fractions of *Nannochloropsis* sp

	NL fraction	**GL fraction**				**PL fraction**				
Fatty Acid	**TAG (%)**	**MDGD (%)**	**DGDG (%)**	**SQDG (%)**	**DGTS (%)**	**PC (%)**	**PE (%)**	**PG (%)**	**PI (%)**	**Total (%)**
C14:0	0.78	5.20	1.04	0.41	0.34	0.24	0.14	0.10	0.71	8.97
C16:0	2.85	3.07	3.99	3.91	0.64	1.31	0.45	3.08	4.62	23.91
C18:0	0.12	-	-	0.05	0.07	0.06	0.04	0.04	0.18	0.55
ΣSFAs	3.75	8.26	5.03	4.37	1.06	1.61	0.63	3.22	5.51	33.43
C16:1	3.24	3.38	5.29	3.10	2.18	4.33	1.50	0.85	5.83	29.70
C18:1	0.12	-	-	-	0.08	0.61	0.09	-	0.63	1.53
ΣMUFAs	3.36	3.38	5.29	3.10	2.26	4.95	1.59	0.85	6.46	31.23
C18:2n6	0.06	0.16	0.13	-	0.12	1.61	0.13	0.04	0.79	3.03
C18:3n6	-	-	-	-	-	0.32	0.05	-	-	0.37
C20:4n6	-	0.18	-	-	0.46	0.19	1.37	-	2.28	4.49
C20:5n3	-	13.82	2.40	-	5.36	0.57	1.40	1.81	1.10	26.47
ΣPUFAs	0.06	14.16	2.53	0	5.95	2.69	2.95	1.85	4.17	34.36
Total FA (%)	7.16	25.80	12.85	7.47	9.27	9.24	5.17	5.92	16.13	99.02
Total Contribution of Fractions (%)	7.16	55.39				36.46				

The profile of fatty acids obtained reveal approximately equal quantities of SFAs, MUFAs and PUFAs (33% w/w, 31% w/w and 34% w/w, respectively). Regarding the fatty acids present in higher amounts, the palmitoleic acid (C16:1) represents 29.7% of the fatty acids while eicosapentaenoic acid (EPA, C20:5w3) represents 26.5% and palmitic acid (C16:0) represents 23.9% of the total fatty acids.

As the calculations of *Nannochloropsis* sp. fatty acids profile were extrapolated based on a study in which the growing conditions were nitrogen replete, the neutral fraction is the minor fraction with only 7% of the total fatty acids, i.e.1%of the total biomass dry weight. EPA, a component of major interest from the biomass, accounts for 26% of the total fatty acids i.e. 3% of the total biomass dry weight.

As expected, there are omega-3 fatty acids present in this microalgae genus. The EPA fatty acid is present in the polar lipids predominantly in form of monogalactosyldiacylglycerol (MDGD), a glycolipid. Also, note that the TAG fraction does not present EPA in its constitution.

Hereupon, studying the composition of each lipid, the fatty acids contributions on the total molar mass were calculated (Table 4.2) using the following equation (4.1) in order to obtain the total percentage of lipids present in the biomass (found this calculations in Annex A).

$$Lipid_i/_{Biomass}\,(\%) = \frac{FA_{Biomass}(\%)}{FA\ (\%)\ in\ Lipid_i} \quad \textbf{(4.1)}$$

Where $FA_{Biomass}(\%)$ is relative to the quantity of the fatty acid in the biomass and $FA\ (\%)\ in\ Lipid_i$ is the contribution of the molar weight of each fatty acid in the lipid.

Table 4.2. Calculated percentages of total lipids based on their composition in fatty acids and other groups. *Experimental Values of previous fatty acids analysis performed in A4F.

Lipid Type	Fatty Acids / Biomass (%)*	Lipids/ Biomass (%)	Lipids/ Total Lipids (%)
TAG	0.91	1.03	5.4
Σ NL	0.91	1.03	5.4
MGDG	3.30	4.94	26.1
DGDG	1.65	3.09	16.3
SQDG	0.95	1.59	8.4
DGTS	1.18	1.70	9.0
Σ GL	7.08	11.32	59.8
PI	2.05	3.10	16.4
PG	0.75	1.02	5.4
PC	1.18	1.62	8.5
PE	0.67	0.85	4.5
Σ PL	4.66	6.58	34.8
Total	12.7	18.9	100.0

It can be concluded from the above table that 1.03% of the total dry weight biomass are in the form of TAG and that 11.32% and 6.58% are in the form of glycolipids and phospholipids, respectively. Evaluating this lipid distribution, within total lipids, this consists of 59.8% glycolipids, 34.8% phospholipids and 5.4% TAG. Lastly, the sum of saponifiable lipids fractions present in this microalgae biomass,GL, PL and NL was found to be 18,9% w/w.

4.2. Proposal of Microalgae Biorefinery Routes

In this section, four proposals of biorefinery routes were built upon theoretical concepts of engineering and bibliographic research to fractionate the compounds present in *Nannochloropsis*

sp. and drawn in SuperPro Intelligen Inc®. The biorefinery process flow diagrams (PFDs) proposed aim at minimizing the capital investment and energy consumption, combining various unit operations such as bead milling, tricanting, centrifugation, membrane filtration, extraction and drying. All of them are commonly used and are highly developed in other industries such as food or pharmaceuticals.

Design improvements, versatility in the control parameters and scale-up, makes the bead mill technique the most suitable disintegration technique [38] in this case as it adds the opportunity to extract different high value-added chemicals of this strain characterized by a very resistant cell wall. Hence, a bead milling equipment is included as the first processing step common to all biorefinery PFDs proposed. Table 4.4 shows the main differences between the biorefineries proposed.

4.2.1. Detailed Description of Biorefinery 1A and 1B

The following description is relative to Figure 4.2 and Figure 4.3 that differ only in a unit operation: ethanol recovery.

After the bead milling step, the output stream obtained would be a suspension of disrupted cells. Considering density differences between the culture medium and the cells debris composed by non-soluble material, the following step is a centrifugation (solid-liquid separation). After this operation two streams are expected to form: a) aqueous supernatant rich in water soluble proteins and carbohydrates and b) a pellet rich in polar lipids and pigments. Although this strain is considered oleaginous, due to the numerous publications targeting induction of lipid overproduction unlike the present case, only nearly 1% of the lipids are in form of TAG's. Since proteins are known as emulsifiers allied to the low percentage of non-polar lipids, it is presumed that an emulsion will be formed.

A recent research [118], suggests a new method to recover proteins by their isoelectric point. Adding acid to the supernatant will lower the pH to their isoelectric point cancelling out their charges and consequently they become insoluble. As proteins form a precipitate, a centrifugation to recover them is necessary.

The remaining aqueous phase is rich in recoverable carbohydrates that might enhance the value of this biorefinery. Carbohydrate purification based on chemical-free, low energy and mild operations is possible through membrane filtration, therefore a 30 kDa ultrafiltration membrane is proposed according to the size and charge exclusion of sugars in the following figure (Figure 4.1).

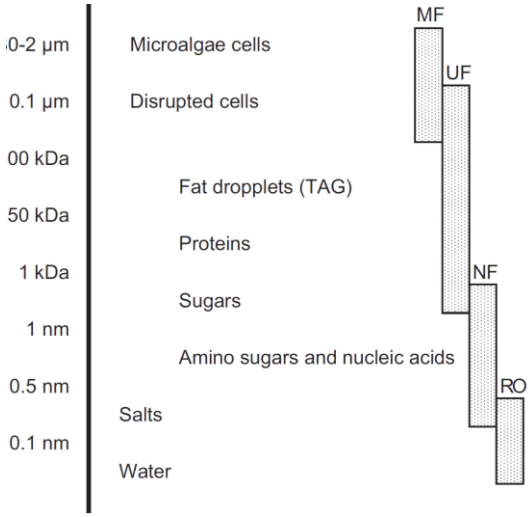

Figure 4.1. Relation of the size of the different microalgae components and membrane process. Source: [113]

Regarding the green pathflow (Figure 4.2 and Figure 4.3)**, i**t is envisaged that the most valuable compounds should be in this solid phase: pigments and polar lipids. It will be also rich in water insoluble proteins, carbohydrates and ashes.

As stated before, it is important to find greener solvents and for that reason n-hexane is no longer an option to extract non-polar compounds. According to the literature[119], it has been demonstrated that the use of terpenes instead of the regular n-hexane for oil extraction is a promising procedure. Since the three terpenes (D-limonene, α-pinene and p-cymene) relevant properties are very similar (**see Annex B** [120]), their prices were compared according to various suppliers of laboratory compounds, and D-limonene was the solvent selected for the extraction. This solvent can be a substitute for petroleum solvents such dichloromethane, toluene, of even the hexane for the extraction of products. According to Table 4.3, D-limonene is non-toxic and does not represent environmental impact, fitting perfectly in green chemistry principles. The major drawback is its high boiling point (175,5ºC) which might represent higher energy consumption for solvent recovery by distillation compared to hexane (69ºC) [121].

Table 4.3. Relevant properties of n-hexane, toluene, dichloromethane and d-limonene [121]

Properties	*n*-Hexane	Toluene	Dichloromethane	*d*-Limonene
Empirical formula	C_6H_{14}	$C_6H_5CH_3$	CH_2Cl_2	$C_{10}H_{16}$
Molecular weight	86.18	92.14	84.93	136.23
Boiling point (°C)	68.7	110.6	39.8–40.0	175.5
Heat of vaporization (kJ/kg)	334	351	28.6	353
Density (g/mL)	0.6603	0.8669	1.325	0.8411
Toxic	Yes	Yes	Yes	No
Environmental impact	High	High	High	Low

To overcome this barrier, membrane filtration technology might be the solution to recover the solvent since it is not toxic and has GRAS rating (“Generally Recognized As Safe”) [122], thus it does not represent a safety problem if there are residues in the final product.

After the extraction using D-limonene, the remaining pellet should be rich in polar compounds such as glycolipids and phospholipids. To extract them, a green polar solvent needs to be selected and it will be ethanol which has been studied as possible green solvent to extract compounds of microalgae [50]. The only difference between biorefinery A and B is the ethanol recovery because it can be done by membrane filtration and by distillation due to the relatively low boiling point (79 ºC).

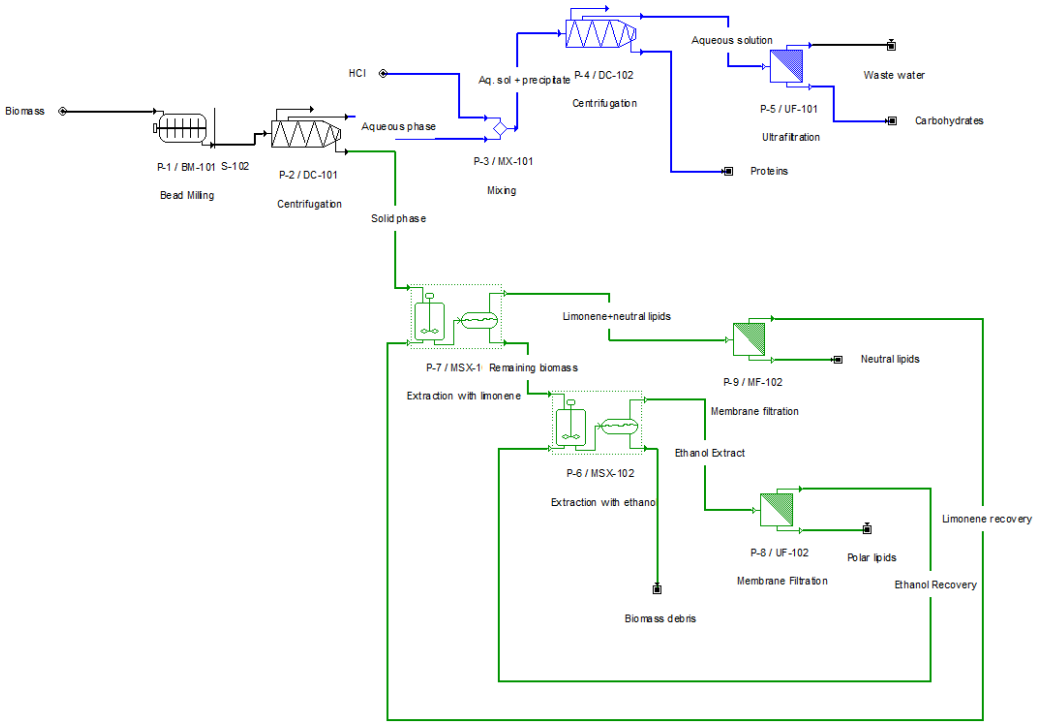

Figure 4.2. Proposal of biorefinery 1A

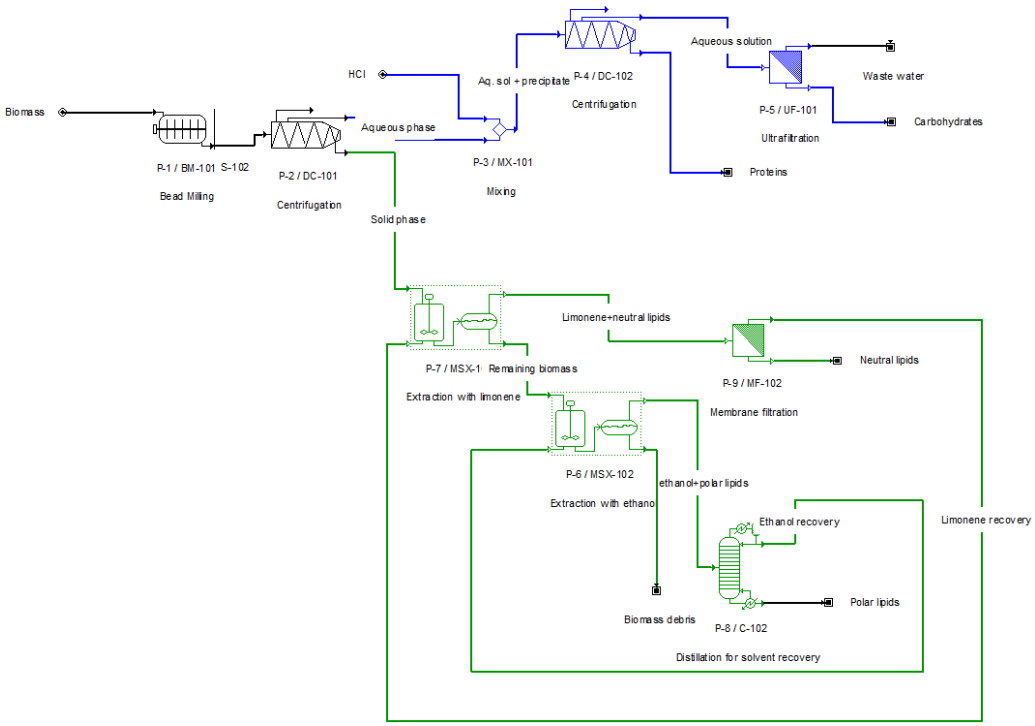

Figure 4.3. Proposal of biorefinery 1B.

4.2.2. Detailed Description of Biorefinery 2A and 2B

The following description is relative to Figure 4.4 and Figure 4.5 and also differ only in a unitary operation: solvent recovery of ethanol extraction.

To obtain three noticeable phases thus enabling a tricanter operation, it is highly recommended to guarantee a high amount of TAG (triacylglyceride) in the total lipids to avoid creation of emulsions. There are two possible ways to ensure a high amount of TAG in the total lipids, the first one relies on nutrient deprivation to encourage microalgae to accumulate a higher percentage of neutral lipids. On the other hand, for a microalgae strain grown under nutrient replete conditions, the addition of TAG from an external source should avoid emulsions as referred before. Thus, a good approach should be to feed the biomass stream with a high TAG content and consequently three different phases should be obtained (oily, aqueous and solid phase). The top layer will be an oil phase which represents itself a final product as a rich oil (red flow pathway in the diagram).

Looking upon a possible emulsion, it is proposed the usage of a vegetable oil as TAG external source to avoid it. The vegetable oil plays an important role in our ordinary diet, consumed directly in the refined or virgin form, it does not need to be separated from the microalgae oils and it could constitute an innovating final product ready to be consumed. From data of the Statistical Institute of Portugal [123] concerning the consumption per capita of fats and oils (kg/ inhab) in 2016**,** olive oil is one of the most sought-after oils in the country. Although the demand of coconut oils is still

lower than the other oils, they have antiviral and antibacterial effects and excellent healing properties. Thus, they are not only used for direct consumption but also for cosmetic industry and infant formulas. Despite its attractive properties, its solid nature at room temperature (melting point ≈25ºC) might be a limitation as well as the distinct and powerful flavor of coconut enhancing only a small number of cooking applications [124]. Considering the barriers mentioned, olive oil was the selected solvent to add to the bead mill.

Regarding density properties, the second layer obtained from the tricanter should be an aqueous phase rich in water soluble compounds which should be processed by membrane filtration processes (blue flow pathway) and dried by spray-drier operations. Although ultrafiltration membranes could retain the remaining proteins and permeate low MW solutes, the presence of salts in the final product might be considered an impurity. A diafiltration mode may solve this potential barrier because of the continuous addition of pure solvent to the retentate feed enhancing the separation of mixtures of salts and small organic solutes such as monosaccharides. This process may involve three steps: pre-concentration step, diafiltration step and post-concentration step. [125] The molecular weight cut-off (MWCO) was chosen according to the previous classification of the components size (Figure 4.1).

The remaining biomass (green flow pathway) should be a solid phase rich in insoluble molecules phase, such as carbohydrates, proteins and lipids. Considering that non-polar compounds were extracted by the olive oil, it is only necessary to perform an ethanol extraction to recover the remaining compounds.

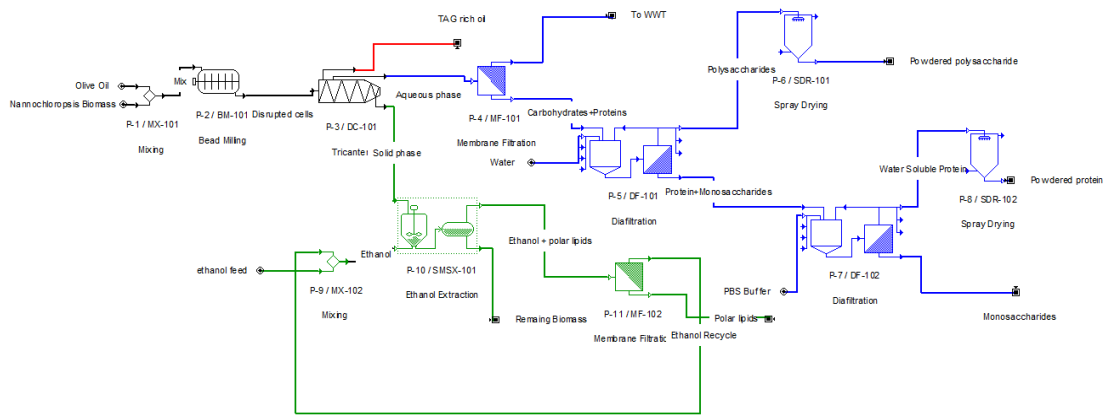

Figure 4.4. Proposal of biorefinery 2A.

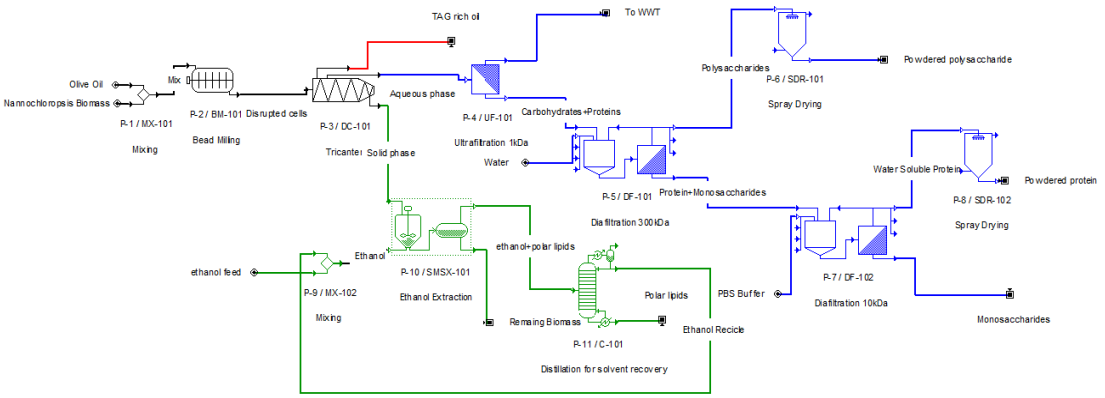

Figure 4.5. Proposal of biorefinery 2B.

Table 4.4. Comparison of the main steps of the biorefineries proposed.

Biorefinery Steps	Biorefinery 1A and 1B	Biorefinery 2A and B
Cell Disruption	Bead Milling	Bead milling
Biomass Dewatering	2-phase centrifuge	3-phase centrifuge
Neutral Lipids Extraction	D-limonene extraction with membrane solvent recovey	Olive Oil extraction
Proteins Extraction	pH-shift	Diafiltraction
Carbohydrates Extraction	Ultrafiltration	Diafiltration
Polar Lipids and Pigments Extraction	Ethanol extraction Bioref. 1A: Membrane filtration Bioref. 1B: Distillation	Ethanol extraction Bioref. 2A: Membrane filtration Bioref. 2B: Distillation

4.3.

Experimental Results

4.3.1. Characterization of *Nannochloropsis* sp. feedstock

For mass balances calculations, it is necessary to have feedstocks full characterization. After harvesting the culture in A4F facilities, the biomass was resuspended in culture medium to obtain a 100 g/L suspension. Firstly, the dry weight was measured, and the value obtained was 12,50 ± 0,01% of dry weight including salt. Then by measuring the salinity, it was possible to conclude that the dry weight excluding the salt quantity (SFDW, Salt-free dry weight) was 8,00 ± 0,01%.

In the following table (Table 4.5), the results of pigments analysis are presented. As it is possible to compare, the theoretical (see section 2.2.2) and experimental values of pigments profiles present slight variations. Limitation of nitrogen, phosphorus, increment of salinity and other variations in culture conditions can cause these variations. For instance, if the nitrogen supply were

limited in proportion to other elements, the photosynthesis might continue but the resultant compounds would include a smaller proportion of nitrogen-rich components, and higher content accessories pigments as carotenoids and more energy-rich components such as lipids and carbohydrates. [126]

Since degradation of carotenoids and chlorophylls is catalysed by oxygen and light, pheophytin a– a degradation product - is present in experimental results possibly due to light and oxygen exposure during the analysis procedures.

Table 4.5. Comparison between theoretical composition of pigments of *Nannochloropsis* sp. and the obtained experimental results.

Pigment	**Theoretical Composition [25]**		**Experimental Results**	
	Pigments/Biomass (%)	Pigments/Total pigments (%)	Pigments/Biomass (%)	Pigments/Total pigments (%)
Chlorophyll a	1.69	54.38	2.3 ± 0.05	68.47
Pheophytin a	-	-	0.30 ± 0.01	8.96
Violaxanthin	0.69	22.21	0.23 ± 0.02	6.98
Vaucheriaxanthin	0.48	15.32	0.20 ± 0.08	6.14
Canthaxanthin	0.11	3.40	0.005 ± 0.005	0.15
Astaxanthin	0.08	2.51	0.08 ± 0.04	2.33
Zeaxanthin	0.02	0.61	0.001 ± 0.001	0.04
Antheraxanthin	0.05	1.58	-	-
Lutein	-	-	0.17 ± 0.04	5.22
Neoxanthin	-	-	0.06 ± 0.06	1.67
Total	3.10	100	3.33 ± 0.02	100

In the last topic, theoretical composition of lipids was calculated according to fatty acids analysis performed in A4F. Since the cultivation parameters may modify the fatty acids profile, a new fatty acids analysis was performed to other similar biomass cultivation to compare the results, see the following table (Table 4.6).

Table 4.6. Fatty acids profile of *Nannochloropsis* sp. obtained in similar cultivation conditions.These values are extrapolations from the previous theoretical profile. *Experimental Values.

	NL fraction	GL fraction				PL fraction				
Fatty Acid	TAG (%)	MDGD (%)	DGDG (%)	SQDG (%)	DGTS (%)	PC (%)	PE (%)	PG (%)	PI (%)	**Total (%) ***
C14:0	0.8	5.1	1.0	0.4	0.3	0.2	0.1	0.1	0.7	8.8
C16:0	3.1	3.3	4.3	4.2	0.7	1.4	0.5	3.3	5.0	25.8
C18:0	-	-	-	-	-	-	-	-	-	-
ΣSFAs	3.8	8.4	5.3	4.6	1.0	1.6	0.6	3.4	5.7	34.6
C16:1	2.9	3.1	4.8	2.8	2.0	3.9	1.4	0.8	5.3	26.8
C18:1	0.5	-	-	-	0.4	2.9	0.4	-	3.0	7.2
ΣMUFAs	3.5	3.1	4.8	2.8	2.4	6.8	1.8	0.8	8.2	34
C18:2n6	-	-	-	-	-	-	-	-	-	-
C18:3n6	-	-	-	-	-	4.6	0.8	-	-	5.4
C20:4n6	-	0.2	-	-	0.4	0.2	1.3	-	2.1	4.2
C20:5n3	-	11.4	2.0	-	4.4	0.5	1.2	1.5	0.9	21.8
ΣPUFAs	-	11.6	2.0	-	4.9	5.2	3.2	1.5	3.0	31.4
Total	7.3	23.0	12.1	7.4	8.2	13.7	5.6	5.7	16.9	100
Total Contribution of Fractions (%)	7.3	50.8				41.9				

Since the samples were run externally in a gas chromatograph, replicates were not generated. In fatty acids analysis, its quantification was achieved almost exclusively by the area normalization. The major drawback of this measurement is the propagation of the error, i.e. the strong interdependence of the results. If the result (area count) for an individual fatty acid was incorrectly estimated, the results for all other analyses are affected.

To achieve the previous fatty acid profile (Table 4.6), extrapolations were performed using the theoretical profile obtained previously, considering the same proportions of the different fractions of lipids. The extrapolation was possible only because the experimental results of fatty acids analysis were very similar to the ones used to obtain the theoretical profile. Thereby, the major fraction of lipids is glycolipid, representing 50,8% of the total lipids.

The fatty acids present in higher amounts in the experimental analysis was also the palmitoleic acid (C16:1) representing 26,8% of the fatty acids while eicosapentaenoic acid (C20:5w3) represents 21.8%, slightly lower than the theoretical value and palmitic acid (c16: 0) represents 25,8% of the total fatty acids.

To complete the characterization and using the same calculations procedures from the previous section, the lipids total fraction in the cell was calculated, see Table 4.7. Those results were then used to obtain a new biochemical composition, to study the biomolecules path during the lab scale tests and further mass balance. (Table 4.8).

Table 4.7. Calculated percentages of total lipids based on their composition in fatty acids and other groups.

Lipid Type	Fatty Acids / Biomass (%)	Lipids/ Biomass (%)	Lipids/ Total Lipids (%)
TAG	0.82	0.92	5.5
Σ NL	0.82	0.92	5.5
MGDG	2.59	3.88	23.2
DGDG	1.36	2.55	15.2
SQDG	0.84	1.39	8.3
DGTS	0.93	1.34	8.0
Σ GL	5.72	9.17	54.7
PI	1.91	2.88	17.2
PG	0.64	0.86	5.1
PC	1.54	2.11	12.6
PE	0.63	0.80	4.8
Σ PL	4.72	6.66	39.8
Total	11.27	16.8	100

Table 4.8. Theoretical and experimental composition of *Nannochloropsis* sp.*These values were adjusted according to the literature, since it was not possible to analyse these fractions.

Biomass Composition		Theoretical Composition (% dry weight)	Experimental Results (%dry weight)
Lipids	Saponifiable Fraction (GL, PL and TAG)	18.9	16.8
	Unsaponifiable Fraction (waxes and sterols)	5.3	3.4 *
	Total	24.2	22.1
Proteins	Soluble	20	23.1
	Insoluble	30	27.2
	Total	50	50.3
Carbohydrates	Polysaccharides	11.2	11.2*
	Monosaccharides	3.7	3.7*
	Total	14.9	14.9*
Others	Pigments	3.1	3.3
	Minerals	7.8	11.4
	Total	10.9	14.7

As extrapolations of the theoretical values were made, similar values of lipid fractions are presented in Table 4.7 with slight variations**:** 0,92% of fatty acids are in form of TAG and 9,2% and 6,7% are in the form of glycolipids and phospholipids, respectively.

The sum of saponifiable lipids fractions that will be considered for further mass balances will be 16,8% w/w.

Full feedstocks biochemical characterization is present in the previous table and it was mainly obtained through biomass analysis. However, some adjustments were made on fractions that

weren't possible to obtain through analysis For instance, according to the literature [127], in *Nannochloropsis* sp. polysaccharides ranged from 74% to 88% of the total carbohydrate. Since there was no induced stress in the culture used for this work, 75% of polysaccharides was the estimated value and the remaining portion of 25% are constituted by monosaccharides. The theoretical value of this fraction is according to Tibetts et al.(2015)[128].

About proteins fraction, the experimental values were similar with the literature. The ashes fraction was higher in experimental results possibly due to the prior resuspension in culture medium rich in minerals.

The total quantification of unsaponifiable lipids was estimated for both theoretical and experimental values by subtraction of the other different fractions from the total biomass.

4.3.2. Bead Milling

Several bottles of the pre-dispersed *Nannochloropsis* sp. were processed by bead mill equipment. Regarding the first trial, the vessel was processed at 8,5ºC through integrated cooling jacket and the biomass was processed in two passes. For the second trial, the product was also processed in two passes but at room temperature. Microscopic observations of the cells and analytical methods such as pigments and proteins analysis were performed to study the different operation conditions and select the most suitable for this project. Towards a better understanding of the results of these trials, the “Sample 1 and 2” are concerned to bead milling processing at low temperature (8ºC) with 1 and 2 passes, respectively. “Samples 3 and 4” are relative to bead milling processing at room temperature with 1 and 2 passes, respectively.

4.3.2.1. Microscopic Observation

The cell disruption was considered successful if all cells were completely disrupted, whereby the cells have lost their bright illumination and round shape. In the next pictures (Figure 4.7, Figure 4.8, Figure 4.9, Figure 4.10), the microscopy results are shown.

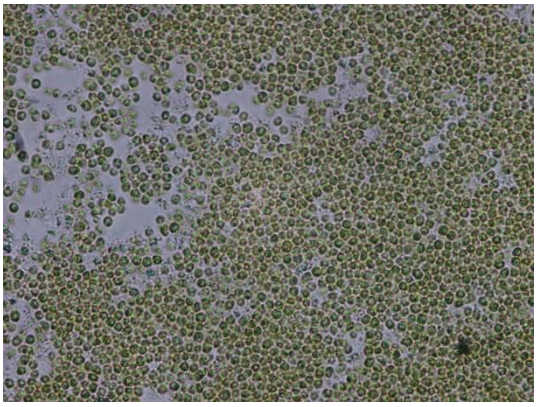

Figure 4.6. Microscopic photograph of reference sample with non-disrupted cells

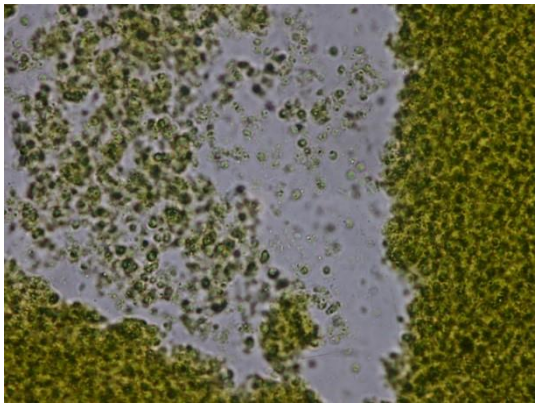

Figure 4.7. Microscopic photograph of sample 1.

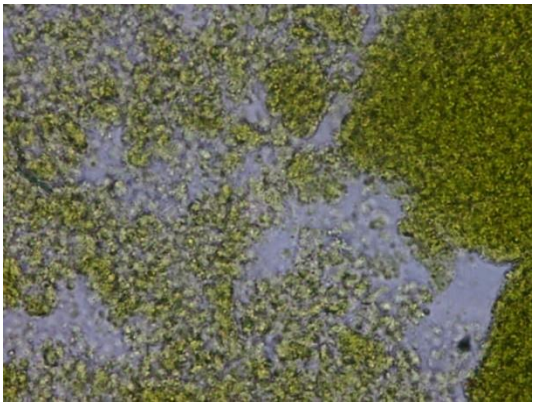

Figure 4.8. Microscopic photograph of sample 2.

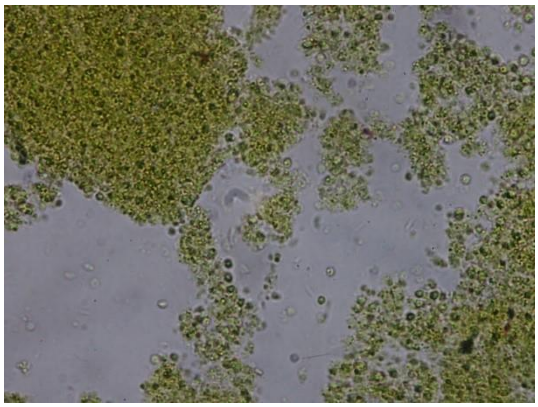

Figure 4.9. Microscopic photograph of sample 3.

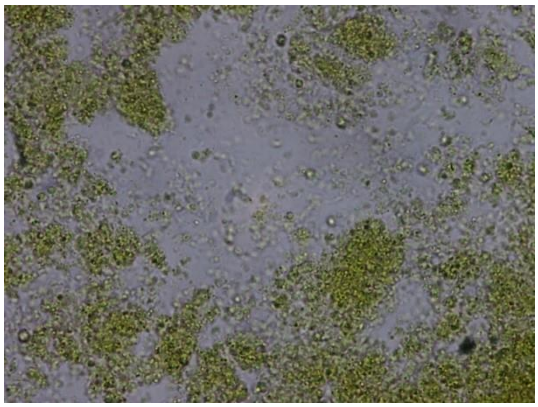

Figure 4.10. Microscopic photograph of sample 4.

According to these microscopic observations, it is possible to conclude that this equipment is quite effective and its efficiency, as expected, increases with the number of passes. After one pass (Figure 4.7 and Figure 4.109), still a few cells were intact between the clusters of ruptured cells. After the two passes test (Figure 4.8 and Figure 4.10), almost all cells were smashed.

4.3.2.2. Cell Disruption Efficiency

To calculate the effieciency of rupture of this processing, the concentration of pigments in each sample was considered. Carotenoids and chlorophyll a when exposed to oxygen, light and temperature form different degradations products, however the method of detection used does not include carotenoids degradation products. Due to this fact, the cell disruption efficiency was calculated for both pigments fraction for further comparison (4.2) and (4.3).

$$\textbf{Cell Disruption Efficiency}_{Carotenoids}\ (\%) = \frac{[Carotenoids]_i}{[Carotenoids]_{ib}} \times 100 \qquad \textbf{(4.2)}$$

Where $[Carotenoids]_i$ is the concentration of carotenoids in the sample i after the bead milling processing and $[Carotenoids]_{ib}$ is the concentration of carotenoids in the initial biomass.

$$\textbf{Cell Disruption Efficiency}_{Chlorophyll\ a}\ (\%) = \frac{[Chlorophyll\ a]_i + [Pheophytin\ a]_i}{[Chlorophyll\ a]_{IB} + [Pheophytin\ a]_{IB}} \times 100 \quad \textbf{(4.3)}$$

Where $[Chlorophyll\ a]_i$ and $[Pheophytin\ a]_i$ are the concentrations of chlorophyll a and pheophytin a in the sample i after the bead milling processing, respectively. $[Chlorophyll\ a]_{IB}$ and $[Pheophytin\ a]_{IB}$ are the concentrations of chlorophyll a and pheophytin a in the initial biomass.

As stated before, bead milling processing is a mechanical treatment and the associated heat transfer and the increase of temperature even containing a cooling jacket is unavoidable. Since pheophytin a is a degradation product of chlorophyll a, the degradation rate was calculated through (4.4), see Table 4.9.

$$Chlorophyll\ a\ Degradation\ (\%) = \frac{[Pheophytin\ a]_i}{[Chlorophyll\ a]_{ib} + [Pheophytin\ a]_{ib}} \times 100 \quad \textbf{(4.4)}$$

Where $[Pheophytin\ a]_i$ is the concentration of pheophytin in the sample i and $[Pheophytin\ a]_{IB}$ and $[Chlorophyll\ a]_{IB}$ is the concentration of pheophytin and chlorophyll a in the initial biomass, respectively.

Table 4.9. Cell disruption efficiency and chlorophyll a degradation results.

Temperature Operation (ºC)	Number of Passes	$\textbf{Cell Disruption Efficiency}_{Chlorophyll\ a}$ (%)	$\textbf{Cell Disruption Efficiency}_{Carotenoids}$ (%)	Chlorophyll a Degradation (%)
8	1	64.9 ± 4.7	67.8 ± 7.0	18.4 ± 3.6
	2	73.8 ± 2.3	83.0 ± 5.5	
23	1	60.1 ± 1.7	59.3 ± 3.3	25.2 ± 3.1
	2	84.8 ± 4.6	84.0 ± 4.6	

Safi et al. (2017) publication using similar conditions of bead milling processing (same microalgae genus, 100 gL-1 biomass suspension and 0,5 mm beads) showed a cell disruption efficiency higher than 95% after 20min of operation [129]. Comparing with the results obtained in this work, they are lower for both pigments calculations than expected but several reasons may have resulted in these values. The uncertainty of the values calculated through standard deviation proved to be high in all the calculations, which could mean that this method might be inappropriate. Also, the figures presented before, show very few non-disrupted cells when processed with two passes, which indicates a high efficiency, possibly near 100%.

There are several other parameters which can affect this efficiency such as the agitator speed, bead density and bead filling (% of the grinding chamber volume). As this experiment could not be performed in A4F premises, it was not possible to optimize the parameters of the process.

Other factor that needs to be considered, is the fact that the cell wall characteristics of microalgae are very diverse based on the species and the cultivation conditions. Regarding this, the efficiency of this unit operation varies as a function of the cell wall structural characteristics of the species selected. [129]

According to results obtained by Postma et al.(2014) [38], pigment release in the bead milling processing was found to be slower than the biomass disintegration as chlorophyll lies within the chloroplast and on the other hand, when the carotenoids degrade are not detected by the method, then this calculations may not be the most appropriate.

Comparing the efficiency obtained in the different number of passes, it can be concluded that by two passes processing, the efficiency increases roughly 20 percentage points which means higher number of products released and consequently, higher profits.

Regarding the temperature conditions, disruption efficiency results seemed to be similar, and comparing the chlorophyll a degradation, it slightly increases when a higher temperature is used. With these results, it is proved that processing at room temperature does not promote a substantially higher degradation of pigments and to spare the higher costs of refrigeration, these conditions may be the most appropriate. Therefore, for further calculations, the conditions process of 23ºC and two passes will be considered.

4.3.3. pH-shift test

According to biorefinery 1A and 1B, after the disruption process and further centrifugation of the biomass processed in two passes at 8ºC and 23ºC, to obtain a supernatant rich in proteins and carbohydrates and a pellet rich in pigments and lipids, a pH adjustment was tested to recover the proteins from the supernatant by precipitation. As reported by Cavonius et al.(2015) [118], the proteins solubility is high between pH 5,5 and 10,5, therefore to establish the most appropriate pH for the process, 3 different pH experiments were tried. From each of the samples, three known volumes of supernatant were collected and subsequently the pH of each was adjusted to 3, 4 and 5. In the following figures (Figure 4.11), it is presented the appearance of each of the samples after the pH adjustment.

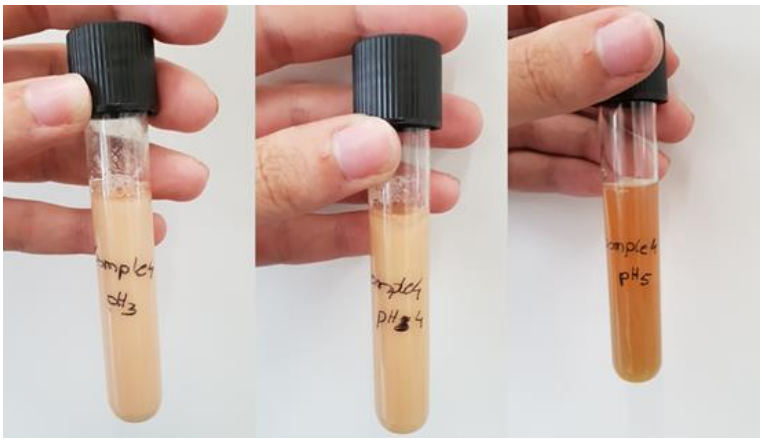

Figure 4.11. Samples after pH adjustment: a)pH at 3; b)pH at 4; c) pH at 5.

As expected, the decrease of pH accentuates the turbidity of the sample which in turn is related to the increase of precipitated solids. After centrifugation (Figure 4.12), the precipitated proteins formed a pellet and, as expected, the pellet at pH 3 visually presents more amount of

precipitate. Moreover, the precipitated solids formed layers of different colours which indicate that they may have different compositions.

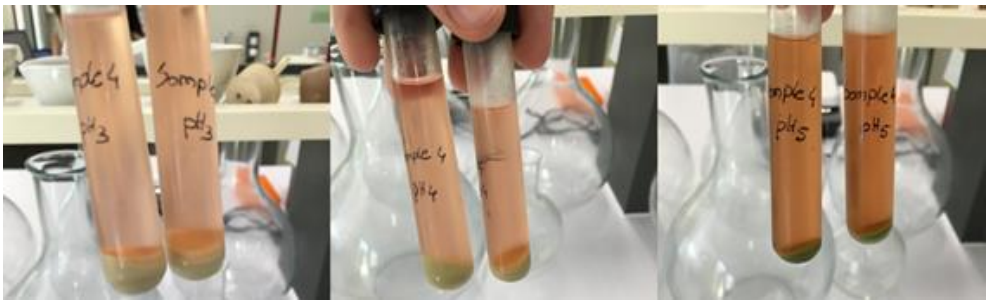

Figure 4.12. Samples after centrifugation: a) pH at 3; b) pH at 4; c) pH at 5.

In the following figure (Figure 4.13), the solid weight results per volume of sample are presented. According to these results, sample 4 which was processed at higher temperature obtained higher percentage of solid recovery. This can be justified by the fact that temperature affects solubility of proteins by modifying surface hydrophobic (protein-protein) and hydrophilic (protein-solvent) interaction. Thereupon, for higher milling temperature, more compounds were dissolved in the aqueous phase during the process, and therefore more matter was available to precipitate in these pH shift tests. [130]

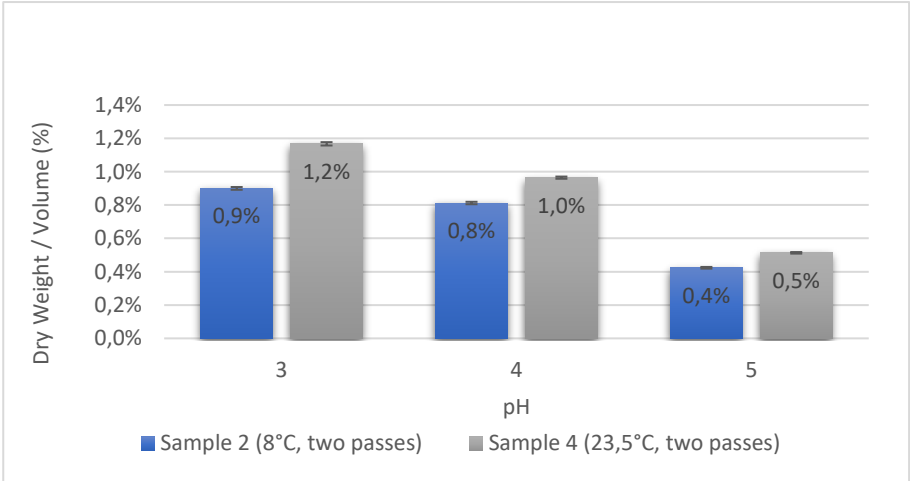

Figure 4.13. Precipitated weight per volume of sample at different pH. The uncertainty was calculated based on the propagation of the error considering the balance and pipette accuracy.

Despite the high amount of precipitate recovered, analysis of protein was performed to confirm if it is constituted by proteins only. The first trial of analysis was not successful since after using the moisture balance to eliminate of humidity, the solid did not redissolve and the spectrophotometry reading was disturbed by turbidity. In light ofthese results, the wet precipitate was resuspended in distilled water and the redissolution was achieved, allowing protein quantification. In the following table, the results of this analysis are presented (Table 4.10).

Table 4.10. Results of protein analysis of the precipitate obtained in the pH-shift test. The uncertainty was calculated based on the propagation of the error considering the balance and pipette accuracy and also the standard deviation of the replicates.

pH	Sample 2 (% g protein/g precipitated)	Sample 4 (% g protein/g precipitated)
3	14.0 ± 0.2	29.6 ± 0.3
4	19.8 ± 0.2	21.4 ± 0.2
5	24.6 ± 0.3	19.4 ± 0.2

This analysis revealed that the precipitate is not constituted only by proteins as desired. According to the Cavonius et al. (2015) [118] research, it may be possible that at low lipid content such as the *Nannochloropsis* sp. used in this work, as fatty acids are mainly present in the form of polar lipids associated with membranes, it is expected to exist an association with proteins through the cytoskeletal lattice and therefore not be separated at the centrifugation step. Also, they concluded by this method that high concentrations of proteins, fatty acids and carbohydrates were found in the precipitates, as well as ashes in reduced amounts. With these results together with the photos shown above, a conclusion can be taken: the precipitate is constituted by several components such as proteins and lipids and form layers which is the consequence of density differences. For future work, this constitution should be studied in order to find the complete specification of this product or even differentially separate the fractions.

4.3.4. Extraction of Microalgae Compounds

In this project, biorefineries using green solvents such as D-limonene, ethanol and olive oil were proposed. Despite the significant number of papers dealing with the use of microalgae as feedstocks, chemicals, or natural products, the extraction using these solvents is not well studied yet since few publications are available.

In this topic, the results of extractions using different solvents relative to different biorefineries are presented. Two fractions of biocompounds were analysed (pigments and fatty acids) and different extraction processes evaluated to identify the best solvents by partition coefficient calculation and by biomass/solvent ratio. For future work, more tests will be required to improve the results known up to now, since the correlation between the chemical structures of the compounds extracted from the microalgae biomass and the solvents and technologies being applied should be properly investigated.

4.3.4.1. D-limonene

D-limonene, considered as a safe agricultural by-product, was used in previous work to extract rice bran oil and compared against hexane, a petroleum product widely used as a solvent for extracting edible oil [131]. After that, this solvent was tested to extract lipids from microalgae and

the results were quite impressive. So, for biorefinery 1A and 1B, an extraction using d-limonene and analysis for further calculations were performed.

As it is well known, the solvent/solid ratio, extraction duration and temperature are key factors in extraction processes, as they affect the kinetic of compounds release from the solid matrix. Thus, it is very important to define the conditions of these variables to maximize the extraction results.

Since temperature and light affects extraction kinetics especially with pigments present in the solid matrix, the trials were performed at controlled temperature and the extraction vessels were conditioned to prevent light from interfering with the operation.

The ratio (biomass : solvent) assayed in the first trial was 1:10 and the trial lasted for 6 hours. Figure 4.14 shows the results of pigment analysis. Since replicates were not generated, the uncertainty considered was overestimated as 5%.

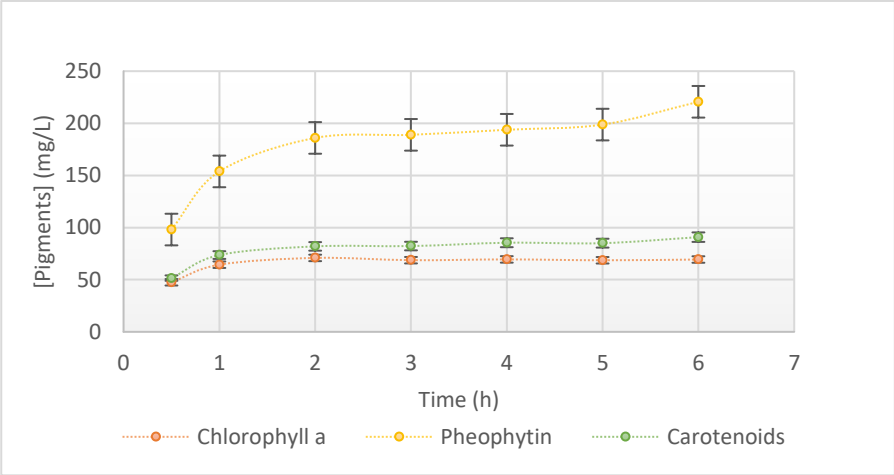

Figure 4.14. Evolution of pigments extraction with d-limonene (ratio of 1:10). The concentration of pigments (mg/L) were plotted against time.

As predicted, the concentrations of pigments in general increased in the beginning of the extraction. Although the extraction procedures were performed to prevent degradation of the pigments, the oxygen present in the atmosphere of the extractor and the fact that D-limonene is an organic solvent might boost the degradation of chlorophyll a as it is visible in the previous figure. Another result to discuss is that the concentrations started to stabilize (after one hour) which points to the fact that the solvent is saturated.

Another indicator of the possible saturation state of this extraction trial is the colour of the obtained extract (Figure 4.15: a)) which was dark green, and the appearance of fresh limonene is colourless. Afterwards, fresh limonene was added to the solid matrix used in the extraction and the appearance was changing to green during manual shaking, thus confirming the saturation (Figure 4.15: b))

a) Solvent and solid matrix appearance after 6h of extraction

b) Solvent appearance after new addition of limonene.

Figure 4.15. Conventional extraction with limonene (ratio of 1:10).

Concerning the previous results, a new ratio was tested. Because D-limonene was found to be expensive when compared with other solvents, such as ethanol, it was decided to not greatly increase the amount of solvent in the process. Thus, a ratio of 1:12 was tested and pigments analyses were performed, see Figure 4.16.

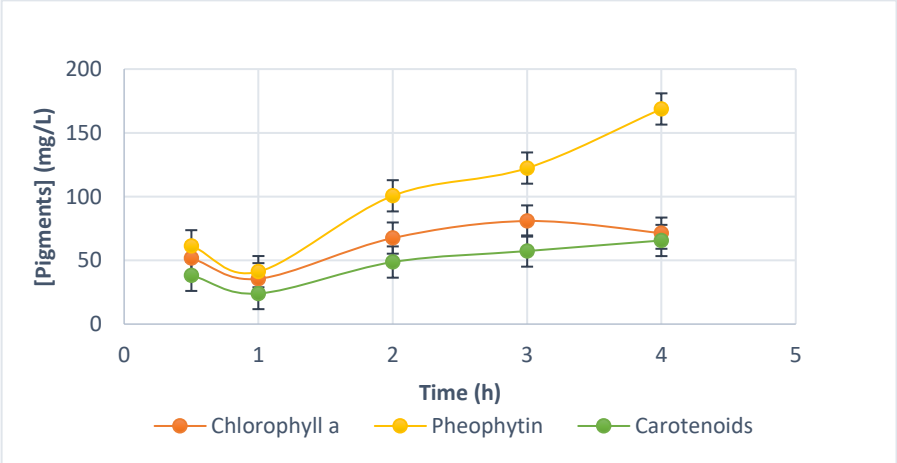

Figure 4.16. Evolution of pigments extraction with d-limonene (ratio of 1:12). The concentration of pigments (mg/L) were plotted against time.

Figure 4.16 shows the evolution of the extraction using D-limonene and in this trial the diffusion behaviour of the pigments were similar, as pheophytin achieved higher concentrations in the final extract. Yet, the concentrations obtained were lower that the obtained in the previous trial and also, varied during the four hours of extraction that could mean that saturation state was not achieved. For further calculations of mass balances these new concentrations were considered.

Fatty acids analysis was also performed to evaluate this operation, the results are presented in the following table (Table 4.11). The weight of sample used in this analysis was 845,5±0,1 mg.

Table 4.11. Results of the fatty acids analysis to the extracts with limonene as solvent. *Weight measured by gas chromatography of the fatty acids detected. **These values were calculated considering the ratio of extraction.

Fatty Acid Detected	Fatty acids/Limonene (% w/w)	Fatty Acids/Biomass (% w/w)**
C14:0	0.02%	0.2%
C16:0	0.03%	0.4%
C16:1	0.04%	0.5%
C18:1	0.01%	0.1%
C20:4w6	0.002%	0.02%
C20:5w3	0.02%	0.3%
Total	0.12%	2%

First, as limonene is considered a non-polar solvent, it was expected that only SFAs and MUFAs were extracted because according to the calculated profile of lipids (Table 4.6), the neutral fraction is not constituted by PUFAs. But PUFAs were indeed extracted with the same efficiency. This can be due to the slightly more polar nature of terpenes when compared with n-hexane. But more likely it could mean that lipids in polar form within the original biomass were no longer in that form in the beginning of the extraction process. The total amount of fatty acids extracted per biomass obtained was 2%, which compared to the expected value of 0,8% of TAG, from the biomass composition indicated in Table 4.7, the efficiency was higher than expected.

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4.3.4.2. Ethanol

As stated before, ethanol is a worthy candidate to be studied as alternative solvent as it is cheap, and it can be produced by fermentation from a large variety of biological materials, and thus considered as “natural” or “bio renewable”. [132]

In this processing step, the aim was to study the extraction of lipids and pigments from this microalgae genus using ethanol. The process developed here may be used as the extraction step obtaining oils with a high content in n-3 PUFA and also pigments for all the biorefineries proposed.

The same procedures of limonene extraction were adopted for the first trial using ethanol, then the ratio (biomass:solvent) tried was 1:10 and lasted for 6 hours. The evolution of the pigments extraction are presented in the following figure (Figure 4.17).

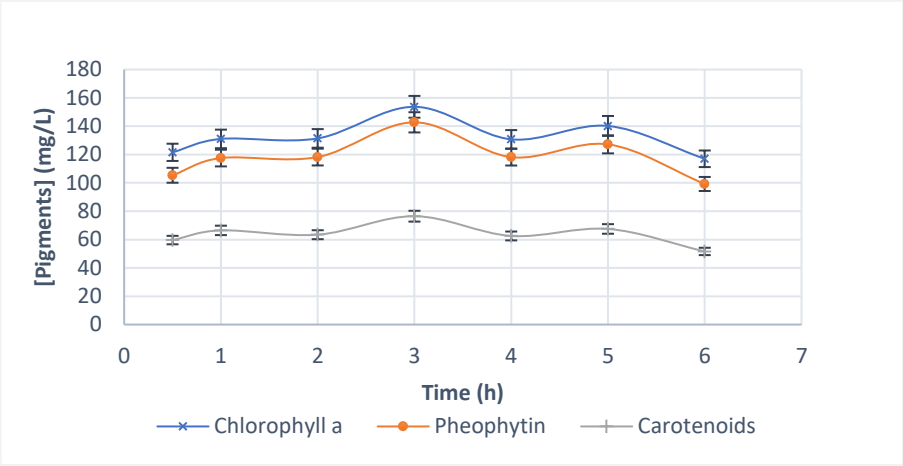


Figure 4.17. Evolution of pigments extraction with ethanol (ratio of 1:10). The concentration of pigments (mg/L) were plotted against time. Since no replicates were generated, an uncertainty of 5% was considered.

Because the solid matrix is very miscible in ethanol, the sampling of this trial could not be done in perfectly homogeneous conditions due to deposition of matter in the bottom of the sample flask. For that reason, the evolution of the pigments extraction presents flutuations. However, there were no significant increases in the pigments concentration, which could mean that the saturation concentration was achieved even before the first analysed time point.

The carotenoids family can be divided in two main groups: the polar carotenoids (xanthophylls) and non-polar carotenoids (carotenes). Since *Nannochloropsis* sp. is constituted by predominantly xanthophylls with hydrophilic behaviour, the carotenoids fraction of pigments was detected in the ethanol extracts. Chlorophyll a contains several polar C-O and C-N bonds, therefore they are easily extracted by ethanol, as well as the pheophytin a which are chlorophyll molecules without the Mg^{2+} ion and two protonated nitrogen atoms instead.

As the polar lipids are only partially miscible in ethanol, the driving force of extraction decreases as the concentration of lipids in the solvent increases and goes to zero at the saturation concentration, therefore a new ratio was tested. Several volumes of ethanol were added to the solid matrix until the extract was colourless (Figure 4.18).

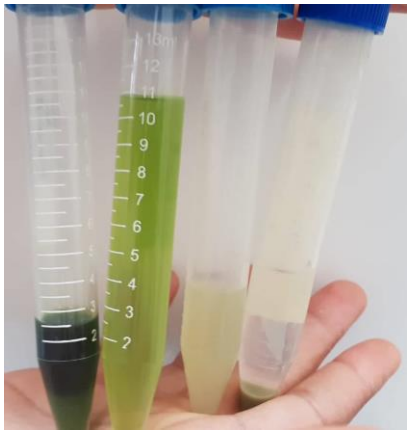


Figure 4.18. Evolution of colour of the extract while adding controlled volumes of ethanol to the solid matrix.

After adding 16 volumes of 1 mL of ethanol to fresh solid matrix, the solvent was colourless, and it was assumed that all the extractable compounds were already dissolved. Then, a new trial was performed in the same conditions, but the ratio was 1:16 and the experiment lasted 3 hours. The results of pigment analysis are present in the following figure (Figure 4.19).

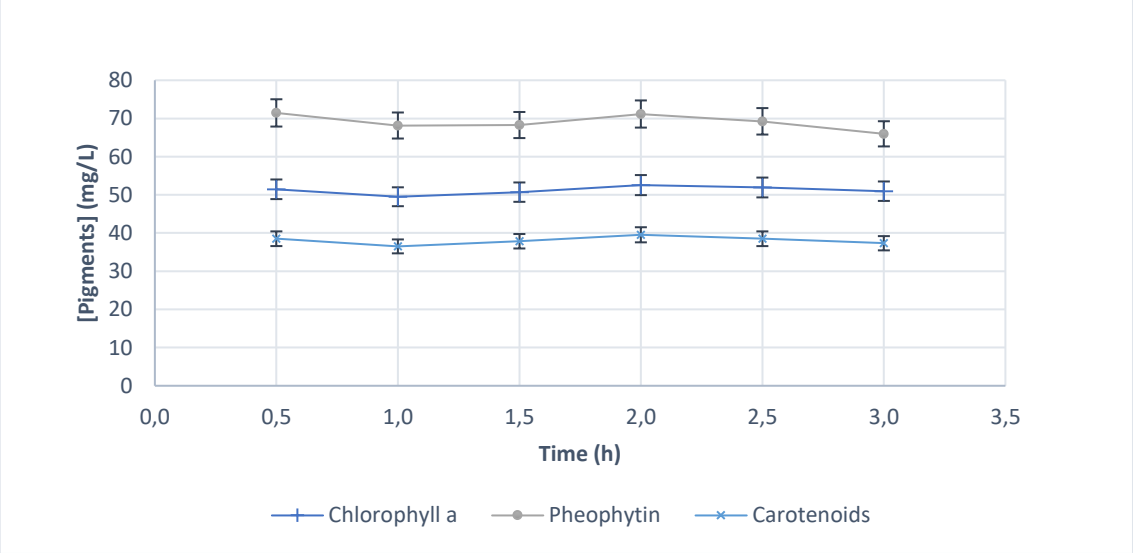


Figure 4.19. Evolution of pigments extraction with ethanol (ratio of 1:16). The concentration of pigments (mg/L) were plotted against time. Since no replicates were generated, an uncertainty of 5% was considered.

Since these trials were performed after few days, the analysis detected the presence of higher concentration of pheophytin a when compared to chlorophyll a concentration. Despite being covered from light, the oxygen is also a potential factor of degradation.

During this trial, some problems occurred with the agitation, as the solvent volume increased, the stirrer had to be changed to a larger one to obtain an extraction efficiency equivalent to the

previous trial, but this did not occur due to vortex formation. The vortex delayed the distribution of the particles into the entire region of solution; hence, it decreased the process of mass transfer. Also, as it is shown in the previous figure, the concentrations of pigments achieved were lower than the ones obtained in the previous trial, which means that the saturation concentrations were not achieved.

To improve this study about the extraction using ethanol, another method was tested. The temperature of the process can also affect the extraction efficiency, modifying the solvent physical properties, for instance reducing the solvent surface tension, increasing the solute's solubility and increment the solute diffusion rate [133].Thereupon, to evaluate the performance of this extraction, a soxhlet extraction using the same ratio (1:16) was performed. A Soxhlet extraction is a common solid-liquid extraction that uses solvents at boiling temperature and low pressures (ambient pressure). It is commonly used for evaluating the performance of other methods [134]**.** The results of pigments analysis are presented in Figure 4.20.

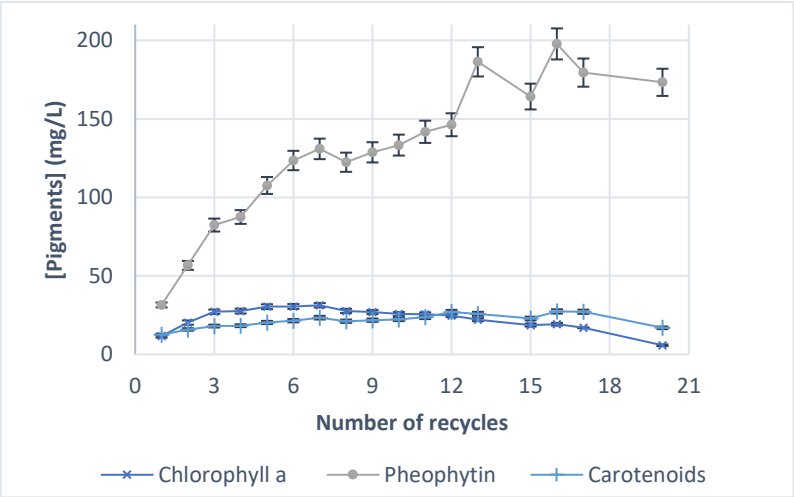


Figure 4.20. Evolution of pigments extraction with ethanol (ratio of 1:16) using a soxhlet extractor. The concentration of pigments (mg/L) were plotted against the number of recycles. Since no replicates were generated, an uncertainty of 5% was considered.

As it is possible to see, the pheophytin a concentration presents a significant increase relative to chlorophyll since it is a degradation product and was formed when their molecules were exposed to high temperatures.

Concerning carotenoids concentration evolution, the thermal degradation and cis-trans isomerization and their extraction seems to reach an equilibrium since their concentration is constant during the recycles. In the final recycles, the concentration drops as the equilibrium is destabilized by the lack of carotenoids left in the solid matrix.

Overall, with this method, a higher concentration of pigments was extracted, with 240mg/L against 158 mg/L of the conventional extraction. However, the main drawbacks of this method are [135]:

- The extraction time is long;
- Agitation cannot be provided in the Soxhlet device to accelerate the process,
- The possibility of thermal decomposition of the target compounds since the extraction occurs at the boiling point of the solvent for a long time.

For these reasons, this method was only tested to emphasise the ethanol efficiency as a solvent because for scale-up purposes this method is not applicable.

In this unit operation, the interest was mainly focused on the extraction polar lipids and for that reason, fatty acids analysis performed to the ethanol extracts and the raffinate solid matrix, the results are exposed in Table 4.12.

Table 4.12. Results of the fatty acids analysis to the extracts with ethanol as solvent (ratio biomass:solvent of 1:16). *Weight measured by gas chromatography of the fatty acids detected. **These values were calculated considering the ratio of extraction.

	Ethanol Extract			Raffinate	
Fatty Acid	**Weight (mg)**	**Fatty acids/Ethanol extract (% w/w)**	**Exctracted Fatty acids/ Biomass (% w/w)****	**Weight (mg)***	**Remaining Fatty acids/Solid (% w/w)**
C16:0	0.07	0.01	0.2	0.2	0.03
C16:1	0.03	0.007	0.1	0.08	0.01
C20:4w6	-	-	-	0.03	0.01
C20:5w3	0.02	0.004	0.06	0.1	0.02
Total	0.1	0.03	0.4	0.4	0.07

Analysing the previous table, it is possible to verify that in sum, the fatty acids available for extraction was in low quantity. This may happen because of the separation of the aqueous phase in which a high amount of lipids present in the membrane may have been attached to the soluble proteins. Also, during the bead milling processing, the presence of water might hydrolysate the lipids into free fatty acids, decreasing their polarity and increasing solubility in D-limonene. For future works, may be advantageous to analyze the aqueous fraction in detail and also the precipitate obtained in topic 4.3.3.

Considering the equation (2.1), the partition coefficient for total fatty acids was calculated and was found to be near 6, which measures how hydrophilic the fatty acids are. However, this value might be higher if indeed, a hydrolysis occurred during the bead milling,

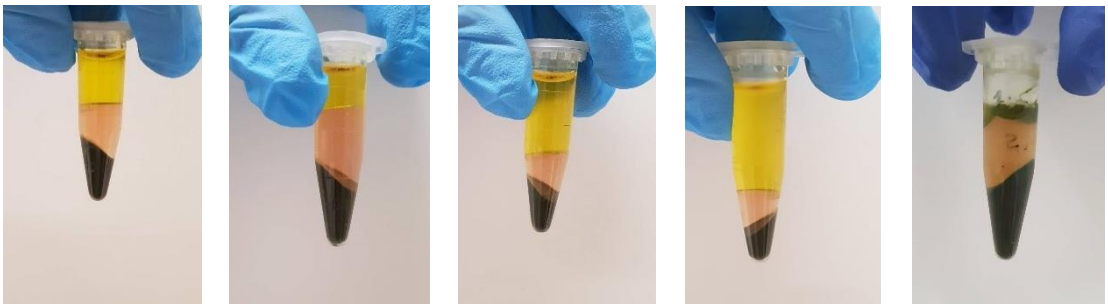
4.3.4.3. Olive Oil

For biorefinery 2A and 2B, it was proposed to add olive oil to the bead mill to form 3 phases:

- Upper phase, organic, constituted of oil and pigments;
- Intermediary phase, aqueous, with soluble compounds;
- Lower phase, constituted of wet insoluble matter.

Five ratios (biomass:olive oil) were tested by adding the solvent to the previously disrupted and suspended biomass and then centrifugated (at 14,000 x g, 10 min). This is not the most

suitable measurement to fully understand this potential extraction since in the proposal, the solvent would be added directly to the bead milling. As it is possible to observe, three phases were formed by this trial as expected, see Figure 4.21.



a) 1:1 b) 2:1 c) 1:2 d) 1:5 e) 5:1

Figure 4.21. Emulsion breaking by adding olive oil to suspended biomass. Ratios tested (biomass : olive oil).

Observing macroscopically, the ratio using less solvent seems to extract a high amount of pigments while the colour of the olive oil of the remaining ratios resembles the original. To select a ratio to proceed, pigments analysis was performed. In the following figure Figure 4.22, the concentration of carotenoids (mg/L) were plotted against the ratios tested.

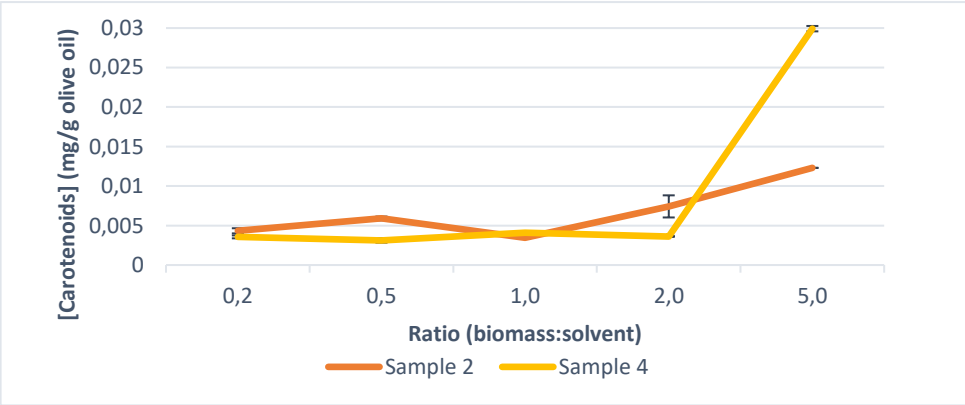


Figure 4.22. Evolution of carotenoids extraction with olive oil at different ratios. The uncertainty was calculated based on the standard deviation in carotenoids concentration values for the replicates in each test.

Although further studies may be performed to select the proper olive oil quantity to add, namely between ration 2:1 and 5:1, the input of solvent in the milling process would enhance the final product if added at higher ratios. This conclusion can be taken only for carotenoids since there was a high concentration extracted by the olive oil, following the previous figure.

Thus, for mass balances of this biorefinery, the higher ratio was considered. In order to be able to characterize this final product, fatty acids analysis was performed. As the olive oil is rich in fatty acids, it was easier to analyse the pellet of biomass after the extraction to prevent possible reading errors.

The results are presented in the following table (Table 4.13) as well as the calculations to discuss the amount of fatty acids extracted.

Table 4.13. Results of the fatty acids analysis to the solid matter after extraction with olive oil. *Weight measured by gas chromatography of the fatty acids detected.

Fatty Acid Detected	Fatty acids/Biomass after extraction (% w/w)	Fatty acids/Initial Biomass (% w/w)	Fatty acids Extracted with olive oil /initial biomass (%w/w)
C14:0	0.5	1.0	0.5
C16:0	1.3	2.9	1.6
C16:1	1.4	3.0	1.6
C18:1	0.4	0.8	0.4
C18:3	0.3	0.6	0.3
C20:4w6	0.2	0.5	0.3
C20:5w3	1.1	2.5	1.4
Total Fatty Acids	5.2	11.3	6.1

The resulting extract can be defined as “flavoured oil” or “gourmet oil” since this final product seems to be enriched with high value fatty acids. As summarized above, the olive oil extracted 6,1 percentage points of fatty acids from the suspended biomass in which 1,4% is the much-sought-after EPA. Like happened with limonene extractions, the polar fraction of glycolipids and phospholipids seems to not be enough to keep the biomolecules in the solid matrix or either in the aqueous phase. Or, if indeed, this were free fatty acids hydrolysed during the bead milling, perhaps they migrated to the olive oil phase because of the loss of the polar lipid fraction. This can lead to increasing acid value of the oil phase, which decrease its value, and should be further investigated.

The selectivity of olive oil is dependent on their types and components inside, resulting in variable extraction efficiency and enrichment factors [136]**.**

For future works, studies about the prediction of the dissolving power of olive oil such as their supramolecular complexity, various extraction methods, target solute, acylglycerol and fatty acid composition should be performed.

4.3.5. Solvent Recovery

The solvent consumption in the chemical industries is increasingly becoming a topic of interest. To minimize its impacts, two recovery processes were studied to know their effectiveness and viability in the proposed biorefineries: membrane filtration and distillation.

4.3.5.1. Membrane Filtration

The membrane filtration process is recognized as one of the key technologies to drive solvent recovery due to its inherent simplicity and energy efficiency. The concentration of the targeted

compounds can be achieved with a simple pressure gradient and without phase changes to both reduce the solvent consumption and achieve high productivity separation [137]. To evaluate the feasibility of this type of solvent recovery in the proposed biorefineries, three steps were performed: membranes stability, solvent permeability and solute rejection.

Prior to using the membrane equipment, the membranes were conditioned by exposure to solvents to check their stability and all of them remained the same in terms of size and appearance after 72 hours of exposure. For that reason, none of them could be ruled out in this first step.

Regarding the membrane solute permeability, it plays a role in the build-up of concentration polarization in pressure-driven crossflow filtration processes, and consequently in the determination of the permeate flux, solute rejection, retentate flux and concentration [138].

The recovery of lipids from a solvent using membrane technology is usually difficult because of their low concentration, often vestigial, and the complexity of the original matrix where they need be recovered from. Also, the characteristics of the membrane must be compatible with the solvent nature. For instance, for d-limonene processing a solvent resistant nanofiltration membrane is required.

4.3.5.1.1. D-limonene

Solvent resistant nanofiltration is a versatile technology that represents a promising alternative to conventional separation techniques and to understand the behaviour of D-limonene using this technology, three membranes were tested. As they showed stability after 72h, the next step was the permeability test using a model solution of D-limonene and 0,1%w/w of palmitic acid (palmitic acid was chosen considering the molar mass and quantity in the cell).

Note that compaction test was not performed for this work, consequently there is a possibility of when the polymeric membrane was put under pressure, the polymers could slightly reorganized and the structure might change, resulting in a lowered volume porosity and increased membrane resistance. [139]

Figure 4.23 shows the evolution of the fluxes of each membrane as a function of the concentration factor, CF.

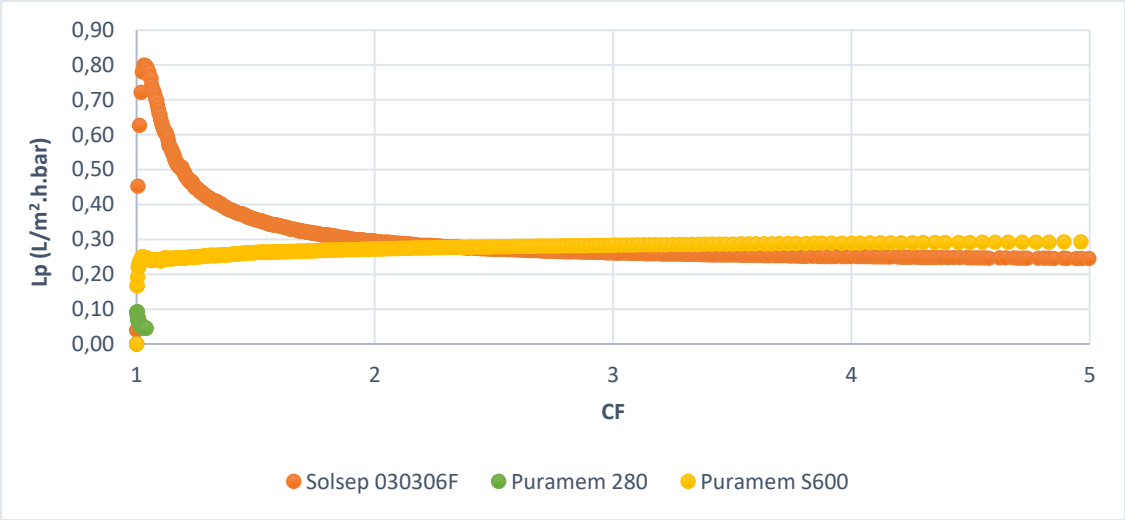


Figure 4.23. Experiments of nanofiltration using a model solution with membranes Solsep 030306F, Puramem 280 and Puramem S600. Permeability (L/m2.h.bar) was plotted against the concentration factor, CF.

During the experiments, Puramem 280 showed low fluxes and after 1h of membrane permeation using the model solution, the test was stopped since the CF was very low (CF=1,03). This behaviour may be expected since this membrane has a low molecular weight cut-off (MWCO) (see Table 3.2) and consequently, may be more sensitive to fouling.

Regarding Solsep 030306F and Puramem S600, they presented higher permeate fluxes and the CF 5 was achieved for both. Both present an initial increase in permeability that in some time start to invert. This flux decline may result from concentration polarization which is related to pore blockage by solute adsorbed on the membrane surface or within pores, and from the formation of a solute layer on the membrane surface, which also offers a resistance to flow in addition to the membrane itself. [140] This non-linear behaviour can also be explained by membrane compaction. The initial behaviour of higher permeabilities achieved using Solsep 030306F is explained by its higher MWCO and its abrupt decrease reveals that this membrane is more unstable in organic solvents and / or more sensitive to fouling compared to Puramem S600.

To enable a conclusion of these results, the mean permeability was calculated, and the results are presented in the following table Table 4.14.

Table 4.14. -Average permeability results of d-limonene using the solvent resistant membranes: Solsep 030306F, Puramem 280 and Puramem S600.

	Average Permeability (L/m^2.h.bar)
Solsep 030306F	0.33
Puramem 280	0.05
Puramem S600	0.26

Indeed, Puramem 280 presented low permeability and for further tests it will not be considered. For solute rejection tests, Solsep 030306F and Puramem S600 were tested using limonene extracts since their permeability values were similar and suitable conclusions cannot be taken.

Figure 4.24 shows the results of the evolution of the fluxes of both membranes testes using limonene extract as a function of the concentration factor, CF. Despite the higher MWCO of Solsep 030306F, this membrane presented low permeability when using real extracts. After 8 hours of test, the CF achieved was only of 1,2 and for that reason the test was stopped. The value of average permeability calculated was 0,03 L/m^2.h.bar which is very low. As stated before using a model solution, this membrane revealed instability or/and fouling sensibility and by using these extracts rich in compounds of varying sizes, these phenomena have likely been intensified, making mass transfer difficult through the membrane.

Concerning Puramem S600, this membrane also revealed stability and the average permeability increased using the real extract, with a value of 0,41 L/m^2.h.bar.

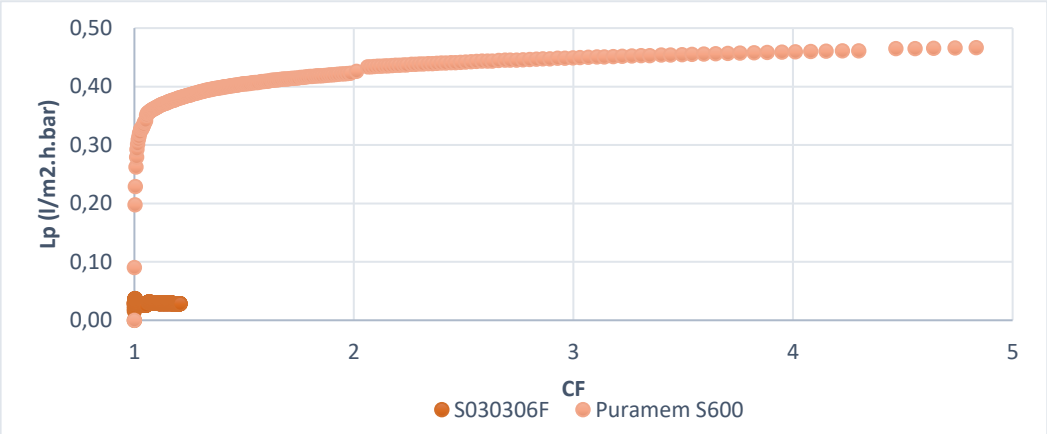


Figure 4.24. Experiments of nanofiltration using limonene extracts with membranes Solsep 030306F and Puramem S600. Permeability (L/m2.h.bar) was plotted against the concentration factor, CF.

Since membrane are supposed to allow the passage of only the smaller solutes, there will be an accumulation of solutes which are largely rejected by the membrane. To evaluate this membranes selectivity, fatty acids and pigments analysis were performed.

Table 4.15 shows the rejection coefficients values of pigments for both membranes tested calculated through equation 3.2. Analysing these results, for both membranes chlorophyll a present high rejection coefficient maybe because of its high molecular weight (molecular weight of ~893,5 g/mol). Regarding Solsep 030306F values, they were lower than Puramem S600 with 2,7% of rejection for carotenoids fraction.

Rejections for fatty acids was also calculated, see Table 4.16.

Table 4.15. Rejection coefficients calculated for chlorophyll a, pheophityn a and carotenoids in D-limonene extract regarding Solsep 030306F and Puramem S600. *For Puramem S600 values, the mean of CF rejection results were calculated. The uncertainty was calculated based on the standard deviation of CF rejection results.

Membrane	$R_{Chlorophyll\ a}$ (%)	$R_{Pheophytin}$ (%)	$R_{Carotenoids}$ (%)
Solsep 030306F	90.1	62.2	2.7
Puramem S600*	99.1 ± 0.5	93.8 ± 1.5	90.4 ± 2.7

Table 4.16. Rejection coefficients calculated for fatty acids regarding Solsep 030306F and Puramem S600. *For Puramem S600 values, the mean of CF rejection results were calculated. The uncertainty was calculated based on the standard deviation of CF rejection results. n.d.= not detected.

Membrane	$R_{C16:0}$ (%)	$R_{C16:1}$ (%)	$R_{C18:1}$ (%)	$R_{C20:5w3}$ (%)	$R_{Total\ FA}$ (%)
Solsep 030306F	n.d.	33.9	n.d.	32.5	14.9
Puramem S600*	19.6 ± 7.8	58.1 ± 5.9	44.7 ± 7.4	73.4 ± 5.2	32.9 ± 5.1

Overall, Puramem S600 had higher observed rejection factors than Solsep 030306F. It has been proved that solute-membrane interactions contribute to the eventual rejection of organic solutes because ideally, all molecules larger than the MWCO of a membrane should be rejected but pH and ionic strength affect the size, shape and flexibility of a solute. Thus, future additional study is required about these complex interactions between the membrane and organic matter chemistry [141].

For further calculations, the Puramem S600 membrane results will be considered due to it higher performance and stability. Also, it presented high rejection coefficient of EPA fatty acid which is one of the most valuable products.

4.3.5.1.2. Ethanol

As stated before, there is an increasing interest in using less toxic and renewable solvents such as ethanol for the extraction of compounds instead of petroleum-derived solvents. Yet, low-cost and low-energy methods will have to be used instead of evaporation or distillation to recover and recycle the solvent to compensate for ethanol's higher latent heat and higher boiling point [128]. Therefore, envisaging the concentration of extracted compounds and the ethanol recovery, membrane processing was carried out.

Although the swelling test did not detect any differences in size and appearance, once submitted to pressure, the membrane's behaviour may be significantly different. To test the efficiency of this unit operations at required conditions for biorefining, several nanofiltration membranes were tested for flux and rejection of selected solutes in ethanol. The same procedures used in membrane filtrations of D-limonene trials were adopted. The results of the evolution of the fluxes of each membrane were plotted against the concentration factor are presented in Figure 4.25**.** The average values of permeability were calculated through equation (3.3) are presented in Table 4.17**.**

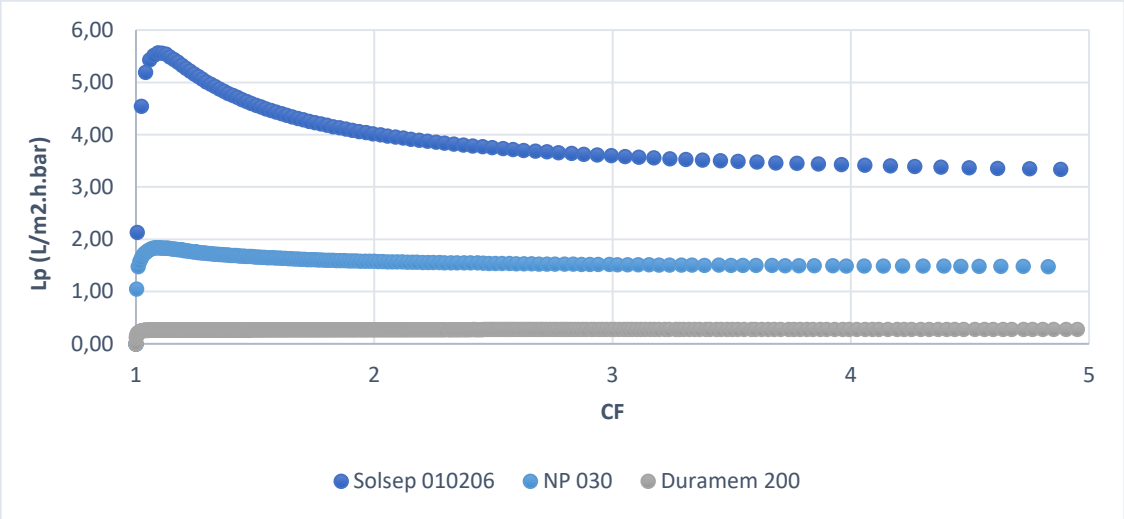


Figure 4.25. Permeability testes of the membranes Solsep 010206, NP 030 and Duramem 200 with ethanol.

Table 4.17. Average permeability results to ethanol using the membranes: Solsep 010206, NP 030 and Duramem 200.

Membrane	Average Permeability ($L/m^2.h.bar$)
Solsep 010206	4.16
NP030	1.62
Duramem 200	0.27

As it is possible to see, Duramem 200 presents a low value of average permeability. This fact might be due to the degradation of the membrane that occured during the trial. The solvent dissolved the active layer of the membrane, consequently the colourless fluxes began to turn yellow. The following figure in which this occurrence is presented, it is possible to see that the brownish layer was almost completely dissolved during this trial with ethanol (Figure 4.26). Although the swelling tests did not detect any undesired behaviour, when the pressure was applied, a higher polymer–solvent interaction might have led to dissolution of the polymeric membrane. The porous structure may have collapsed, changing thereby the permeability. [142]

a) membrane before the trial b) membrane after the trial; c) the permeate with the active layer dissolved

Figure 4.26. - Degradation of the membrane Duramem 200 during permeation test with ethanol.

For this reason, this membrane was no longer considered for the next steps.

Regarding the Solsep 010206 and NP 030 results, it is not possible to relate the difference of permeability with the pore size because despite the membrane Solsep 010206 possesses lower pore size, higher permeabilities were obtained. This could be explained based on interaction of solvent-membrane and the solvation of the pores: for membranes with some hydrophilic character, the hydration of the pore wall occurs, leading to a decrease on their effective pore size. [143]

Also, the compaction phenomena could be a factor of permeability variation since this step were overpassed. This might prove that NP030 is more sensitive to this phenomenon because as stated before the reorganization of the polymers affects the fluxes by higher membrane resistance.

However, no conclusion can be taken with only these tests since the extracts composition also affects the membrane efficiency.

The results of the permeability using the ethanol extracts rich in pigments and polar lipids are presented in the following figure (Figure 4.27).

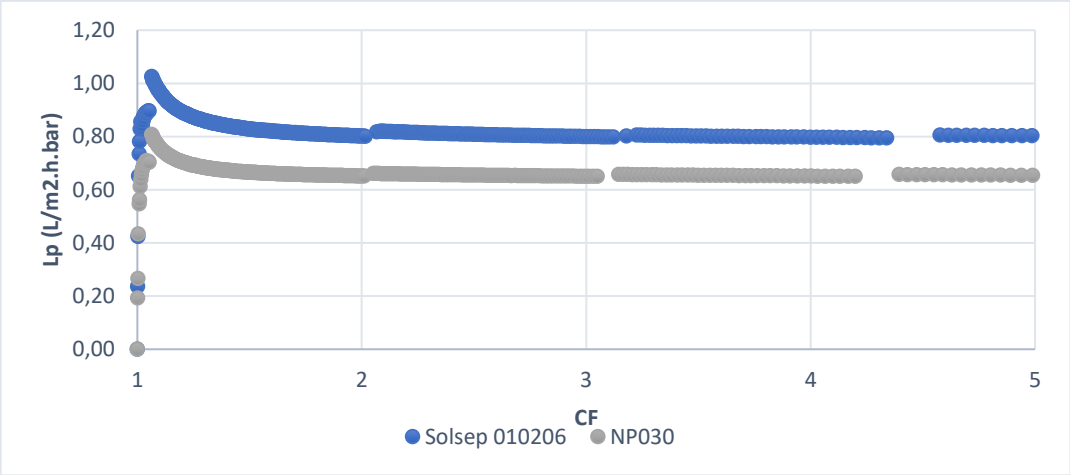


Figure 4.27. Experiments of nanofiltration using ethanol extracts with membranes Solsep 010206 and NP030. Permeability (L/m^2.h.bar) was plotted against the concentration factor, CF.

Analysing these results, it is possible to conclude that the average permeability for both membranes faced a drop to 0,83 (L/m^2.h.bar) concerning Solsep 010206 and for NP030 of 0,67(L/m^2.h.bar). This might be due to fouling phenomenon.

In order to assess if the membrane filtration technology enhances the concentration of high value compounds, the rejection coefficients for pigments and fatty acids were calculated by results of extracts analysis. These results are present in Table 4.18 and Table 4.19.

Table 4.18. Rejection coefficients calculated for chlorophyll a, pheophytin a and carotenoids regarding Solsep 010206 and NP030. The mean of CF rejection results was calculated to obtain these values. The uncertainty was calculated based on the standard deviation.

Membrane	$R_{Chlorophyll\ a}$ (%)	$R_{Pheophityn}$ (%)	$R_{Carotenoids}$ (%)
Solsep 010206	71.2 ± 3.0	64.1 ± 3.9	73.1 ± 3.2
NP 030	23.5 ± 3.7	16.5 ± 4.2	29.9 ± 3.2

Table 4.19. Rejection coefficients calculated for fatty acids regarding Solsep 010206 and NP 010206. The mean of CF rejection results was calculated to obtain these values. The uncertainty was calculated based on the standard deviation of CF rejection results.

Membrane	$R_{C16:0}$ (%)	$R_{C16:1}$ (%)	$R_{C20:5w3}$ (%)	$R_{Total\ FA}$ (%)
Solsep 010206	94.3 ± 9.4	83.8 ± 8.4	84.7 ± 8.5	87.6 ± 8.8
NP 030	4.4 ± 0.4	5.0 ± 0.5	8.9± 0.9	5.3 ± 0.5

Among both membranes, the Solsep 010206 presents the highest rejection for the all compounds analysed. At first sight, the pore size of the membranes might be the driving force for this

large difference of rejection since NP030 is constituted by larger pore size. However, the mechanism of transport of solutes through the membranes is not solely supported on size-exclusion phenomena. Actually, the solute-membrane interactions might be affected by solute characteristics such as: their size; the molecular geometry which is very different between all the solutes tested; dipole moment or Van der Waals interactions). The interactions will depend also on the membrane surface chemistry and structure[144].

For future works, relevant parameters should be optimized, such as: the membrane material, temperature, pH, ionic strength and transmembrane pressure. Also, the fluid dynamic conditions employed shall be studied for determination of the conditions for mass transfer near the membrane interface and, subsequently, the local concentration of the various chemical species present in the media [144].

Regarding the measurements to obtain these results, there is a high uncertainty associated, due to the fact that the preparation of the methyl ester derivatives of fatty acids (FAME) for analysis by gas chromatography might be affected by the presence of ethanol. Since ethyl esters will also be formed in the sample preparations, they will be also detected in the gas chromatography analysis. For that reason, the amount of sample was limited to 10% in molar mass of ethanol relative to the amount of methanol used in the procedures. Thus, the uncertainty considered was 10% of the values obtained.

4.3.5.2. Distillation

The distillation process is a very old and common separation technology for separating liquid mixtures. Since ethanol has a relative low boiling point, this unit operation should be considered for solvent recovery. Extracts rich in polar lipids and pigments were subjected to distillation process to measure the pigments degradation due to high temperatures exposure. Also, a fatty analysis was performed on the distillate sample to confirm if there would be no dragging during the process. Chlorophyll a degradation (%) of the concentrate was calculated through the equation (4.4).

The chlorophyll degradation was found to be 28,7 ± 2,6% which is quite high since this concentrate is supposed to be a final product, therefore this value will be considered for mass balances calculations. This phenomenon occurs due to high temperatures causing the loss of the central magnesium atom of the porphyrin ring of chlorophyll which is replaced by two hydrogen atoms to form pheophytin. Following this reaction, an undesirable colour change from bright green to olive brown will occur [145], which possibly would devalue this final product when processed with this unit operation.

Concerning the distillate samples, fatty acids and pigments analysis did not reveal any dragging. However, for comparisons between distillation and membrane filtration for solvent recovery, mass balances and pre-sizing of equipments must be completed.

4.4.1. Mass Balances

The operation of any facility requires the characterization of the process through mass balances. This stage aims to account for the material flow throughout the biorefinery and is therefore
4.4. essential for the realization of the technical and economic project of the facility. First, the desired annual capacity is defined. It is intended to process 270 tonnes of biomass per year. Considering that the plant operates 350 days a year for 24 hours a day, it is necessary to process 32 kg per hour. The remaining 15 days of the year are for cleaning and maintenance of equipment. For mass balances calculations, the principle of mass conservation was followed, described by the equation (4.5).

$$m_{in} - m_{out} + m_{generated} - m_{consumed} = m_{accumulated} \quad \textbf{(4.5)}$$

Where the m_{in} is the inlet mass, m_{out} is the outlet mass, $m_{generated}$ is the mass generated during a reaction, $m_{consumed}$ is the mass consumed during the reaction and $m_{accumulated}$ is the mass accumulated in the system.

This principle was applied to any defined boundary and has been applied to account for the mass flows of the overall process as well as for each unit operation of the process. In these biorefineries, there are no reactions between the compounds of the biomass, except the degradation of chlorophyll a to pheophytin a but it was assumed that the $m_{generated}$ and $m_{consumed}$ are equal, cancelling each other. In fact, the difference in molecular weight between the compounds is 2,5% and the fate of the lost magnesium cation must be determined in every unit operation. For a steady-state process the accumulation term will be zero. Thus, the equation (4.6) was considered.

$$m_{in} = m_{out} \quad \textbf{(4.6)}$$

First of all, the inlet stream per compound was calculated by the feedstock full characterization obtained in the experimental results as well as the fraction of dry weight and the facility capacity per hour (kg/h). For bead milling processing, the cell disruption efficiency calculated in the previous section was used to predict the non-disrupted cells weight.

For each unit operation, the efficiencies obtained in the last section were considered but for those in which it was not possible to test at laboratory scale, values from the literature were assumed. Table 4.20 shows the assumptions considered for biorefinery 1 (A and B) and 2 (A and B).

Table 4.20. Theoretical values assumed in the mass balances.

Biorefinery	Operation	Compound	Yield/Rejection Coefficient (%)	Source
1A and 1B	pH-shift	Proteins	81%	Experimental
1A and 1B	pH-shift	Lipids	40%	[119]
1A and 1B	pH-shift	Carbohydrates	90%	[119]
1A and 1B	pH-shift	Ashes	85%	[119]
1A and 1B	Ultrafiltration	Proteins	89%	[113]
1A and 1B	Ultrafiltration	Carbohydrates	36%	[113]
1A and 1B	Ultrafiltration	Lipids	23%	[113]
1B and 2B	Distillation	Ethanol Recovery	97%	Aspen Plus ®
2A and 2B	Ultrafiltration	< 200 Da	76%	[146]
2A and 2B	Ultrafiltration	>200 Da	28%	[146]
2A and 2B	Diafiltration 1	NaCl	40%	[126]
2A and 2B	Diafiltration 1	400-1000Da	90%	[126]
2A and 2B	Diafiltration 1	200-400Da	85%	[126]
2A and 2B	Diafiltration 1	100-200Da	50%	[126]
2A and 2B	Diafiltration 1	>100Da	20%	[126]
2A and 2B	Diafiltration 2	Proteins	100%	[147]
2A and 2B	Diafiltration 2	Monosaccharides	13%	[147]

For each biorefinery, multiple and different products streams were achieved in mass balances. In order to recognize and to aid further discussion, Table 4.21 discerns and describes each stream. In Table 4.22 and Table 4.23**,** the global balance for each biorefinery is presented.

Table 4.21. - Products description of each biorefinery. * Relative to A **Relative to B

Biorefinery 1		**Biorefinery 2**	
Stream	**Description**	**Stream**	**Description**
Product 1	Solid obtained after pH-shift	**Product 1**	Olive Oil rich TAG
Product 2	Concentrate rich in carbo-hydrates after UF	**Product 2**	Permeate after UF
Product 3	Permeate after UF	**Product 3**	Permeate after DF
Product 4	D-limonene concentrate rich in lipids after NF	**Product 4**	Permeate after DF
Product 5	D-limonene Purification	**Product 5**	Protein concentrate after DF
Product 6	Raffinate after ethanol ex-traction	**Product 6**	Raffinate after ethanol ex-traction
Product 7 *	Ethanol concentrate rich in lipids after NF	**Product 7 ***	Ethanol concentrate rich in lipids after NF
Product 8 *	Ethanol Purification	**Product 8 ***	Ethanol Purification
Product 7 **	Ethanol concentrate rich in lipids after NF	**Product 7 ****	Ethanol concentrate rich in lipids after NF

Table 4.22. Global mass balance concerning biorefinery 1A and 1B.*Relative to biorefinery 1A.** Relative to biorefinery 1B.

Compounds	Inlet (%)	Product 1(%)	Product 2(%)	Product 3(%)	Product 4(%)	Product 5 (%)	Product 6(%)	Product 7(%) *	Product 8(%)*	Product 7(%)**
Lipids (Unsaponifiable)	0.86	22.83	0.12	0.09	2.00	22.20	0.67	41.88	30.95	39.97
C14:0	0.08	2.03	0.01	0.01	-	-	-	-	-	-
C16:0	0.23	5.79	0.03	0.02	0.02	0.83	0.12	18.85	6.29	16.82
C16:1	0.24	6.16	0.03	0.02	0.08	0.52	0.07	16.66	17.80	16.74%
C18:1	0.06	1.83	0.01	0.01	1.95	21.74	-	-	-	-
C18:3	0.05	1.56	0.01	0.01	-	-	-	-	-	-
C20:4w6	0.04	1.14	0.01	0.01	-	0.002	-	-	-	-
C20:5w3	0.20	5.36	0.03	0.02	0.04	-	0.15	8.27	8.26	8.22
Chlorophyll a	0.18	-	-	-	15.27	1.25	0.09	2.13	4.76	2.52
Pheophytin a	0.02	-	-	-	65.52	38.99	-	10.32	31.94	13.55
Carotenoids	0.06	-	-	-	15.12	14.46	0.46	1.89	-	2.18
Insoluble Proteins	2.12	-	-	-	-	-	48.49	-	-	-
Soluble Proteins	1.90	24.02	0.62	0.02	-	-	-	-	-	-
Polysaccharides	0.78	16.68	1.41	0.04	-	-	-	-	-	-
Monosaccharides	0.26	5.56	0.19	0.08	-	-	-	-	-	-
Ashes	0.91	7.05	-	0.37	-	-	7.71	-	-	-
Water	87.5	-	97.54	93.30	-	-	-	-	-	-
NaCl	4.50	-	-	6.00	-	-	-	-	-	-
Impurities	-	-	-	-	-	-	14.43	-	-	-
Non-disrupted Biomass	-	-	-	-	-	-	27.82	-	-	-
Total Stream (kg/h)	32.1	0.7	5.8	24.1	0.05	6E-03	1.4	0.06	0.01	0.08
Total (kg/h)			32.1							

Table 4.23. Global mass balance concerning biorefinery 2A and 2B. *Relative to biorefinery 2A.** Relative to biorefinery 2B

Compounds	Inlet	Product 1 (%)	Product 2 (%)	Product 3 (%)	Product 4 (%)	Product 5 (%)	Product 6 (%)	Product 7* (%)	Product (%) 8*	Product (%) 7**
Lipids (Unsaponifiable)	0.86	48.91	-	-	-	-	2.37	43.60	34.45	42.23
C14:0	0.08	4.46	-	-	-	-	0.60	-	-	-
C16:0	0.23	13.15	-	-	-	-	0.21	17.56	6.02	15.83
C16:1	0.24	13.67	-	-	-	-	0.14	16.92	18.56	17.16
C18:1	0.06	3.67	-	-	-	-	0.50	-	-	-
C18:3	0.05	2.73	-	-	-	-	0.37	-	-	-
C20:4w6	0.04	2.14	-	-	-	-	0.29	-	-	-
C20:5w3	0.20	11.13	-	-	-	-	0.38	11.31	11.59	11.35
Chlorophyll a	0.18	0.03	-	-	-	-	2.00	2.79	6.41	3.33
Pheophytin a	0.02	0.06	-	-	-	-	0.25	6.09	19.34	8.07
Carotenoids	0.06	0.06	-	-	-	-	0.79	1.74	3.63	2.02
Insoluble Proteins	2.12	-	-	-	-	-	41.37	-	-	-
Soluble Proteins	1.90	-	0.20	2.28	4.67	-	-	-	-	-
Polysaccharides	0.78	-	0.20	2.26	4.65	-	-	-	-	-
Monosaccharides	0.26	-	0.20	2.19	0.02	0.76	-	-	-	-
Ashes	0.91	-	0.01	0.40	0.06	-	14.70	-	-	-
Water	87.5	-	98.04	-	90.60	-	-	-	-	-
Salt	4.50	-	1.36	92.87	-	99.24	-	-	-	-
Impurities	-	-	-	-	-	-	12.31	-	-	-
Non-disrupted Biomass	-	-	-	-	-	-	23.73	-	-	-
Total Stream (kg/h)	32.1	0.3	25.8	0.7	3.1	0.4	1.6	0.1	2E-02	0.2
Total (kg/h)		32.1								

The products 5 (biorefinery 1) and 8 (biorefinery 1 and 2) are concerning the permeate of membrane filtration and they were considered a product to avoid accumulation in mass balances. For future developments of this project, the recovery and posterior reuse of organic solvents must be investigated as it is a very important aspect to be taken into account. Usually, the application of a single stage organic solvent nanofiltration process is only possible to be applied in case of relatively easy fractionation of the compounds, so, most of the times, multiple membrane modules have to be integrated to carry out more complex separations to fulfil the technical, economic, and environmental requirements. [148]

In this project, two methods of solvent recovery were tested, and the mass balances results discern between membrane filtration (orange column) and distillation (green column). Although in lab scale there was no dragging of compounds to the distillate, in industrial scale this might not happen. Also, a possible thermal degradation of oil and pigments and an incomplete elimination of ethanol are the major disadvantages of this technology, besides the large amount of energy used in these processing steps.

In contrast, membrane filtration technology can be processed at low temperatures, preserving the components of interest, obtaining a more stable product, with higher quality. For future work, an ideal membrane should be selected combining specific properties such as high oil rejection and permeate flux, as well as thermal, mechanical and chemical resistances. [149]

In facilities design, energy balances are made to determine the energy requirements of the process: the heating, cooling and power required. In plant operation, an energy balance (energy audit) on the plant will show the pattern of energy usage and suggest areas for conservation and savings.[150] For future developments of this project, energy balances must be calculated for estimations of operation costs for concrete comparisons between these two technologies.

Regarding biorefinery 1, the EPA fatty acids seems to be extracted mostly as a form of precipitated solid in the aqueous phase after the pH-shift, as most of the other fatty acids. Despite the high rejections demonstrated by experimental results, the weight of product obtained by membrane filtration with D-limonene as solvent is low (product 4). The extraction does not seem to have the efficiency expected, since the allocation of most of the target solute was not in the path of this solvent, as it was expected (see discussion in section 4.3). Also, this product is rich in pheophytin a due to the degradation of chlorophyll a that occurs during the extraction process, demonstrated in the experimental results.

About the raffinate obtained after ethanol extraction (product 6), it is rich in insoluble proteins that were assumed to not be extracted by this solvent due to their non-polar behaviour, however for future works it is necessary to analyse this fraction to confirm these assumptions. Likewise, the components within the non-disrupted cells are not in the range of the solvent due to the cell wall barrier. During the protein analysis to the bead milling processed biomass, it was found out that only 41% is composed by proteins against 50% of the initial biomass which might mean that 9 percentage points were denaturated/destroyed during the processing or/and the foam formed during the homogenization of the samples might be rich in proteins and consequently they were not detected by the analysis. For this reason, for mass balance it was considered that this fraction is a loss during the cell disruption processing and it will not be extracted by any solvent.

Concerning biorefinery 2, it is possible to observe that the olive oil (product 2) is rich in valuable fatty acids such as EPA which might become a highly valuable and profitable product. These long chain omega-3 fatty acids provide significant health benefits to the human population, particularly in reducing cardiac diseases such as arrhythmia, stroke and high blood pressure. Also, the fact that global fish stocks are declining and cannot provide a sustainable source of omega-3 fatty acids, makes this vegetable oil which is already largely consumed more attractive and heathier. [151] The complete properties of the oil product should be determined in future, in order to characterize the possible application in the food markets.

Like in biorefinery 1, the raffinate after ethanol extraction is rich in insoluble proteins, non-disrupted cells and impurities caused by bead milling processing. Although this solid product might be interesting for animal feed since it is rich in proteins that could be a partial replacement for conventional proteins, the fact that non-disrupted cells are also present in a small fraction and the microalgal cell wall is largely indigestible by monogastric animals, it is relevant to development and search for adequate technologies to improve microalgal nutrient bioavailability in animals. [152]

4.4.2. Biorefinery Design and Economical Approach

The main objective of this step is to obtain the essential parameters for CAPEX estimation cost. Industrial plants are built to make a profit, and an estimate of the investment required is needed before the profitability of a project can be assessed.

A cost estimation (± 20-30%) is required during the feasibility stage of a design project, usually it is sufficient for the initial cost estimation study by designing the major equipment, reducing the calculation time required for more detailed estimates. Detailed (and more accurate) cost estimates are usually required after the detailed design work has been completed, including the design and sizing of all equipment, determination of pipework layouts, and specification of the control and instrumentation schemes. [153]

For a pre-project evaluation, only the major equipment was considered, including centrifuges, extraction tanks, flash distillation column and membrane filtration systems. Ancillary equipment such as process piping, and insulation needs to be estimated for future works, after the total cost of the major items is known.

Centrifuges

Centrifugation is an operation aimed at separating two phases of a liquid-solid mixture by the difference in densities between the components. This is used by the action of centrifugal force in situations where the gravitational force is not sufficient to promote separation. The efficiency of this unit operation depends on the size of the particles, the viscosity and the difference between the densities, and the efficiency is greater the larger the particles, the lower the viscosity of the liquid phase and the larger the difference in density between phases. The size of this equipment was calculated through the measurements described in Coulson & Richardson's Chemical Engineering Design [150]. In the general case, two equations (equations (4.7) and (4.8)) were applied in order to calculate the area of gravitational settler.

$$Q = 2u_g\Sigma \tag{4.7}$$

$$u_g = \frac{\Delta\rho {d_s}^2 g}{18\mu} \tag{4.8}$$

Where Q is the volumetric flow of liquid through the centrifuge (m^3/s), u_g is the terminal velocity of the solid particle settling under gravity through the liquid (m/s), Σ sigma value of the centrifuge which is the equivalent area of a gravity settler (m^2), $\Delta\rho$ is the density difference between solid and liquid (kg/m^3), d_s is the diameter of the solid particle (m), μ is the viscosity of the liquid $Nm^{-2}s$ and g the gravitational acceleration (m/s^2).

The results of are presents in Table 4.24.

Table 4.24. - Area of gravity settler required for the centrifuges. *To simplify the calculations, this centrifuge design was calculated using the same procedures.

Equipment	Σ (m^2)
2-phase centrifuge	10.7
2-phase centrifuge	10.1
3-phase centrifuge*	12.8

Tanks

For estimation costs of the equipment for extractions, only the vessels were considered. The geometric relations of the sizing of this type of equipment consists in the calculation of the total volume, considering a residence time of the extractions (experimental results), and the incoming flow rate in the tank. Considering a relation between height and radius of 3, the following equations (equations (4.9) and (4.10)) were used for design calculations.

$$V_{total} = F\tau \quad \textbf{(4.9)}$$

$$V_{total} = 3\pi r^3 \quad \textbf{(4.10)}$$

Where V_{total} is the total volume of the tank, F is the inlet flow rate, τ is the residence time and r is the radius.

Table 4.25. Size of the tanks required for solvent extraction and pH-shift technique.

Biorefinery	Equipment	Radius (m)	Height (m)
1A and 1B	pH-shift tank	0.25	0.76
1A and 1B	D-limonene extraction	0.46	1.4
1A and 1B	Ethanol extraction	0.48	1.4
2A and 2B	Ethanol extraction	0.51	1.5

Membrane Filtrations

Experimental testing is generally needed in the scale-up and design of conventional and cross-flow filtration systems, especially when organic solvents are involved. The starting point for the calculations is data on flux rates and rejections for various membranes as a function of concentration and other parameters such as temperature, transmembrane pressure and crossflow velocity. However, since in the present work it was not possible to collect this data by experimental testing, theoretical values were assumed [154]. The membrane area required was the parameter

to evaluate the investment cost as well as the nature of the membrane, for instance for the membrane required to process the extract composed by D-limonene needs to be solvent resistant. The concentration factor considered for all of the filtrations was 5. Through the equation (4.11), the required area of membrane was calculated and the resulted are exposed in Table 4.26.

$$A = \frac{V_o - V_F}{\mathrm{T} J_{avg}} \tag{4.11}$$

Where V_o is the initial volume, V_F is the final volume (V_o/CF_F while CF_F is the concentration factor), T refers to processing time and J_{avg} the average flux rate.

Table 4.26. Membrane area required for filtrations processing.

Biorefinery	Type of system	Membrane Area (m^2)
1A and 1B	Ultrafiltration	0.42
1A and 1B	Nanofiltration	0.73
1A	Nanofiltration	0.43
2A and 2B	Ultrafiltration	0.32
2A and 2B	Diafiltration	0.20
2A and 2B	Diafiltration	0.19
2A	Nanofiltration	0.55

Flash Distillation

The sizing of this equipment is influenced by the properties of the gaseous phase, more precisely its diameter, which should be large enough to slow the rate at which the particles are being deposited. The following equations (equations (4.12) and (4.13)) were used to calculate the required parameters to estimate a cost. [150]

$$u_t = 0{,}07 \left(\frac{\rho_L - \rho_v}{\rho_v}\right)^{\frac{1}{2}} \tag{4.12}$$

Where u_t is the settling velocity (m/s), ρ_L is the liquid density (kg/m^3) and ρ_v vapour density (kg/m^3).

$$D_v = \sqrt{\frac{4V_v}{\pi u_s}} \tag{4.13}$$

Where D_v is the minimum vessel diameter (m) and V_v is the vapour volumetric flow-rate (m3/s). $u_s = u_t$ if demister pad is used and $0{,}15u_t$ for a separator without a demister pad.

To obtain the physical properties required for these calculations, a simulation in the software Aspen Plus ® were performed. As the feed rate is low comparing to common chemical industries, the minimum diameter of the separator is 0,14 m, thus, for cost estimation, the smaller diameter was considered.

Economical Approach

For each biorefinery, a cost estimation based on the pre-sizing of the major equipment was performed (Table 4.27). From the economical point of view, the biorefinery 2 shows lower CAPEX than biorefinery 1 due to the high costs of centrifugal equipment. Since the density of marine microalgae varies from 1030 to 1100 kg/m3 and as result it is required a larger area of gravitational settlement because of the identical densities of the cell and the medium. [155]

As expected, the membranes resistant to organic solvents presents higher CAPEX than the conventional ones. The fact that in biorefinery 1 was proposed two extractions using organic solvents also contributes to the higher CAPEX when compared to biorefinery 2.

As shown in Table 4.27, the estimated CAPEX for ethanol recovery by membrane filtration and distillation is equal. Although more investigation is required to confirm which technology is the most suitable for this biorefinery paths, the membrane technology seems to be more attractive due to the lower operation costs associated when compared with distillation.

Table 4.27. Main economical results of the total CAPEX required for both biorefineries.

Biorefinery 1					**Biorefinery 2**				
Equipment	Flow rate (kg/h)	Specification	**CAPEX (€)**	Source	**Equipment**	Flow rate (kg/h)	Specification	**CAPEX (€)**	Source
2-phase centrifuge	32	Gravitational Area = 2.16 m^2	**95000**	[153]	**3-phase centrifuge**	39	Gravitational Area = 2.5 m^2	**111000**	[156]
Tank	31	Volume = 0.015 m^3	**2500**	A4F	**Ultrafiltration System**	30	Membrane area = 0.321 m^2	**22000**	A4F
2-phase centrifuge	31	Gravitational Area = 5.1 m^2	**155000**	[156]	**Diafiltration System 1**	26	Membrane area = 0.204 m^2	**22000**	A4F
Ultrafiltration System	30	Membrane area = 0.42 m^2	**22000**	A4F	**Diafiltration System 2**	21	Membrane area = 0.194 m^2	**22000**	A4F
Extractor 1	20	Volume = 0.095 m^3	**55000**	[156]	**Spray Drying 1**	22	-	**50000**	A4F
Extractor 2	20	Volume = 0.103 m^3	**56000**	[156]	**Spray Drying 2**	3	-	**20000**	A4F
Nanofiltration system 1	19	Membrane area = 0,.73 m^2	**25000**	A4F	**Extractor**	25	Volume = 0.125 m^3	**60000**	[156]
Nanofiltration system 1	19	Membrane area = 0.43 m^2	**25000**	A4F	**Nanofiltration system 1**	23	Membrane area = 0.55 m^2	**25000**	A4F
Distillation	19	Minimum diameter = 0.14m	**5000**	A4F	**Distillation**	23	Minimum diameter = 0.14m	**25000**	A4F
Total CAPEX			435500*		**Total CAPEX**			332000*	

Figure 4.28 shows a detailed cost analysis, where the major equipment necessary for a biorefinery counts less than 5% of the total costs in projections made for different markets in South

Spain. Thus, to a concrete comparison between the two biorefineries, the OPEX needs to be also studied in future work.

Concretely, analysing the food additives cost analysis regarding the target market considered in this project, the consumables (membranes) and solvents represent a large part of the OPEX. However, since in the last 20 years the membrane filtration technologies have been widely investigated, new technologies as low-fouling, reversible spiral and capillary ultrafiltration have been set up and developed, improving the life-time of membranes and cleaning efficiency.

In summary, despite the long road ahead to reach maturity concerning the possible paths of biorefinery, it was shown that valuable products can be achieved with green and sustainable technologies.

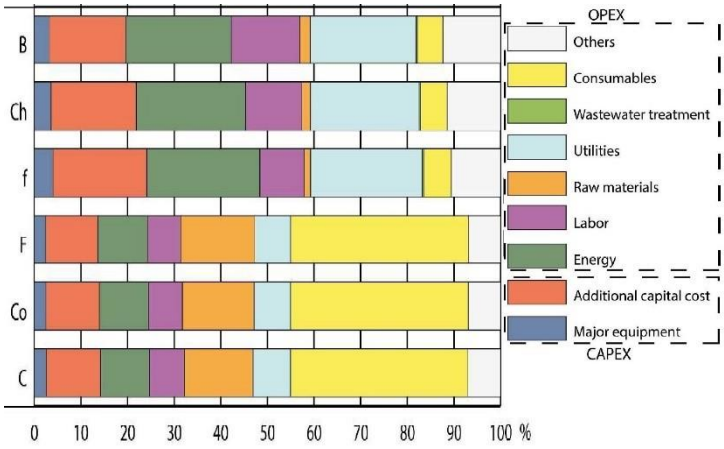


Figure 4.28. Cost analysis for projections on biorefineries in South Spain. B: Biofuels, Ch:Chemicals, f:Food-feed, F:Food aditives , Co:Cosmetic-Health care, C:complete biorefinery. Adapted from [6]

Chapter 5

Conclusions

For *Nannochloropsis* sp. biorefinery, several solvents were found in the literature to extract lipids and pigments. However, in order to improve products and systems from an environmental protection perspective and targeting food safety issues, D-limonene, olive oil and ethanol were tested on lab scale to study their efficiency.

First of all, the detailed biochemical characterization of this genus was performed, and it was found that nearly 3% of the total biomass dry weight is composed by EPA fatty acids, a valuable product highly demanded for its benefits for human health.

The method to disrupt the cell selected for both biorefineries was the bead milling processing which as studied in the literature, can achieve an efficiency of above 95% and has several parameters to optimize. However, in this work, through pigment analysis, the cell disruption efficiency was found to be nearly 85% when processed by two passes. These results might be due to the utilized method, since pigments release was found to be slower than the biomass disintegration, as chlorophyll lies within the chloroplast (a compartment within the cell compartment) and on the other hand, when the carotenoids degrade, they are not detected by the method used, which means these calculations may not be the most appropriate methodology. In the photos taken during the trials, there are very few non-disrupted cells visible after two passes in the equipment, which suggests that the efficiency might be near 100%, altough rupture of chloroplasts is also difficult to observe with this technique.

The temperature of operation in the bead milling was also tested at 8ºC and room temperature and it does not seem to enable substantially significant pigment degradation when using the higher temperature. Thus, it would be appropriate to process microalgae biomass at room temperature in order to save resources.

Concerning biorefinery 1, the EPA fatty acids seem to be extracted mostly as a form of precipitated solid in the aqueous phase after the pH-shift. Because of their allocation in the membrane, they are mainly present in the form of polar lipids, and an association with proteins is expected to occur through the cytoskeletal lattice and, therefore, not be separated at the centrifugation step. Also, the stratification of the precipitate confirmed that it was not composed only by proteins. There is also a pending question as to whether the bead milling process in the presence of aqueous culture medium could promote the hydrolysis of polar lipids during the process, and resulting free fatty acids could display the same behaviour.

Despite being a non-polar solvent, PUFAs were detected in the fatty acids analyses in the extracts using D-limonene. This can be due to the slightly more polar nature of this terpene when compared with other solvents or/and the presence of water in the bead milling processing might set off lipid hydrolysis, consequently the free fatty acids might lose their polar moiety and it would be easier to dissolve in this solvent. Membrane filtration using this solvent was also tested and Puramem 600 was found to be promising as a solvent resistant nanofiltration membrane with rejection coefficients of above 90% for pigments and 73% for EPA fatty acids (in unknown lipid form, i.e., unknown molecular weight and polarity).

The partition coefficient of ethanol was found to be near 6 but without the fully understanding of the lipids path in the biorefinery, this value might not be realistic, therefore, a complete prediction of partitioning taking into account all the possible reactions of lipids, can lead to further developments in design, scaleup and process optimization of this process.

Membrane filtration was also studied using ethanol and the coefficients of rejection obtained were high when processed with Solsep 010206, with above 70% for chlorophyll a and carotenoids and nearly 85% for EPA fatty acids (again in unknown lipid form, i.e., unknown molecular weight and polarity). To compare with this technology, a distillation was performed and, although in lab scale there was no dragging of compounds to the distillate, in industrial scale this might happen and would require investigation. Also, the thermal degradation of oil and pigments and an incomplete elimination of ethanol are the major drawbacks of this technology.

Regarding biorefinery 2, only the tricanter operation was tested by adding olive oil to the disrupted cells suspension. This solvent extracted 56% of EPA fatty acid from the available fatty acids present in the initial biomass. This extract can be defined as “flavoured oil” or “gourmet oil” since it is enriched with high value fatty acids.

Biomass/solvent ratios were tested in the three extraction trials and by pigments analyses, it was discovered that the best ratios were 1:12 for D-limonene extractions, 1:16 for ethanol and 5:1 for olive oil. However only two trials for each were performed, and in order to truly understand the potential of these solvents, other ratios should be tested to fine-tune the parameter.

A cost estimation of CAPEX of each biorefinery (± 20-30%) was performed by pre-designing the major equipments, and biorefinery 2 was found to have a lower cost. This result combined with the highly profitable olive oil extract makes this proposal a promising route to achieve a viable biorefinery.

Future research must pursue two major points: to increase the efficiency of the extraction using the solvents tested by fully understanding the behaviour of the most valuable compounds and develop novel technologies or cheaper filtrations systems (membranes) for fractionation of compounds.

Concerning the economical approach, the next stage in evaluating and comparing the profitability of the biorefineries products is to compare the total cost of production (for example, per tonne produced) with the current market price. It is also necessary to estimate future demand for the product, and to determine the trend in the selling price over several previous years. The operating costs by energy balances should also be considered when the process routes are being evaluated, and they can significantly influence the final choice that is made.

In summary, the results obtained reveal very promising first steps in key process sections for potential complete *Nannochloropsis* microalgal biorefineries. However, future work is still needed to consolidate the generated know-how.

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ANNEXES