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ANA MARGARIDA FERREIRA MARTINS

BIOREFINARIA MARINHA: VALORIZAÇÃO DE ALGAS PARA PRODUTOS DE VALOR ACRESCENTADO

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Engenharia Química, realizada sob a orientação científica da Professora Doutora Sónia Patrícia Marques Ventura, Professora Auxiliar do Departamento de Química da Universidade de Aveiro, e do Professor Doutor João Manuel da Costa e Araújo Pereira Coutinho, Professor Catedrático do Departamento de Química da Universidade de Aveiro.

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Ao núcleo mais forte.

o júri

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agradecimentos

"We rise by lifting others." – Robert Ingersoll. And I was definitely lifted by others… to those I want to thank.

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palavras-chave

Biorefinaria marinha, algas, pigmentos, clorofilas, carotenoides, ficobiliproteinas, processamento a jusante, solventes alternativos

resumo

O paradigma do consumo está a mudar. Os consumidores estão mais atentos ao que consomem e a questões relacionadas com a sustentabilidade desses produtos. Esta mudança foi motivada pelo cuidado de saúde associado ao consumo de produtos naturais, mas também pela necessidade de implementar recursos e processos mais sustentáveis como forma de combater as mudanças climáticas, sem comprometer a qualidade e o preço do produto final. Os pigmentos são uma das classes de compostos que podem ser obtidos a partir de biomassa, sendo reconhecidos pelas suas inúmeras atividades biológicas. Pelas suas propriedades espectrais e biológicas, os pigmentos podem ser aplicados em diversos setores de atividade que vão desde a alimentação humana, à cosmética, nutracêutica e medicina. Contudo, as metodologias convencionais para obtenção de pigmentos exigem frequentemente o uso de equipamento dispendioso e/ou o uso de solventes orgânicos considerados tóxicos. Nesta tese, processos de extração e purificação alternativos para recuperar os pigmentos de algas foram desenvolvidos e propostos. Metodologias simples e eficientes, maioritariamente baseadas em solventes aquosos, foram usadas para obter um ou mais pigmentos de uma mesma alga. Em todos os capítulos apresentados, um conjunto de solventes foi testado assim como foram estudadas as condições operacionais de forma a atingir os melhores rendimentos e/ou purezas. Soluções aquosas de líquidos iónicos tensioativos mostraram um enorme potencial, sob várias perspetivas, na obtenção de pigmentos hidrofóbicos tais como clorofilas e carotenoides. Por outro lado, a precipitação induzida de proteínas provou ser uma boa alternativa na precipitação seletiva (e consequente purificação) de ficobiliproteinas. Os impactos económicos e ambientais foram estudados e resultados encorajadores foram obtidos de ambos as perspetivas, levando-nos a acreditar no sucesso da implementação industrial de alguns dos trabalhos apresentados.

keywords

Blue biorefinery, algal biomass, pigments, chlorophylls, carotenoids, phycobiliproteins, downstream processes, alternative solvents

abstract

The consumption paradigm is changing. Consumers are more attentive to what they consume and to the sustainability questions addressed to those products. This change was driven by the healthy label given to natural products but also by the urgent need to find more sustainable sources of raw materials and to implement processes as a way to mitigate climate changes, without compromising the quality and affecting the price of the final product. Pigments are compounds that can be obtained from various sources of biomass, being recognized by their numerous biological activities. Due to their spectral and biological properties, pigments can find application in many fields, from food to cosmetics, nutraceutics, and medicine. However, conventional methodologies to obtain pigments demand very often the use of costly equipment and/or toxic organic solvents. In this work, alternative methodologies to extract and purify pigments from algae of were developed. Simple and efficient methodologies mostly based on aqueous solvents were used to recover one or more pigments from algae within a blue biorefinery framework. In all chapters, a variety of solvents were screened, and operational conditions were studied and optimized in order to reach high yields of extractions and/or purities. Aqueous solutions of tensioactive ionic liquids have shown great potential for the recovery of hydrophobic pigments such as chlorophylls and carotenoids. In the other hand, induced precipitation of proteins is shown to be a good alternative for the selective precipitation (and further purification) of phycobiliproteins. Economic and environmental impacts were assessed and encouraging results were obtained from both perspectives, leading us to believe in the potential for the industrial implementation of some of the results here obtained.

List of Abbreviations

ANOVA	Analysis of variance
BCA	Bicinchoninic acid
CCRD	Central composite rotatable design
Chl	Chlorophyll
CMC	Critical micelle concentration
C-PC	Phycocyanin from cyanobacteria
DAD	Diode array detector
fuco	Fucoxanthin
GENIALG	GENetic diversity exploitation for Innovative Macro-ALGal biorefinery
HPLC	High performance liquid chromatography
IL	Ionic liquid
LC-MS	Liquid chromatography coupled to mass spectrometry
LED	Light-emitting diode
LSC	Luminescent solar concentrator
MS^2	Tandem mass spectrometry
PEG	Polyethylene glycol
PPG	Polypropylene glycol
R-PC	Phycocyanin from red macroalgae
R-PE	Phycoerythrin from red macroalgae
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLR	Solid-liquid ratio
UF	Ultrafiltration
UHPLC-MS	Ultra-high performance liquid chromatography coupled to mass spectroscopy
UV	Ultraviolet
UV-Vis	Ultraviolet visible

List of Symbols

$\$_{biom}$	Cost associated with the acquisition of biomass
$\$_{prod}$	Commercial market price of a certain product
$\%_{IL}$	Volume fraction in percentage of the aqueous solution of ionic liquid in solution
$\%_{oil}$	Volume fraction in percentage of oil in solution
$^1O_2$	Singlet oxygen
Abs_{280}	Absorbance at 280 nm
Abs_{412}	Absorbance at 412 nm
Abs_{565}	Absorbance at 565 nm
Abs_{617}	Absorbance at 617 nm
C_{IL}	Concentration of ionic liquid in water
CO_2 eq	Carbon dioxide equivalent
CoG	Production cost
C_{prod}	Amount of product in the biomass
EU	Euro
Φ_Δ	Quantum yield of singlet oxygen
K	Partition coefficient
K_{ow}	Octanol-water partition coefficient
Ln (A_0/A)	Napierian logarithm of the ratio of the initial absorbance and the absorbance at a certain time
Log_{Kow} OR LogP	Logarithm of the octanol-water partition coefficient
m/z	Mass-to-charge ratio
miLogP	LogP prediction developed at Molinspiration
n	Number of variables
NMVOC eq	Non-methane volatile organic compounds equivalent
ºC	Degree celsius
oil eq	Oil equivalent
p	Pellet
P	Permeate
p	p-value

R	Retentate
Rp	Resuspended pellet
rpm	Rotation per minute
S	Supernatant
SO_2 eq	Sulfur dioxide equivalent
t	Time
US $	United States dollar
v:v	Volume fraction
V1, V2, V3	Experimental values in response surface methodology for validation of the optimum conditions
w:v	Weight/volume fraction
wt %	Weight Fraction percentage
X1, X2, X3, X4	Independent variables in the response surface methodology
Y	Dependent variable in the response surface methodology
α	Multiplier of the production costs
α, β, γ	Subunits of phycobiliproteins
α-helix	Alpha helix in secondary structure of proteins
β_0	Intercept
β_1	R-PE content
β_2	Overall recovery yield
β_3	Materials cost
β_4	Process duration
β-sheets	Beta sheets in secondary structure of proteins
θ	Ellipticity
λ	Wavelength
λ_{max}	Wavelength of maximum absorbance

List of Chemicals

$(NH_4)_2SO_4$	Ammonium sulfate
$[C_{10}C_1im]Cl$	1-decyl-3-methylimidazolium chloride
$[C_{12}C_1im]Cl$	1-dodecyl-3-methylimidazolium chloride
$[C_{14}C_1im]Cl$	1-methyl-3-tetradecylimidazolium chloride
$[C_{16}C_1im]Cl$	1-hexyl-3-methylimidazolium chloride
$[C_{16}py]Cl$	Hexadecylpyridinium chloride
$[C_2C_1im]Cl$	1-ethyl-3-methylimidazolium chloride
$[C_4C_1im]Cl$	1-butyl-3-methylimidazolium chloride
$[C_6C_1im]Cl$	1-hexadecyl-3-methylimidazolium chloride
$[C_8C_1im]Cl$	1-methyl-3-octylimidazolium chloride
$[N_{1,1,1,10}]Br$	Decyltrimethylammonium bromide
$[N_{1,1,1,10}]Cl$	Decyltrimethylammonium chloride
$[N_{1,1,1,12}]Br$	Dodecyltrimethylammonium bromide
$[N_{1,1,1,14}]Br$	Tetradecyltrimethylammonium bromide
$[N_{1,1,1,16}]Br$	Hexyltrimethylammonium bromide
$[N_{1,1,1,2(OH)}]Cl$	Cholinium chloride
$[N_{1,1,1,6}]Br$	Hexadecyltrimethylammonium bromide
$[N_{1,1,1,8}]Br$	Octyltrimethylammonium bromide
$[N_{4,4,4,4}]Cl$	Tetrabutylammonium chloride
$[P_{4,4,4,14}]Cl$	Tributyltetradecylphosphonium chloride
$[P_{4,4,4,4}]Cl$	Tetrabutylphosphonium chloride
Brij L4	Polyethylene glycol dodecyl ether
EO	Ethylene oxide
NaOH	Sodium hydroxide
NaPA 1200	Poly(acrylic acid) sodium salt with average molecular weight of 1200 $g.mol^{-1}$
NaPA 8000	Poly(acrylic acid) sodium salt with average molecular weight of 8000 $g.mol^{-1}$
PEG 10000	Polyethylene glycol with average molecular weight of 10000 $g.mol^{-1}$
PEG 800	Polyethylene glycol with average molecular weight of 800 $g.mol^{-1}$

Pluronic L81	Polymer composed of PEG-PPG-PEG-blocks with approx. 2800 $g.mol^{-1}$
Pluronic P 17R4	Polymer composed of PPG-PEG-PPG-blocks with approx. 2700 $g.mol^{-1}$
Pluronic P123	Polymer composed of PEG-PPG-PEG-blocks with approx. 5800 $g.mol^{-1}$
Pluronic PE 6200	Polymer composed of PPG-PEG-blocks with approx. 2450 $g.mol^{-1}$
Pluronic PE 6400	Polymer composed of PPG-PEG-blocks with approx. 2900 $g.mol^{-1}$
Pluronic PE 6800	Polymer composed of PPG-PEG-blocks with approx. 8000 $g.mol^{-1}$
PO	Propylene oxide
PPG 400	Polypropylene glycol with average molecular weight of 400 $g.mol^{-1}$
SDS	Sodium dodecyl sulfate
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
Triton X-114	Polyoxyethylene(8) octylphenyl ether
Tween 20	Polyethylene glycol sorbitan monolaurate
Tween 80	Polyethylene glycol sorbitan monooleate
Zn(II)chlorin-e6	Zinc(II) complex of chlorin e6

List of Figures

List of Tables

Index

CHAPTER 1. Introduction

1.1. General introduction

Probably as far as History goes, plant-derived biomass (and biomass extracts) have been used not only as human food, but also as sources of bioactive ingredients for cosmetics, perfumes, medicines, dyes, energy and building materials, being all biomass segments used according to their potential. Along with the tremendous growth of the petroleum era and development of petroleum-based processes and products, many of the older processes were replaced, creating a huge number of solutions, services, products and opportunities, in larger scale and lower times. However, this linear economy strategy, that starts (most of the times) with fossil-based feedstocks and relies on reagents and wastes that are often highly reactive and toxic,[1] represents a huge global problem to human health, climate changes, and the depletion of fossil resources.[2]

The concerns about climate change are not new. There are international deals and policies which intend, by 2050, to reduce to zero the greenhouse gas emissions from the European Union, which is still far from becoming a reality.[3] The increase of human population, as well as their demands for products and services, make the challenges previously mentioned even more challenging. It is clear the need of disruptive technologies developed by industry and academia that will lead the world back to biomass-based processes without giving up from the current performance.[1] An approach to achieve a regenerative economy, based on natural preservation, an economy that increasingly promotes the conversion and valorisation of residues into products with added value, following a circular economy strategy.[4] This concept intends to close of the loops, generating revenue streams from materials that were no longer valuable and reducing the consumption of raw materials towards a more sustainable future (Figure 1.1.1).[1,4]

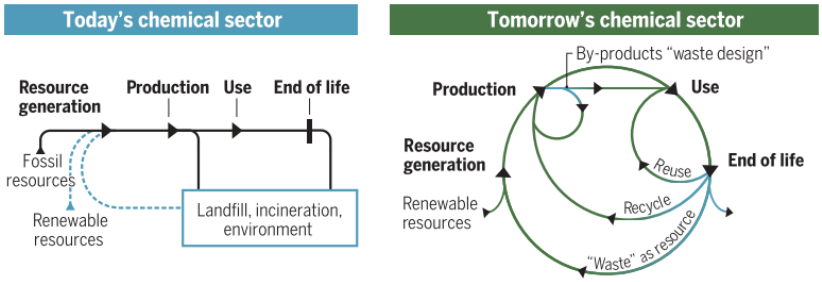


Figure 1.1.1. Schematic representation of linear (today's) chemical sector and circular economy (tomorrow's chemical sector).[1]

To develop greener and more sustainable processes, all incomes and outcomes should be taken into consideration as a whole, and best practices should be adopted.[5] This includes as income: the raw material, the energy consumption, the solvent and as outcome wastes and by-products, the extract, and the process itself. Briefly, the raw material should be natural and renewable and should not be over-exploited. The solvent, if needed, should be mainly composed of water, natural, of natural origin, based on renewable or agro-sourced materials, should be safe, non-toxic, and non-allergenic. The energy consumption should be minimized (through process optimization or reduction of operational units, for example) and reused as much as possible. The process should be safe, robust, and controlled, should provide high yields and high-quality extracts, being less time consuming, with less economic and environmental impacts. The extract should be functional, safe, and of high-quality, keeping the native molecular structure. The wastes should be reduced to its maximum and all products should be seen as by-products through the development of pathways for the complete valorisation and exploitation of a raw material, under the biorefinery framework.[2]

Biorefinery is a key tool towards the development of a circular economy and is itself circular by nature. The term ''biorefinery'' derives from the classical petroleum refinery concept[6] and refers to biomass conversion to obtain energy/biofuels and high-value products through processes and equipment for biomass transformation.[7] It is considered one of the most promising ways to create a biomass-based industry and has been spotlighted as an alternative solution to escape from the non-renewable and strong fossil-based economy. Considering the renewability, amount, and diversity of biomass available, there is a clear opportunity to develop processes to generate new products with industrial interest.[6] Besides, a biorefinery should follow a cascade-based

strategy, in which there is a maximization of the use of the biomass and the minimization of wastes. In this "resource-efficient" policy there are the pre-treatment and separation of biomass components in a "primary biorefinery" and the subsequent conversion ("secondary biorefinery") up to the end products.[8] To reach a maximum economical valorisation of a biomass, it is important to know the size market and their value for each set of products (Figure 1.1.2). Although the market demand for energy production is high, their economic value is very low, at least when compared to products with application in the pharmaceutical and cosmetic sectors or as fine chemicals.[8] This means that all procedures should be considered in an integrated process in which all the valuable compounds present in the biomass should be recovered, being the remaining biomass used for energy production.[9]

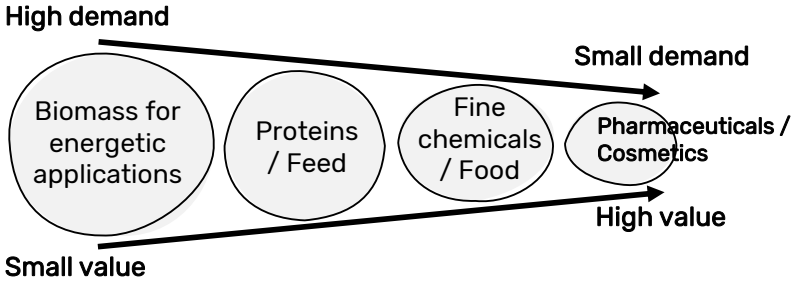


Figure 1.1.2. Market value *versus* market size of products.[8]

Most of the biodiversity on Earth is found in the oceans, oceans that represent most of the Earth's surface. As consequence, an incredible number of different bioactive substances can be found,[10] from those traditionally used as human food to conventional medicines used since ever.[11] Over the last decades there has been a change in the market trends with consumers showing an increasing interest in healthy and more natural-based (functional) ingredients.[12] Along with the need of changing the world's economy, initially mentioned, this trend has forced researchers and industries (often in collaboration) to explore new opportunities for the development of novel products derived from a sustainable biomass production from non-traditional sources.[11,13] Indeed, marine resources are one of the pillars of the *Agenda 2030* for Sustainable Development, also included in the Horizon Europe program for 2021-2027.

Within the ocean, algal strains are characterized by high biodiversity with outstanding potential for many purposes.[10] Like plants, algae first need sunlight, carbon dioxide, and water so that they can convert the sunlight energy into chemical energy during

photosynthesis, being this feature transversal to all these organisms.[14,15] Algal organisms are divided in two main groups: macroalgae, also known as *seaweeds*, which are multicellular organisms that can reach 60 meters in length; and microalgae, known by their microscopic size, but also very rich in different secondary metabolites and bioactive compounds.[16] Additionally, cyanobacteria is a group of photosynthetic bacteria which is prokaryote, unlike the common macro- and microalgae which are eukaryotes. Although some controversy exists due to the assumed restraint for the term algae to eukaryotes, some authors also name cyanobacteria as *blue-green algae*, since they have an autotrophic mode of growth (common to eukaryotic plant cells).[17] For this reason, throughout this work cyanobacteria will be included under the general umbrella of *algae*.

Algae have many potential advantages compared to plants because of their faster growth rates and the possibility of cultivation on non-arable land areas, lakes, or oceans.[18] The use of algae is spread worldwide and under different forms and applications, being in the past essentially used as animal feed, human food, or fertilizer. More recently, from the technological point of view, algae have been used as sources of colourants, phycocolloids, thickening formulants, and gelling agents in food industry.[19] Nowadays, its exploitation is seen as a potential source of new chemicals, many of which with biological activities already recognized that can be applied in pharmaceutical, nutraceutical, and cosmetic formulations.[11] Among *added-value compounds* that can be recovered from algae, pigments, terpenoids, proteins, sulfated polysaccharides, agar, alginate, and polar and non-polar lipids are some examples with high industrial applicability.[20] Included in the pigments class are the carotenoids, chlorophylls, and phycobiliproteins.[21] These pigments are indispensable for the photosynthesis to occur, and their specific content in each macroalga allow them to be distinguished in several groups.[22] The green colour of green macroalgae results from the content of chlorophylls *a* and *b*, the brown colouration of brown macroalgae is related with the dominance of carotenoids, while the red macroalgae are characterized by the presence of reddish compounds, the phycobiliproteins.[22]

In the photosynthetic organisms, chlorophylls play an essential role, since they allow those organisms to live thought photosynthesis processes. Chlorophylls are part of reaction centers responsible for the light harvesting, electron transference, protection

against light induced oxidations, and regulation of the construction of the photosynthetic apparatus.[23] Chlorophylls mainly absorb light in the blue and red electromagnetic spectrum, and poorly absorb the green part of the spectrum, being the green colour reflected which gives the characteristic green colour to chlorophyll-containing cells.[24]

There are more than one hundred chlorophyll-like structures that share the same skeleton. The most common chlorophyll structures are composed of a porphyrin ring, four nitrogen atoms with a magnesium atom chelated (Mg^{2+}) at the center and a hydrophobic phytol tail, making them fat-soluble pigments. The types that more often occur in nature are chlorophyll *a* and *b.* These two molecules differ in their structure, being that chlorophyll *a* and chlorophyll *b* contains a methyl group ($-CH_3$) and a formyl group (-CHO), respectively, attached to one pyrrole ring. There are other chlorophyll-like molecules that frequently occur such as pheophytin (free of the central magnesium), chlorophyllide (free of the phytyl group), pheophorbide (free of both central magnesium and phytyl group), among other derivatives that result of adding or deleting groups or atoms from the initial structure.[23]

Chlorophylls have been used as food additives, however, more interesting applications have been proposed, namely for pheophorbide and phytochlorin (other chlorophyll derivatives) in photodynamic therapy, imaging, solar energy conversion, and hydrogen production. In terms of biological activities, chlorophylls and their derivatives have been pointed out as antimutagenic, chemopreventive, antioxidant, anti-inflammatory, and gut microbiota regulator, which significantly increase their potential in high-end applications, and thus the interest in their extraction and purification.[25]

Carotenoids are pigmented molecules, mainly synthesized by plants, algae and microorganisms that are involved in photosynthetic machinery and play a key role in photo-protection against damage. Although carotenoids are not produced by humans, their consumption plays an important role as precursors of vitamin A.[26] Carotenoids belong to the category of tetraterpenoids and their colour can vary from brown, red, orange, and yellow. These are usually lipophilic due to the presence of long unsaturated aliphatic chains and can be divided into two major groups, namely carotenes and xanthophylls. Carotenes are strongly non-polar hydrocarbons, such as α-carotene, β-carotene, and lycopene, while xanthophylls contain oxygen atoms giving them a higher

polarity, being here listed as examples the lutein, zeaxanthin, astaxanthin, fucoxanthin, and peridinin.[26] Carotenoids are known by their antioxidant function (and prevention of oxidative stress), immune response, and pro-vitamin A activity, that can work in the prevention of different types of cancer as well as in the prevention of cardiovascular, gastric, and eye related diseases.[27,28]

Due to their similar polarity and location within the cells, chlorophylls and carotenoids are commonly extracted using the same solvents which are, most often, organic solvents. Among them, ethanol, acetone, methanol, dimethyl sulfoxide, dioxane, dimethylformamide, and hexane are the most used for the extraction of these two types of pigments.[24,29] Given the high volatility of most of the solvents described, and the (eco)toxicity and negative impacted towards human health and environment from many of them, restrictions to their use, or even their elimination from some processes, have been imposed.[2] Additionally, the fact that chlorophylls and carotenoids are extracted with the same solvents leads to a lower selectivity of the extraction and requires the development of further steps of purification. Conventionally, chromatographic techniques[30,31] and saponification reactions are applied.[32–35] However, the complexity of the systems, the need of specific equipment, and the multiple steps needed to obtain pure compounds seem to be economically significant, leading to high energy consumption and costly processes. Lastly, low purity levels are often achieved when the simplest processes are applied, or low yields of pigments are obtained when the most complex methods are employed.[24,30,36–38]

Phycobiliproteins are the last class of pigments to be considered in this work, and unlike chlorophylls and carotenoids, phycobiliproteins are water-soluble coloured proteins. Phycobiliproteins, which include phycoerythrin, phycocyanin, and allophycocyanin, are the major class of pigments found in red macroalgae and can also be found in microalgae and cyanobacteria.[39] These fluorescent proteins are predominant light-harvesting protein complexes organized *in vivo* in supramolecular structures called phycobilisomes, located in the stroma of the cells (Figure 1.1.3 A).[40] The presence of phycobiliproteins in some organisms allows the transfer of light energy in spectral zones that cannot be used by chlorophyll *a*, thus allowing the photosynthesis and the survival of living organisms even at low light intensities.[40] The phycobilisome works as an energetic funnel, allowing the energy transfer through chromophores to the reaction centers.[41]

All phycobiliproteins have the same monomer as basic unit, composed of α and β subunits. Each monomer can carry either one, two, or three chromophores, depending on the molecular species. In the specific case of phycobiliproteins, the chromophores are called phycobilins, which are open-chain tetrapyrroles. Depending on the phycobiliprotein, different phycobilin combinations may occur leading to their specific spectral and optical identity:[42] phycoerythrin with maximum absorption wavelengths (λ_{max}) ranging between 490 and 570 nm (with a three-peak absorption maxima at 565, 539, and 498 nm);[43] phycocyanin (λ_{max} = 610-620 nm), allophycocyanin (λ_{max} = 650-655 nm), and phycoerythrocyanin (λ_{max} = 560-600 nm), as depicted in Figure 1.1.3 B.[44] Giving that, phycobiliproteins differ in the amino-acid sequence, number of chromophores *per* subunit and type of chromophores.

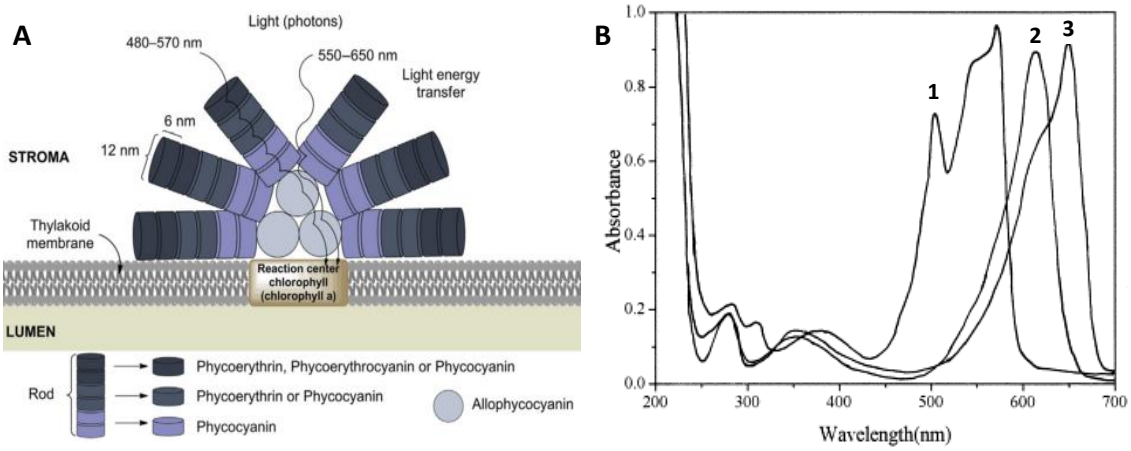


Figure 1.1.3. (A) Schematic representation of phycobiliproteins in a common phycobilisome in red macroalgae[40] and (B) Absorption spectra of (1) phycoerythrin, (2) phycocyanin, and (3) allophycocyanin from *Porphyra yezoensis* in water.[45]

Phycoerythrin is the main pigment found in red macroalgae. Depending on the species, different forms of phycoerythrin can occur: R-phycoerythrin (R-PE) for Rhodophyta or red macroalgae; B-phycoerythrin for Bangiales; and C-phycoerythrin for Cyanobacteria.[40,46] R-PE is generally composed of $(\alpha\beta)_6\gamma$ complexes (α, 18–20 kDa; β, 19.5–21 kDa; and γ, 30 kDa),[44] with a total molecular weight around 240 kDa. The increment of the γ subunit in R-PE in comparison with other phycobiliproteins confers an additional stability, since this subunit is located in the center of the molecule linking the $(\alpha\beta)_3$ trimers.[47] R-PE is recognized by its higher stability towards several denaturant agents, such as temperature and pH.[44,48]

Apart from its great optical properties, the high solubility in water, stability, and protein nature of R-PE has lead much attention from the food (applied as red fluorescent colourants especially for jellified desserts and dairy products and for the production of functional food), pharmaceutical, cosmetic, and textile industries.[40,44] R-PE is also studied in the medical field, namely in clinical medicine, diagnosis, and biomedical research,[49] due to its excellent optical and spectroscopic properties, high absorption coefficient, and high fluorescence yield. It has been used as fluorescent probe in flow cytometry, microscopy, immunochemistry, and in biomedical reagent formulations.[44] It possesses some biological activities that can be useful in the pharmaceutical field, namely their role as antioxidant, antidiabetic, immunosuppressive and antihypertensive chemicals. It was also reported its anti-cancer activity, in which R-PE can help to improve the selectivity of photodynamic therapy, activity tested recently in mouse tumour cells and human liver carcinoma cells.[50] Lastly, a very promising application for phycobiliproteins has been found in the renewable energy field. Since phycobilisomes are photosynthetic antenna complexes, their incorporation in Luminescent Solar Concentrators (LSCs) as optically active centers is an alternative to the semiconductor materials. They allow the most efficient capture and concentration of the natural light towards the photovoltaic devices for energy conversion due to their ability to absorb light from the sun in different wavelengths, according to their content in chromophores, namely in wavelengths that are not absorbed by chlorophyll *a*.[51,52]

Since phycobiliproteins are hydrophilic proteins, the conventional solvents used in their extraction are water or different buffers after a previous step of cell wall lysis, being buffers used to maintain the pH.[53,54] However, the low yields of extraction and the lack of selectivity of the processes decrease the biomass potential and the quality of the extracts obtained.[55,56]

Considering the lack of selectivity of the processes reported, the use of other solvents has been envisioned. In 2015, Martins et al.[57] tested a large range of ionic liquids (ILs) aqueous solutions as alternative solvents/disruption agents. Through their use, the authors concluded on the promising behaviour of using aqueous solutions of ILs, in particular, cholinium chloride, instead of the conventional buffers, since an improvement of 46 % on the yield of extraction was obtained.[57]

Although the use of aqueous solutions of ILs on the extraction of pigments from algae is a new matter of interest, the application of ILs as alternative solvents to extract high value compounds from biomass is not new,[58] being alkaloids,[59] flavonoids,[60] terpenoids,[61] proteins,[57] and lipids,[62] some of the examples. Given that, the use of ILs as solvents can greatly enhance the biomass commercial potential by their application on the extraction and fractionation of different bioactive compounds with industrial/commercial interest, under the biorefinery framework, as was already done for microalgae.[63,64]

ILs are salts with low-charge density and low symmetry among their ions. These features lead to decreasing their melting points in comparison with common salts, allowing often these to be liquid at room temperature. Besides, ILs being composed of a large organic cations and an organic or inorganic anions, and due to the very high number of combinations of ions that can be formed, their properties can be tuneable to a specific application, which is recurrently described as their "designer solvent" nature.[65] ILs are also recognized by some unique properties justified by their ionic character, namely their negligible vapour pressure, low flammability, high thermal and chemical stabilities, broad liquid temperature range, high ionic conductivity, and high solvation ability for organic, inorganic and organometallic compounds.[65] Taking into account their unique characteristics, their role on the extraction processes is crucial since different interactions can occur among the solvent and the compound to extract (e.g. van der Waals, as π···π, hydrogen-bonding, and Coulombic interactions[58]) that may improve their selectivity to different molecules according to the chosen IL. Moreover, the use of aqueous solutions of ILs instead of pure ILs have proved that, for some biomolecules, higher yields of extraction are achieved, due to the higher solubility of the compounds in the solvent (hydrotropic nature of ILs in water).[66,67] Nevertheless, the use of ILs as extractive solvents can also lead to the cell membrane disruption, a phenomenon that also contributes to a more efficient extraction of biomolecules.[68] Aqueous solutions of ILs seem to be a more sustainable choice, since these allow the simultaneous decrease of the viscosity, and the amount of IL to be used in a process, thus of their environmental and economic impacts of the integrated downstream process, going towards the fulfilment of the principals of green chemistry.[58] In spite of all the advantages described, some ILs are still focus of controversy mainly due to their cost and sustainability, the

latest normally attributed to their toxicity.[65] There are some approaches to counter these disadvantages, namely the preference for aqueous solutions and more benign ILs, allied with the possible recovery of ILs for their reuse in other cycles of extraction. The use of aqueous solutions of ILs have as main solvent the water, the most biocompatible, greenest and cheapest solvent. Then, more benign and cheaper ILs are being developed such as carboxylate-, amino-acid-, carbohydrate-, and cholinium-based ILs that can be used as more sustainable alternatives.[58] Lastly, although there is still a big lacuna in the field, many efforts have been done to recover the target molecules from the IL, allowing thus its reuse in new cycles of extraction. Depending on the IL used or depending on the compound extracted, different techniques can be applied, for instance hydro-distillation, back-extraction using industrially-approved organic solvents, precipitation with water or using an anion-exchange resin.[58]

Even if the extraction process is efficient, normally it lacks selectivity, leading to a low purity of the extracts. Solutions of purified phycobiliproteins are expensive and their prices keep increasing, not only considering the markets already established worldwide like the natural food colourants, but also markets that are now in their infancy with very good economic and industrial perspectives. As an example of the market cost and benefit analysis, the current scenario of the natural food colourants market is facing an outstanding increase worldwide. According to recent literature,[69] the food colour business reached US $ 1.3 billion with a 6.8 % growth rate annually, by 2016, expecting at the current growth rate to reach US $ 1.77 billion by 2021. In this sense, different methodologies have already been proposed to isolate and purify phycobiliproteins, and in particular, R-PE. Usually, these methodologies are a combination of techniques to reach higher purity levels. The purity level (also called as purity factor or purity index) is usually defined as the ratio between the R-PE content and the content of the total proteins represented by $Abs_{565}/Abs_{280,}$ where the highest is the purity level (factor/index), the purest is the extract/final product in R-PE.[55] The conventional purification methodologies normally involve a preliminary step using a precipitation with a concentrated solution of ammonium sulfate.[40] In this step, most target proteins will precipitate, while several contaminants will remain in solution. This allows a partial purification by the precipitation of the biggest molecules. Therein, the precipitate is re-dissolved in a fresh buffer solution and a chromatographic method is used.[40] Many

different protein features can be exploited by chromatographic methods in order to purify R-PE: ion-exchange chromatography,[43,49,76,53,56,70–75] based on net charge; gel filtration chromatography,[55,70,74,75,77–79] based on size; and hydrophobic chromatography,[49,53,56,72,73] based on the reversible interaction between the protein and hydrophobic ligand bounds and the matrix of the column. Other methods were also reported, in particular the hydroxyapatite chromatography,[54,55,72,77,79] which is considered a "pseudo-affinity" chromatography or "mixed-mode" ion exchange, and finally, preparative electrophoresis methodologies,[48] based on ionic charge.

In general, good purity indexes were achieved, however in some cases the yield in R-PE can decrease more than 90 %,[56] representing significant losses of the target compound. Besides, the several steps of chromatography lead to a difficult scale up and to a relevant increase of the costs of the process. Moreover as the number of purification steps increases, although the purity of R-PE is potentially increased, the content of R-PE decreases due to the multiple steps of purification, as reported in 2014 by Cai and co-workers.[79] It is thus necessary to search for more efficient technologies of purification, but with industrial potential. A few methods were reported and proved to be apt for scale-up task, but the data available on the resolution ratio reported is still relatively low.[75] Taking into consideration all information here described, it can be concluded that these drawbacks in the purification steps make pure pigments more expensive and are demanding for new and simpler, faster and more efficient downstream technologies.

1.2. Scopes and objectives

As previously discussed, the development of sustainable industrial processes able to answer the needs of a bioeconomy based on marine resources are still a goal to achieve. An algal-based sustainable process should include the:

i) cultivation or harvesting of the biomass, where strategies should be adopted to induce or maximize the production of primary and secondary unique metabolites in these organisms, boosting their industrial potential.[80]

ii) cell disruption, usually applied to induce the rupture of the cell wall, which is the main obstacle for the solvent to reach the compound to be extracted. It can be done using maceration and milling, being often applied with liquid

nitrogen to achieve greater results,[48,57,81] cycles of freezing and thawing,[49,76] ultrasonication- and/or enzyme-assisted extraction.[82–84]

iii) extraction of the compounds in which occurs the disruption of cell membranes and the solubilization of the target compound, being the solvent a key parameter in the performance of the solid-liquid extraction, namely the type and concentration of the solvent.[57,85] Other parameters regarding the extraction itself should not be forgotten, namely the time of extraction,[57] pH,[57] temperature,[83] solid-liquid ratio,[53] solvent nature and concentration,[57] pressure, and agitation.[48]

iv) purification, depending on the purity level required to the application.[86]

Based on these, Figure 1.2.1 attempts to explain how algal-based sustainable processes should be like in a circular bioeconomy. This means the integration of all steps previously described (cultivation or harvesting, cell disruption, extraction of the compounds, and purification) considering:

i) clever and responsible biomass utilization with its maximum valorization (and not by over-exploitation), in a biorefinery chain to obtain different products until the final applications (that can be fertilization or energy production) – blue path;

ii) minimization of the consumption and maximum recovery of solvents, materials, equipment, and energy – green path;

iii) reuse of the final products – yellow path;

iv) minimization of the wastes – brown path.

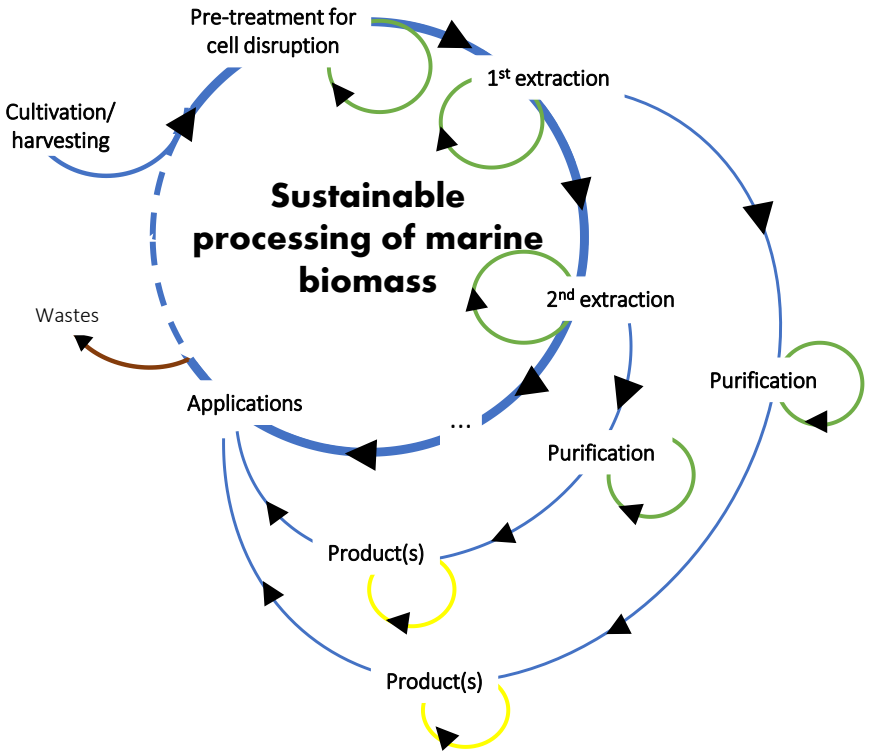


Figure 1.2.1. Algal-based sustainable conversion process.

Nowadays, the extraction and purification steps are pointed out as the major bottlenecks of the bio-based processes, since it is still difficult to separate the various compounds without damaging other bioactive fractions, while keeping high yields of extraction, and high economic and environmental efficiency.[86] However, there is an urgency to define routes to provide a maximum value without compromising the biomass viability for the next processing steps, which can pass through the use of more selective and biocompatible solvents. In this context, this thesis aims the development of new/improved processes of extraction and purification of bioactive compounds from algae using more sustainable and simpler (*i.e.* easier to implement) extractions and purification technologies. Summing up, the various works composing this thesis will focus the different approaches required to create a sustainable process:

(i) the development of alternative but efficient methodologies of extraction and purification of pigments,

(ii) the assessment of strategic methods to reuse as much as possible the solvents by separating them from the pigments;

(iii) the study of potential applications for the pigments recovered, particularly, their use as photosensitizers in photodynamic therapy, and finally

(iv) to envision the industrial potential of the processes developed, by the evaluation of their environmental and economic impacts.

In the end, this thesis should be a contribution towards the sustainable development of the concept of Blue Biorefinery following as much as possible the guidelines of Circular Economy, since it encloses the process development, the solvents reuse, the search for new applications, while maintaining a low environmental footprint and economic impact, as depicted in Figure 1.2.1.

This study is a contribution to the comprehension of the main mechanisms behind the processing of algae (Figure 1.2.2), allowing the development of rules that could be further applied to other algae species and bio-based compounds, that would eventually lead to the development of the blue biorefinery concept and of a bio-based economy.[87]

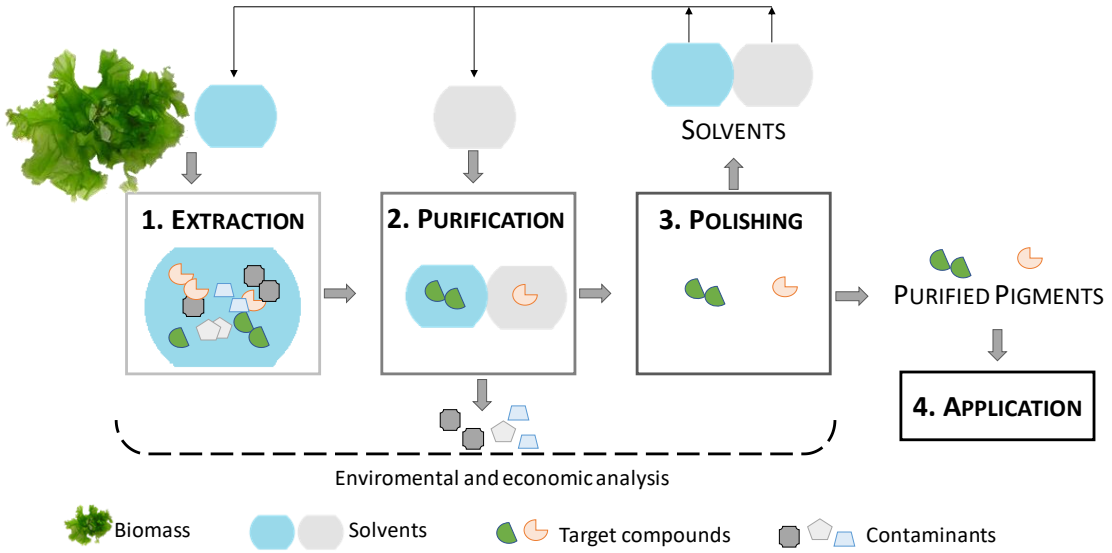


Figure 1.2.2. Representation of the steps in algae processing focused on this thesis.

Summing up, this thesis, schematically represented in Figure 1.2.3, is divided in 6 Chapters described as follows.

Chapter 1: includes the state of the art of this thesis, considering the bioeconomy, principles of sustainable/green processes, biorefinery and the potential of algae and algae pigments, and describes the scopes and objectives of this work;

Chapter 2: encloses the development of alternative methodologies of extraction of pigments from the green macroalgae *Ulva* spp. using aqueous solutions of ILs, together with economic analysis, based on the published paper "Extraction of

chlorophyll from wild and farmed *Ulva* spp. using aqueous solutions of ionic liquids", Separation and Purification Technology, 2020, DOI: 10.1016/j.seppur.2020.117589.

Chapter 3: describes the development of four different alternative methodologies for the purification of chlorophylls and xanthophylls:

Chapter 3.1: Use of organic systems and liquid-liquid extraction to purify chlorophyll and xanthophyll from the green macroalgae *Ulva rigida*, based on the paper "Recovery of pigments from *Ulva rigida*", Separation and Purification Technology, 2020, DOI: 10.1016/j.seppur.2020.117723.

Chapter 3.2: Extraction of pigments using aqueous solutions of ILs and liquid-liquid extraction as an approach to purify chlorophyll and xanthophyll from *Spirulina maxima*, while recovering the IL in new cycles of extraction, together with studies on the potential of a chlorophyll derivative in photodynamic therapy, based on the paper "Recovery of chlorophyll *a* derivative from *Spirulina maxima*: Its purification and photosensitizing potential", ACS Sustainable Chemistry & Engineering, 2021, DOI: 10.1021/acssuschemeng.0c07880.

Chapter 3.3: One-step approach to extract and purify chlorophyll and fucoxanthin from the brown macroalgae *Saccharina latissima* using systems composed of oil and aqueous systems of ILs, together with environmental and economic analysis, based on the paper "Extraction and fractionation of pigments from *Saccharina latissima* (Linnaeus, 2006) using an ionic liquid+oil+water system", ACS Sustainable Chemistry & Engineering, 2021, DOI: 10.1021/acssuschemeng.0c0911.

Chapter 3.4: Use of aqueous solutions of ILs as eluents to obtain purified pigments from the microalgae *Isochrysis galbana*, based on the paper "Ionic liquids as eluents in solid-phase extraction to purify pigments recovered from *Isochrysis galbana*", Chemical Engineering Journal, submitted, 2021.

Chapter 4: development of alternative methodologies of purification of phycobiliproteins from the red macroalgae *Gracilaria gracilis,* together with environmental and economic analysis, based on the paper "Sustainable strategy based on induced precipitation for the purification of phycobiliproteins", ACS Sustainable Chemistry & Engineering, 2021, DOI: 10.1021/acssuschemeng.0c09218.

Chapter 5: final remarks and future perspectives.

Chapter 6: details regarding the scientific contribution of this thesis. This list includes the publications, patents, book chapters, internships, and other activities done during and under the scope of the present thesis.

Chapter 7: detailed list of references used to support this entire work.

Chapter 8: experimental details of the works presented in Chapters 2, 3 (3.1, 3.2, 3.3, 3.4), and 4.

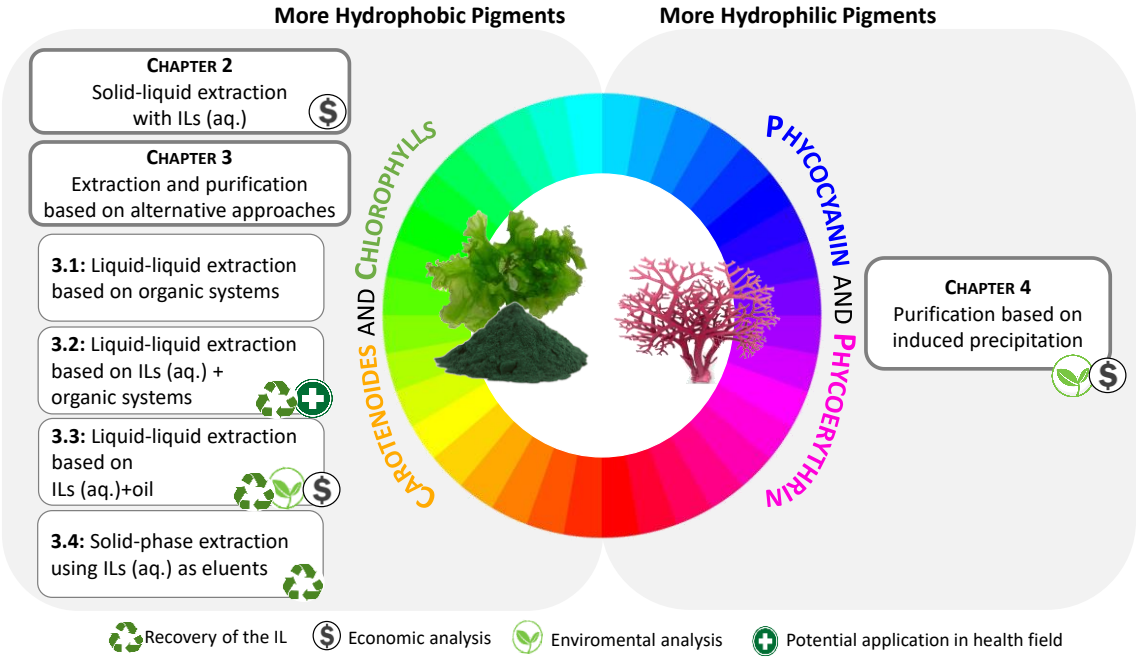


Figure 1.2.3. Summary of the work developed under the scope of this thesis, based on the development of different approaches, for the recovery of different pigments, from different algae.

CHAPTER 2. Extraction of pigments from biomass with alternative solvents

2.1 Extraction of chlorophyll from wild and farmed *Ulva* spp. using aqueous solutions of ionic liquids

This chapter is based on the published manuscript:

Margarida Martins, Andreia P.M. Fernandes, Mario A. Torres-Acosta, Pi N. Collén, Maria H. Abreu, Sónia P.M. Ventura,* "Extraction of chlorophyll from wild and farmed *Ulva* spp. using aqueous solutions of ionic liquids", Separation and Purification Technology, 2020, DOI: 10.1016/j.seppur.2020.117589.

*Contributions: M.M. and A.P.M.F. acquired the experimental data. M.M. performed the data analysis. M.A.T.-A. assessed the economic impact. M.M. wrote the manuscript with substantial contributions from the remaining authors.

Abstract

Products extracted from natural resources are an increasing trend in several fields promoted by consumer demand. Allied to the importance attached to the concept of "natural product" should be the way the "natural product" is obtained. In this work, chlorophyll was extracted from batches of wild-harvested and farm-raised green macroalgae *Ulva* spp. from two different European locations, Portugal and France. The performance of different aqueous solutions of tensioactive compounds such as ionic liquids and common surfactants in the yield of extraction of chlorophyll was studied and the operational conditions of extraction were optimized. The effect of drying the biomass in the yield of extraction of chlorophyll was evaluated as well as the effect of both locations (and the specific conditions of each location in terms of nutrients, water temperature and light intensity) in chlorophyll production. After optimization of all operational conditions, a maximum yield of extraction of 5.96 $mg_{chl}.g_{dry\ biomass}^{-1}$ was obtained using 250 mM of tributyltetradecylphosphonium chloride ($[P_{4,4,4,14}]Cl$). The use of this solvent has allowed the development of a cost-effective (conclusion obtained after the economic analysis) and efficient process capable of maintaining the stability of the final product for more than one month.

Keywords: Green macroalgae, *Ulva* spp., geographic location, chlorophyll, surfactants, tensioactive ionic liquids, economic analysis.

Introduction

Blue biotechnology is emerging as a solution to reduce the world's need of synthetic compounds from non-renewable raw materials. In this sense, the development of sustainable and integrated biorefineries based on abundant marine materials scarcely used is essential.[88] Macroalgae are an example of such a biomass, which did not have up to now, a multi-application approach of the biomass, being macroalgae used mainly for polysaccharide extraction for human food, pharma, cosmetics or even biomaterials. However, this type of biomass can benefit from its integration in processes answering the biorefinery challenges, by combining the extraction of added-value molecules with high-volume/low-cost applications as feed, plant biostimulants or even energy and bioplastics.[8]

Due to their market value and usually lower contents in the biomass, added-value molecules should be the first molecules to be considered in a biorefinery chain.[8] The added-value molecules present in macroalgae represent a large plethora of chemicals, with a wide range of properties, from antioxidant, to anti-inflammatory, and anti-tumoral, with potential in biomedical, pharmaceutical, and cosmetic industries.[89] In macroalgae, lipids, carbohydrates, proteins, minerals and pigments are included in the most valuable bioactive compounds, which can supply consumer current demands for natural products. At the same time, these have been reported for the environmental aspects of sustainability allowing consequently to boost new economies and industrial sectors.[90] Pigments are extremely important for macroalgae since they ensure the light capture required for photosynthesis.[91] Besides, they have a significant number of applications attributed, these potentially including food and textile dyes, but also cosmetic and pharmaceutical products.[21,92]

The role of chlorophyll in the harvesting of light and in conversion of energy of absorbed photons to chemical energy[93] is already well-established. Moreover, their benefits to human health have been reported considering its antioxidant,[94] anti-inflammatory,[95] and anti-tumour activities.[96] Although chlorophyll extraction from living matrices is not new,[97] the reported methodologies are in their vast majority based on the use of hazardous and volatile organic solvents or mechanical treatments that lead to the increase of temperature and partial thermo-degradation.[98,99]

The use of water-based solvents at room temperature appears as a more sustainable and biocompatible approach. To use water as solvent to recover hydrophilic compounds is easy; the challenge is to use water to extract hydrophobic molecules like chlorophyll.[100] Some articles dealing with extraction of hydrophobic pigments already suggest the use of aqueous solutions of tensioactive compounds as extracting solvents.[101] Tensioactive compounds tend to form micelles above the critical micelle concentration, creating a friendly environment for solvation of hydrophobic molecules in water. Besides common surfactants, some ionic liquids (ILs) also have this tensioactive feature.[102] ILs are salts with special interest due to their tunable nature. This results from the correlation between the IL structure-properties-application, allowing them to be recognized as "designer solvents" with affinity to a large set of biomolecules.[57,103]

The main objective of this work is aligned with the objectives defined on GENIALG (GENetic diversity exploitation for Innovative macro-ALGal biorefinery), an European project with several academic and industrial participants from all around Europe. This project focuses on specifically two macroalgae species, the Sac*charina latissima* (or sugar kelp) and *Ulva* spp. (or sea lettuce), two of the species with high biomass yield of production. Under the ambit of this European project, the intention is "to boost the Blue Biotechnology Economy by designing high-yielding seaweed cultivation systems and more sustainable downstream processes". In this sense, the objective of this work encloses the optimization of more sustainable extraction methodologies by replacing the conventional volatile and toxic organic solvents usually used to recover the pigments by more selective solvents, mainly composed of water, that provide higher yields of extraction and higher stability to the pigments, and that simultaneously lead to more sustainable and profitable processes with industrial potential. More specifically, aqueous solutions of common surfactants and tensioactive ILs were used in the extraction of dry and fresh samples of *Ulva* spp. harvested in different locations, namely Portugal and France.

In this work, the extraction of chlorophyll from *Ulva* spp. from two different geographic locations was investigated. Several aqueous solutions of common surfactants and tensioactive ILs were studied and the results obtained compared with the data obtained for a conventional organic solvent, in this work, ethanol. Moreover, the process

conditions of solid-liquid ratio (SLR), time of extraction, concentration of tensioactive, type (dry or fresh) and geographic location (farm-raised @ Portugal and wild-harvested @ France) of the biomass were optimized. Then, the stability of the chlorophyll content extracted was also studied. Finally, the economic evaluation of the traditional *versus* the alternative extraction process was performed, where different scenarios were evaluated in such costs.

Experimental

Biomass

The biomass used in this work was kindly provided by two different companies, ALGAplus (Ílhavo, Portugal) and Olmix (Bréhan, France). ALGAplus farms *Ulva rigida* at Ria de Aveiro lagoon (40°36'44.7" N, 8°40'27.0" W) in coastal Portugal under the European Union organic aquaculture standards (EC710/2009). This aquaculture is performed in a land-based integrated multi-trophic aquaculture system (meaning that the nitrogen input is higher than in the outside natural lagoon due to the use of effluent water from fish production). Olmix harvests *Ulva* spp. in the north Brittany coast near Plestin-les-Grèves (48°40'49.9" N, 3°35'40.1" W), France. Dry and fresh biomass samples from the portuguese company were harvested in September 2018 and June 2018, respectively, and fresh biomass from the French company was harvested in July 2017, being these three samples studied. Fresh biomass was washed at least three times with tap and distilled water and kept frozen (-20 °C) until needed.

Chemicals

Absolute ethanol (HPLC grade, CAS 64-17-5) was purchased from Fisher Scientific being used as a standard organic solvent. Tensioactive compounds in aqueous solution were used on the extraction of chlorophyll from the green macroalgae. The series of 1-alkyl-3-methylimidazolium chloride as 1-hexyl-3-methylimidazolium ([C_6C_1im]Cl, 98 wt%, CAS 171058-17-6), 1-methyl-3-octylimidazolium chloride ([C_8C_1im]Cl, 99 wt%, CAS 64697-40-1), 1-decyl-3-methylimidazolium chloride ([$C_{10}C_1$im]Cl, 98 wt%, CAS 171058-18-7), 1-dodecyl-3-methylimidazolium chloride ([$C_{12}C_1$im]Cl, > 98 wt%, CAS 171058-18-7), 1-methyl-3-tetradecylimidazolium chloride ([$C_{14}C_1$im]Cl, 98 wt%, CAS 171058-21-2), 1-

hexadecyl-3-methylimidazolium chloride ([$C_{16}C_1$im]Cl, 98 wt%, CAS 61546-01-8) were all acquired from Iolitec. The tributyltetradecylphosphonium chloride ([$P_{4,4,4,14}$]Cl, 95 wt%, CAS 81741-28-8) and the decyltrimethylammonium chloride ([$N_{1,1,1,10}$]Cl, 98 wt%, CAS 10108-87-9) were purchased from Iolitec and Tokyo Chemical Industry, respectively. The dodecyltrimethylammonium bromide ([$N_{1,1,1,12}$]Br, 99 wt%, CAS 1119-94-4) and tetradecyltrimethylammonium bromide ([$N_{1,1,1,14}$]Br, 98 wt%, CAS 1119-97-7) were acquired from Alfa Aesar, while the hexadecyltrimethylammonium bromide ([$N_{1,1,1,16}$]Br, 99 wt%, CAS 57-09-0) was purchased from Merck. The surfactants sodium dodecyl sulfate (SDS, 99 wt%, CAS 151-21-3), and polyoxyethylene(8) octylphenyl ether (Triton X-114, 100 wt%, CAS 9002-93-1) were purchased from Acros Organics. The chemical structures of the tensioactive compounds used are depicted in Figure A.1 in Appendix A.

Chlorophyll extraction

Before the extraction, the samples were frozen with liquid nitrogen and ground in a coffee grinder until powder (< 0.5 mm). The drying procedure of the dry samples of macroalgae was carried out by ALGAplus, in which the algae were washed with seawater, centrifuged to remove excess water and then dried in a forced air-tunnel at a set temperature of 25 ºC until reaching a moisture content of 10–11 %. The dried samples were milled to obtain powder and sieved (< 1 mm).

The extractions were performed at room temperature (20–25 ºC) under a constant agitation of 80 rpm. Ethanol was used in parallel as a control solvent. Initially, solutions of 250 mM of the tensioactive compound in water (common surfactants and tensioactive ionic liquids) were used[68] at an incubation time of 30 min and a solid-liquid ratio (SLR) of 0.01 $g_{biomass}.mL_{solvent}^{-1}$. The type of solvent, SLR, solvent concentration, and time of extraction were systematically changed as they were optimized. All assays were performed at least in triplicate. In order to remove the cell debris, a centrifugation step was added in a Thermo Scientific Heraeus Megafuge 16R centrifuge at 4700 *g* for 30 min at 4 ºC.

Chlorophyll quantification

The absorption spectra were measured between 200 and 700 nm using a UV-Vis microplate reader (Synergy HT microplate reader – BioTek) in a period inferior of hour after the extraction process. The chlorophyll content was quantified at 667 nm being the interference of the solvents considered and the chlorophyll concentration calculated according to a calibration curve previously prepared. The results are expressed in terms of yield of extraction ($mg_{chl}.g_{dry\ biomass}^{-1}$).

Chemical stability of the extracts over time

Extracts obtained with the most promising solvents at the optimized conditions were analysed in terms of their stability over time at 25 °C and 4 °C, protected from light, for the wild-harvested and farm-raised algae. The assay was done during 33 days by analysing the percentage of chlorophyll loss.

Statistical analysis

Analysis of variance (ANOVA) was performed using the BIOESTAT 5.3 to compare the significance of the obtained extraction yields for each operational condition and solvent at a time, using a degree of significance of 95 % ($p < 0.05$, $n = 3$). This analysis was always performed considering a comparison of significance in the yield of extraction of chlorophyll for the same algae, solvent and parameter tested.

Economic analysis

The economic evaluation performed focused mainly on the material consumption between the IL and ethanol process options. Production costs were calculated *per* milligram of chlorophyll produced (Cost of goods *per* milligram, $CoG.mg^{-1}$). To calculate the production costs, the following equation (Eq. 1) was employed:

$$\mathrm{CoG.\,mg^{-1}} = \frac{\sum_{i=1}^{n} \frac{Use\ of\ material_i}{Batch} \times \frac{Price\ of\ material_i}{Unit\ of\ material_i}}{\frac{Amount\ of\ chlorophyll}{Unit\ of\ dry\ biomass} \times Mass\ used\ of\ dry\ biomass} \qquad \textbf{Eq. 1}$$

This evaluation consisted of two analyses. Firstly, a deterministic analysis where the $CoG.mg^{-1}$ is calculated using the best conditions selected after the experimental work

was performed. Then, a sensitivity analysis was performed to determine the impact of the material costs (higher or lower than the base cost) and the concentration of IL applied. These variables were defined in the equation presented. It can be seen from Eq. 1 that the only cost related variables are the price of the materials. For this analysis, the price of the IL considered was of 409.3 EU.kg^{-1} (Ionic Liquid Technologies, Heilbronn, Germany) and for ethanol 75 EU.L^{-1} (Fischer Scientific, Portugal).

Results

Screening of alternative solvents and operational conditions optimization: comparison of fresh and dry algae

A comparison among fresh and dry samples of farm-raised *Ulva rigida* from the same location was done, being the screening of aqueous solutions of different alternative solvents and the optimization of the process operational conditions performed. Ethanol was studied simultaneously as an example of a conventional solvent reported for the chlorophyll extraction.[101]

In the screening of the alternative solvents (Figure 2.1.1) common surfactants and tensioactive ILs, namely imidazolium-, phosphonium-, and ammonium-based ILs were studied in a concentration of 250 mM, SLR of 0.01 $g_{biomass}.mL_{solvent}^{-1}$ for 30 min. The effect of the alkyl chain length was studied for imidazolium- and ammonium-based ILs. However, the aqueous solutions of SDS and [$P_{4,4,4,14}$]Cl stand out as the most efficient solvents with similar or even higher results than the ones reported for ethanol. For fresh biomass, the results obtained follow the trends previously described for other biomolecules.[68] For the dry algae, aqueous solutions of SDS showed a colour change of the extract, probably due to chlorophyll degradation.

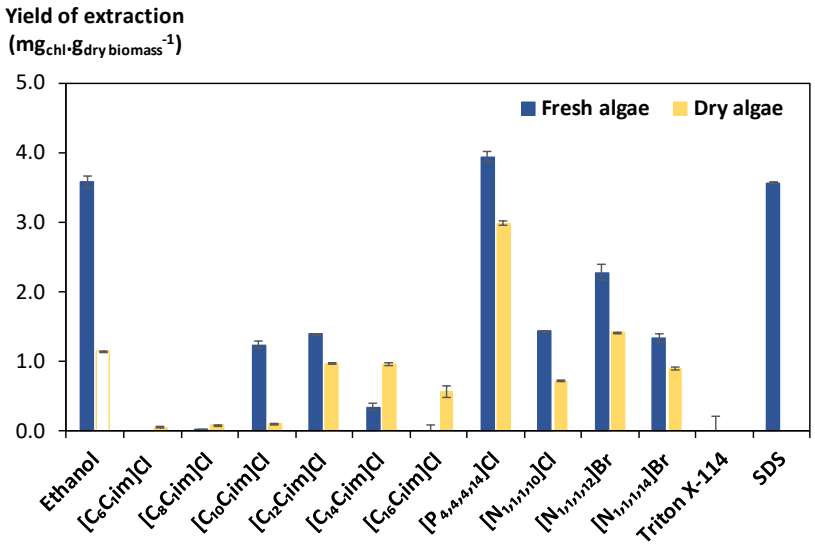


Figure 2.1.1. Yield of extraction of chlorophyll using fresh and dry farm-raised *Ulva rigida* regarding the screening of aqueous solvents of different tensioactive solvents.

The study proceeded with the optimization of the most relevant operational conditions, namely the SLR, solvent concentration in water, and time of extraction (Figure 2.1.2 A, 2.1.2 B, and 2.1.2 C, respectively). For the dry algae, aqueous solutions of SDS were not considered for the reasons discussed above. In any case, the extraction yield obtained using the fresh biomass was always the highest. Moreover, even in the case of dry algae, the yield of extraction of chlorophyll is more than the double using the aqueous solution of [$P_{4,4,4,14}$]Cl (250 mM) instead of ethanol (Figure 2.1.1), which is a consequence of the poor capacity of ethanol to penetrate the dry biomass.

Regarding the effect of the SLR (Figure 2.1.2 A), the choice falls on the condition that uses the least amount of solvent for the highest yield of extraction of chlorophyll possible. When fresh biomass is used, the yield of extraction is maximum for SLR of 0.04 and 0.02 $g_{biomass}.mL_{solvent}^{-1}$ for [$P_{4,4,4,14}$]Cl and SDS, respectively. Meanwhile, when using dry algae, the maximum of chlorophyll extracted was observed for a SLR of 0.013 $g_{biomass}.mL_{solvent}^{-1}$ for [$P_{4,4,4,14}$]Cl. This means that, when the dry biomass is used, to achieve the highest yield of extraction, more volume of solvent is needed. This could be justified by the impact that the drying process may have on the structures of chloroplasts or thylakoidal membranes, but it may also be explained by the negative impact towards the chlorophyll structure.

After selecting the most efficient SLR as being 0.04 and 0.013 $g_{biomass}.mL_{solvent}^{-1}$, for fresh and dry biomass, respectively, the effect of $[P_{4,4,4,14}]Cl$ concentration was tested and for that, aqueous solutions of the IL in concentrations between 50 and 500 mM were tested (Figure 2.1.2 B). The main results suggest that the yield of extraction increases with the tensioactive concentration up to 250 mM, a profile that is independent of the biomass being fresh or dry, for both solvents. Interestingly, this same trend was previously observed found for the extraction of green fluorescence protein from recombinant *Escherichia coli* cells.[68] As a third condition, it was studied the time of extraction as depicted in Figure 2.1.2 C. From the experimental data, it is possible to observe an increase in the yield of extraction up to 60 min, for both fresh and dry biomass.

In general, even after the parameters of extraction optimization, a lower performance regarding chlorophyll extraction from dry biomass when compared with fresh biomass is evident and agrees with data already reported in literature for carotenoids.[104,105] As mentioned before, this can be due to the structural changes in membranes, hindering the extraction of chlorophyll, but also due to the photosystem degradation that many times is irreversible even after rehydration, making this biomass less useful for photosynthetic pigments extraction.[106]

Comparison of fresh algae from different geographic locations

Fresh wild-harvested *Ulva* spp. from the north of France and farm-raised *Ulva rigida* from an aquaculture environment in Portugal were compared (Figure 2.1.3). From the results obtained, it seems that the chlorophyll content in the wild-harvested algae is higher than the farm-raised algae, by *circa* 2 $mg_{chl}.g_{dry\ biomass}^{-1}$. As already discussed by Powley and collaborators,[107] these differences may be attributed to the different habitat conditions, mainly in terms of light intensity, but also temperature and nutrients supply (e.g. phosphorus and nitrogen) of both locations that will interfere with the chlorophyll production.[108–110] Moreover, in the case of farm-raised *Ulva*, we are sure of dealing with only one species (*Ulva rigida*), while in wild-harvested biomass it is possible to have a mixture of different *Ulva* species as well as a small percentage of other contaminant species.

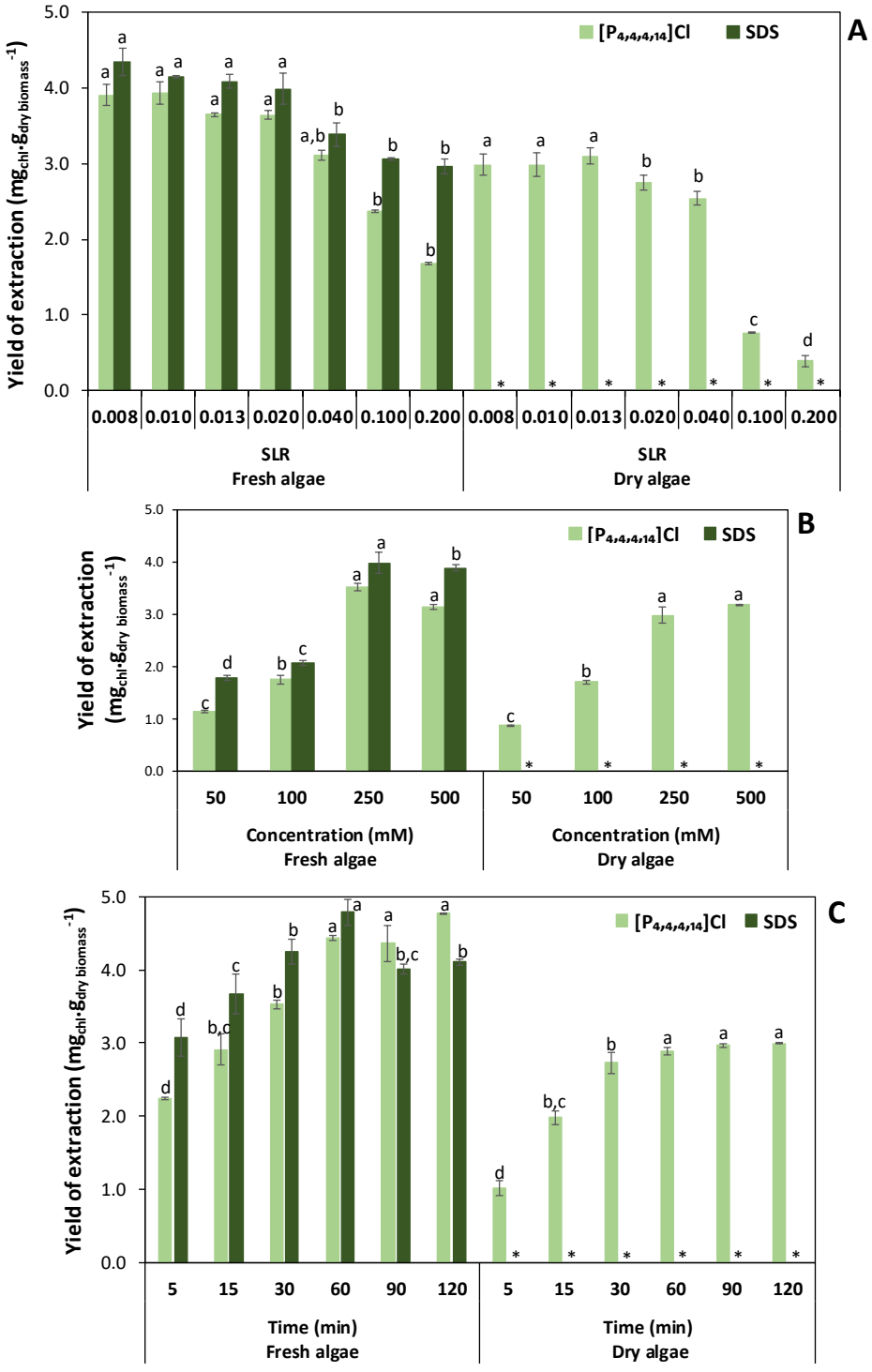


Figure 2.1.2. Yield of extraction of chlorophyll using fresh and dry farm-raised *Ulva rigida* regarding the effect of operational conditions: (A) SLR, (B) solvent concentration, and (C) time of extraction. *SDS was not considered for dry algae. Different letters represent statistically different values ($p < 0.05$). Equal letters in the same column represent statistically equivalent values.

Despite this difference, the same trends were identified for the different alternative solvents and operational conditions under study (Figure 2.1.3). As previously seen for the fresh farm-raised algae, the $[P_{4,4,4,14}]Cl$ and the SDS stand out as the best solvents in the wild-harvested algae. The SLR study revealed a different maximum, being 0.01 and 0.013 $g_{biomass}.mL_{solvent}^{-1}$ for $[P_{4,4,4,14}]Cl$ and the SDS, respectively (Figure 2.1.3 B), which may be related with the different chlorophyll contents found in the two samples. The optimum solvent concentration of the alternative solvent in water using fresh and dry algae is still the same, 250 mM. Finally, a decrease in the time of extraction was observed for the wild-harvested algae to 30 min for $[P_{4,4,4,14}]Cl$, in comparison with the 60 min obtained for the SDS and as well as for both solvents when the farm-raised algae is used.

Chlorophyll stability over time

Given that small differences in terms of yield of extraction were seen using both aqueous solutions of $[P_{4,4,4,14}]Cl$ and SDS, the chlorophyll stability was studied in both solvents. In this case, the stability of chlorophyll extracted with ethanol (standard solvent), and aqueous solutions of both $[P_{4,4,4,14}]Cl$ and SDS, was studied for 33 days, at 25 °C and 4 °C and in the absence of light. The results are displayed in terms of chlorophyll content loss being the chlorophyll content periodically measured (Figure 2.1.4).

Despite the conclusions previously reported for the effect of temperature,[111] in this case the results are not so different. In all cases, the stability seems to be affected by the solvent. In general, the aqueous solutions of SDS provide the lowest stability, with losses in the chlorophyll content up to 40 %, which may justify the lower contents of chlorophyll described during the optimization of the extraction process. In the other hand, the ethanol seems to have a slightly better performance maintaining the stability of the chlorophylls over time, which may be contradicted by the maintenance of the pigments at low temperature (4 °C), for which the results representing the IL as solvent are better (case of farm-raised) or similar (wild-harvested) when compared to the traditional solvent.

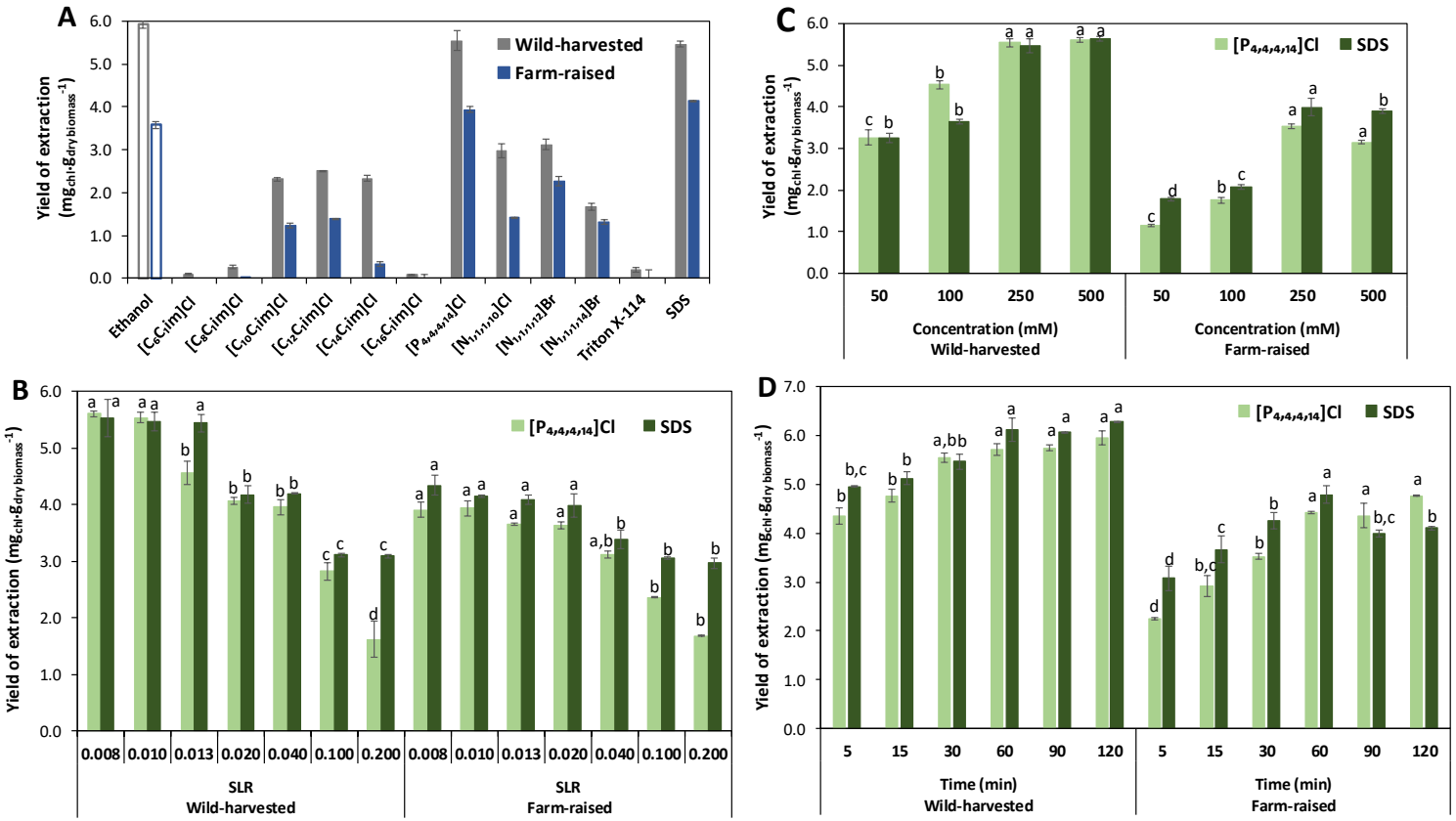


Figure 2.1.3. Yield of extraction of chlorophyll using fresh wild-harvested and farm-raised *Ulva* spp. regarding the (A) screening of alternative aqueous solvents; and the effect of operational conditions such as the (B) SLR, (C) solvent concentration of alternative solvents in water, and (D) time of extraction. Different letters represent statistically different values ($p < 0.05$). Equal letters in the same column represent statistically equivalent values. Results obtained for the farm-raised biomass are also here displayed to facilitate the comparison.

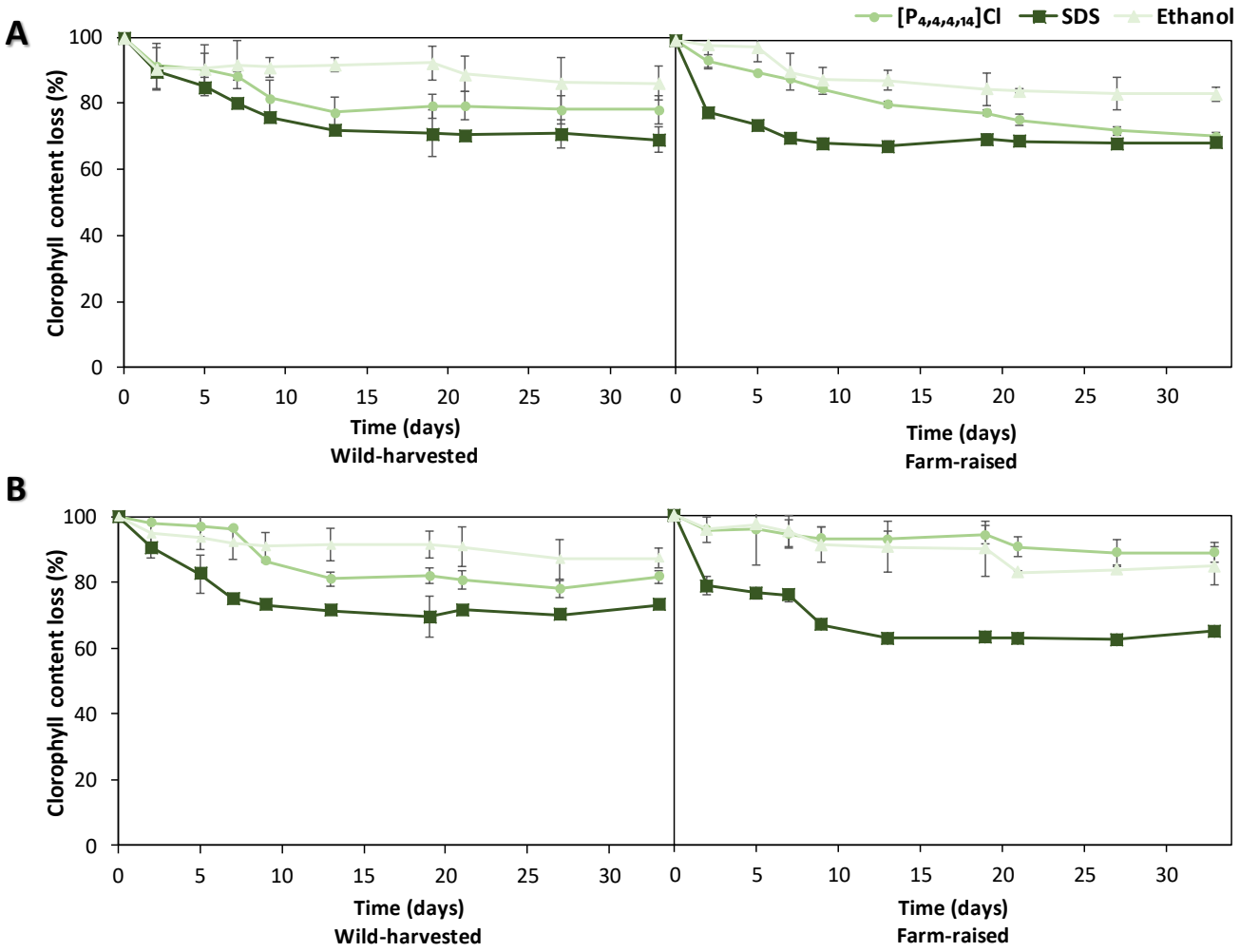


Figure 2.1.4. Chlorophyll stability represented by the chlorophyll content loss in the extract over time and in the dark, at (A) 25 °C and (B) 4 °C.

Economic analysis

In addition to the yields of extraction and stability of the products, to define the most efficient downstream process and industrially more appropriate, an economic evaluation is required. In this work, the processes with the best results in terms of yield of extraction and chlorophyll stability were selected (*i.e.* those based in ethanol and $[P_{4,4,4,14}]Cl$). Results for both analyses are summarized in Figure 2.1.5. The deterministic analysis comprises the calculation of the production costs using the optimum conditions previously determined during the optimization step. For both, it was at 30 min, SLR of 0.01 $g_{biomass}.mL_{solvent}^{-1}$, with 250 mM for $[P_{4,4,4,14}]Cl$ and ethanol 100 %. In general, the results suggest that the $[P_{4,4,4,14}]Cl$ has a lower production cost when applied on the extraction step (1.7 times lower) (Figure 2.1.5 A). This makes the use of $[P_{4,4,4,14}]Cl$ as a more attractive approach. Indeed, despite the higher cost of the IL when compared to the ethanol, in the alternative process using IL much less material is used, which decrease the cost of the alternative downstream process. This specifically contradicts the general assumptions normally found in literature, and shows that the cost of the IL is not the only condition to be considered in the analysis of a process but also the amount of solvent employed, the operational conditions, the yields of extraction and the stability of the products obtained.

After selecting the most cost-efficient and sustainable process, the one based on $[P_{4,4,4,14}]Cl$, a sensitivity analysis (Figure 2.1.5 B) was performed. As previously indicated, a sensitivity analysis details the impact that changes in the process parameters have on the production costs. This analysis is done by the representation of different scenarios for the conditions selected as most important for each process. In this work, it was studied the effect of variations in the materials costs (50, 100 and 150 %) and concentration of $[P_{4,4,4,14}]Cl$ (100, 250 and 500 mM). Considering the use of 250 mM and 100 % of materials costs as the base scenario, the results indicate that the largest impact is provided by the $[P_{4,4,4,14}]Cl$ concentration employed (which is typically observed for other liquid-liquid or solid-liquid extractions),[112] closely followed by the cost variation of the IL. A critical aspect of the concentration effect is that as it changes, the yield of chlorophyll obtained *per* mass unit of biomass is also affected (Figure 2.1.3 C). This

means that the solvent concentration has a combined effect from a change in the amount of IL being used and the amount of product generated as a result of the extraction efficiency. From these results, it can also be concluded that the use of less IL (100 mM), even with a reduced extraction yield, will assure lower production costs.

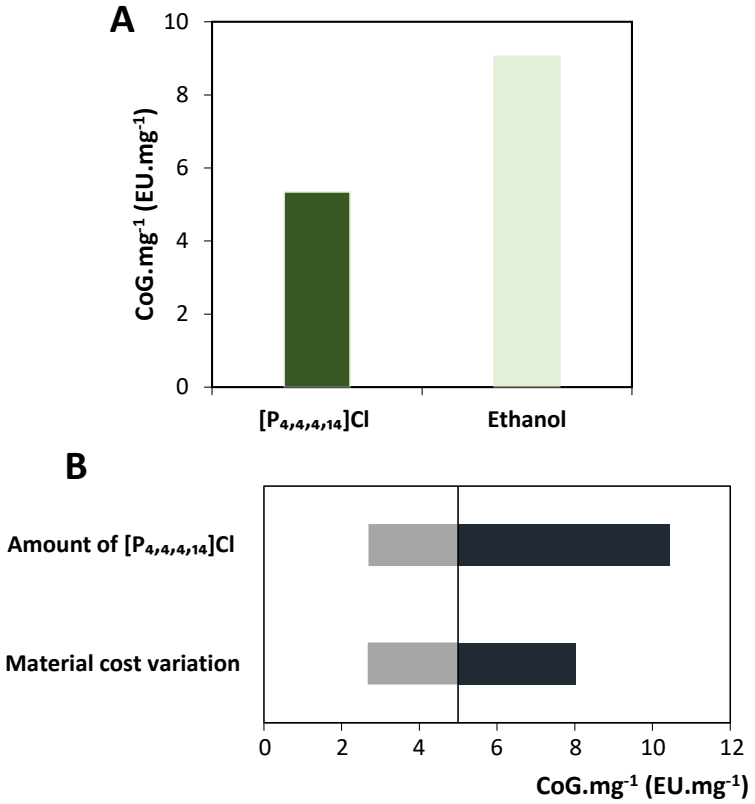


Figure 2.1.5. Economic evaluation considering (A) the comparison of the extractions performed with pure ethanol and the aqueous solution of $[P_{4,4,4,14}]Cl$ for the wild-harvested algae, and (B) the amount of IL and material cost variation in the economic impact on the alternative process suggested in this work.

Considering its high extraction performance, good chlorophyll stability and lower cost of the IL-based process when compared with the ethanol-based process, the final process was defined. Industrially, a complete downstream process should be considered, including the optimized solid-liquid extraction of chlorophyll from *Ulva* spp., a recovery of chlorophyll from the aqueous solution of $[P_{4,4,4,14}]Cl$, and lastly the recycling of solvents. As an alternative, and considering the literature background, a back-extraction may be optimized and strategically applied on the recovery of chlorophyll from the aqueous solution of $[P_{4,4,4,14}]Cl$, allowing at the same time, the

aqueous solution of $[P_{4,4,4,14}]Cl$, now free of chlorophyll, to be reused in the solid-liquid extraction step.[103]

Conclusions

In this work aqueous solutions of tensioactive ILs and common surfactants were used, and compared with ethanol as a conventional solvent, to extract chlorophyll from different batches of *Ulva* spp. Operational conditions of extraction, such as SLR, solvent concentration in water, and time of extraction were also considered. Although the differences between the dry and fresh samples from the same location and the wild-harvested and farm-raised *Ulva* spp. biomass on the chlorophyll content, the process of extraction optimization was successfully applied independently of the type of biomass. The best operational conditions were fixed at 250 mM of $[P_{4,4,4,14}]Cl$ in aqueous solution, for 30 min with a SLR of 0.01 $g_{biomass}.mL_{solvent}^{-1}$ for the fresh wild-harvested algae from the north of France, being a maximum yield of extraction of 5.96 $mg_{chl}.g_{dry\ biomass}^{-1}$ obtained. In the end, the IL-based extraction process has proved to be the most efficient and less expensive according to the economic analysis, while maintaining the stability of the final product for more than one month.

CHAPTER 3. Alternative approaches for the purification of chlorophyll and xanthophyll

3.1 Recovery of pigments from *Ulva rigida*

This chapter is based on the published manuscript

Margarida Martins, Rui Oliveira, João A.P. Coutinho, M. Amparo F. Faustino, M. Graça P.M.S. Neves, Diana C.G.A. Pinto, Sónia P.M. Ventura,* "Recovery of pigments from *Ulva rigida*", Separation and Purification Technology, 2020, DOI: 10.1016/j.seppur.2020.117723.

*Contributions: M.M. and R.O. acquired the experimental data. M.M. performed the data analysis. M.A.F.F., M.G.P.M.S.N., and D.C.G.A.P. assessed the UHPLC-MS experimental data. M.M. wrote the manuscript with substantial contributions from the remaining authors.

Abstract

Pigments, such as chlorophylls and carotenoids, have important applications in various fields, such as colourants in food, cosmetic or textile industries and in biomedical applications. Both pigments have an important role in the photosynthetic process and can be found in the marine green macroalgae genus *Ulva*. In this work, an integrated downstream process was developed to extract chlorophylls and carotenoids from those macroalgae. The solid-liquid extraction was optimized. For that, several conditions were tested, namely the use of different mechanical processes (maceration, microwave- or ultrasound-assisted extraction), type of solvent, number of consecutive extractions, solid-liquid ratio, and the design of the extraction process using a mechanical treatment. Using the extract obtained, a liquid-liquid extraction system composed of ethanol, hexane, and water was then studied. Different mixture points within the biphasic region were tested in terms of their ability to selectively fractionate chlorophylls and xanthophylls to opposite phases in a single step. The optimization and implementation of a simple, fast and efficient downstream process to separate both classes of pigments such as chlorophylls and xanthophylls from green macroalgae is reported. It could be applied to fractionate other extracts with similar compositions obtained from other natural sources.

Keywords: Green macroalgae, *Ulva rigida*, solid-liquid extraction, liquid-liquid extraction, chlorophyll, xanthophyll.

Introduction

Oceans contain nearly 200,000 identified species, but the actual number may be in the order of millions. Included in the organisms identified are the macro and microalgae. Although macroalgae are still an under-explored resource,[21] over the past few years, the commercial application of algae-based products is gaining relevance in different fields. Macroalgae have been used since long as food,[113] however, the markets are expanding and other commercial applications are envisioned.[21,114,115] Included in the most valuable bioactive compounds are the pigments, namely phycobiliproteins, chlorophylls and carotenoids, with the latest two classes of pigments being the focus of this work.

Chlorophylls are photosynthetic pigments used by photoautotrophic organisms, such as plants and algae, to absorb light and to produce, in combination with the fixation of carbon dioxide, the carbohydrates needed for the growth of plants and algae.[93] In green macroalgae, the most common types of chlorophylls present are the *a* and *b*.[116] Structurally, chlorophylls are composed by a reduced porphyrin ring, with a central magnesium atom, and a long hydrophobic tail (phytol), which confers them low solubility in water.[117] However these macrocycles cannot be considered entirely hydrophobic due to the presence of ester and carbonyl polar functions in their structures.[118] Chlorophylls and derivatives are used in the food industry as natural colourants in foods and beverages,[119] however other important features are being reported, namely their antioxidant,[94] anti-tumoral,[120,121] and antimicrobial activities. A recent study proved the possibility of chlorophylls to be used as precursors of photosensitizers for photodynamic therapy, namely for cancer treatment and inactivation of microorganisms.[122]

Carotenoids, divided into xanthophylls and carotenes,[123] are another group of photo-pigments present in brown macroalgae and green algae, although in lower amounts.[21,124] These photo-pigments absorb light at different wavelengths than those absorbed by chlorophylls, allowing the supplementation of the light captured by algae and in this way helping them to survive even at low (sun)light intensities.[21] Among the carotenoids, xanthophylls due to the presence of polar functional groups (e.g. hydroxyl, carbonyl, carboxyl or epoxide) in the polyunsaturated hydrocarbon chain are less hydrophobic molecules than carotenes with no oxygen function. These pigments are

even less hydrophobic than chlorophylls (miLog*P* around 9.8, whereas for fucoxanthin is around 8.5).[26]

Species from the genus *Ulva*, besides several other interesting compounds,[114,125,126] present a significant content of chlorophylls and carotenoids, as recently found by Abd El-Baki et al.[95] However, despite their high commercial value and the increased consumer demand for natural products, few studies report simple and efficient purification processes able to recover both classes of pigments at high purity levels, as required by some of their applications.

Various studies have been reported considering the extraction and purification of pigments from macroalgae. In case of the pigments fractionation, paper,[127] thin layer,[128] and liquid chromatographic[97] techniques are often used. Nevertheless, some are delicate, expensive, and difficult to apply at an industrial scale. A US patent from 1946[129] proposed a procedure to purify carotene from chlorophyll extracted from green leaves by the saponification of chlorophyll at high temperature after their extraction using organic solvents and an alkali salt. Then, by the addition of water a two-phase system is formed, in which the saponified chlorophyll was no longer soluble in organic solvents but in the aqueous phase, promoting thus their separation from the carotene concentrated in the organic phase. Although this procedure is quite simple, the chlorophyll content obtained at the end of the process is not ready to be used. Moreover, if in one hand, the heating step increases the cost of the overall procedure, in the other hand, it may cause pigment degradation.[129]

In this work, the extraction and separation of carotenoids and chlorophylls from the green macroalgae *Ulva rigida* was studied. A process in two steps was designed and adequately optimized considering: (i) the solid-liquid extraction of pigments, with the maximization of the chlorophylls yield, from the fresh biomass, followed by (ii) the purification of both classes of pigments using a liquid-liquid extraction system. In step (i), the parameters under optimization were the application or not of mechanical processes (maceration, microwave- or ultrasound- assisted extraction), type of organic solvent, the effect of consecutive extractions, the solid-liquid ratio, and the combination of mechanical-assisted methods to improve the action of the most efficient organic solvent. Step (ii) comprised the application of a liquid-liquid extraction system composed of two common organic solvents and water, in which the mixture point was

the main parameter studied. After optimization, a low-cost downstream process, simple, efficient, and easily scaled-up, was designed. The process here proposed (patent CI-19-006) could have a crucial role in the improvement of aquaculture infrastructures by the transfer of marine technology, following the demands of Sustainable Development Goals to Oceans (*Conserve and sustainably use the oceans, seas and marine resources for sustainable development* - Goal 14).

Experimental

Biomass

Fresh *Ulva rigida* was collected from March to June of 2016 (different batches) at a land-based integrated aquaculture system by ALGAplus Ltda, a company specialized in the production of marine macroalgae, located in Ílhavo, Portugal. ALGAplus farms *Ulva rigida* at Ria de Aveiro lagoon (40°36'44.7" N, 8°40'27.0" W) in coastal Portugal under the European Union organic aquaculture standards (EC710/2009). This aquaculture is performed in a land-based integrated multi-trophic aquaculture system (meaning that the nitrogen input is higher than in the outside natural lagoon due to the use of effluent water from fish production). After the harvesting of the macroalgae, the samples were washed and stored in a freezer at -20ºC until utilization.

Chemicals

Several organic solvents were used on the extraction of pigments from the biomass. Ethanol (CAS 64-17-5), hexane (CAS 110-54-3), dimethyl sulfoxide (CAS 67-68-5), and acetonitrile (CAS 75-05-08) were acquired from Fisher Scientific. Cyclohexane (CAS 110-82-7) and dodecane (CAS 112-40-3) were purchased from Sigma-Aldrich, while acetone (CAS 67-64-1) was purchased from VWR^{TM}. Heptane (CAS 142-82-5) and methanol (CAS 67-56-1) were acquired from Labsolve and Chem Lab, respectively. All the mentioned chemicals used are HPLC-grade.

Solid-liquid extraction

Frozen macroalgae samples were firstly grounded in liquid nitrogen (particle size < 0.5 mm) and homogenized in different pure organic solvents, in triplicate and with a solid-liquid ratio (SLR) of 0.01 $g_{fresh\ biomass}.mL_{solvent}^{-1}$. The extraction was performed in an

incubator shaker (IKA KS 4000 ic control) at 250 rpm, for 30 min at room temperature (20–25 °C) and protected from light exposure, being these initial conditions adopted from Martins et al.[57] At the end of the solid-liquid extraction, the obtained green organic-based extract was centrifuged in a Thermo Scientific Heraeus Megafuge 16R Centrifuge at 4700 *g* for 30 min at 4 °C. The pellet was discarded while the green supernatant was collected. The absorption spectra were determined for each extract in the interval between 200–700 nm in a UV-Vis microplate reader (Synergy HT microplate reader – BioTek) and the chlorophyll concentration was calculated using a calibration curve previously determined (R^2 = 0.9805). The results are depicted in terms of yield of extraction of chlorophyll ($mg_{chl}.g_{fresh\ biomass}^{-1}$).

Microwave- and ultrasound-assisted extractions

Ungrounded algae samples were used to study the microwave- and ultrasonic-assisted extraction using a SLR of 0.01 $g_{fresh\ biomass}.mL_{solvent}^{-1}$ for different organic solvents. The microwave-assisted extraction was performed using a Milestone Microsynth MLS Ethos 1600 microwave at 300 W for 1 min. The ultrasound-assisted extraction was performed using an ultrasonic bath Sonorex Digitec DT 100 for 10 min. The mentioned conditions of extraction were adopted from Picot et al[130] and adapted to avoid overheating of each system. Other times of extraction for microwave and ultrasound-assisted extraction were not studied in this work. In future works this variable should be consider, but with small extraction times to avoid overheating. After both mechanical treatments, in which temperatures were carefully controlled to avoid passing 40 °C, the green solution was centrifuged and analysed as described.

Pigments fractionation by a liquid-liquid extraction

A liquid-liquid extraction was performed at room temperature (20–25 °C) until the equilibrium is achieved using a system composed of water + hexane + pigment-rich ethanolic extract. Different mixture points, covering the biphasic region (previously described by Moriyoshi et al)[131] were studied. The content in pigments was determined for each phase by ultra-performance liquid chromatography - tandem mass spectrometer (UHPLC-MS).

Ultra-performance liquid chromatography coupled mass spectrometer (UHPLC-MS) analysis

The UHPLC-MS was performed in a Thermo Scientific LC-MS Ultimate 3000RSLC. The separation of the compounds was carried out with a gradient elution program at a flow rate of 0.3 $mL.min^{-1}$, at 30 ºC, by using a Hypersil Gold C18 column (150x2.1 mm; 5 µm, Thermo Fisher). The injection volume in the UHPLC system was 3 µL and the mobile phase consisted in formic acid 0.1 % (A) and acetonitrile (3):methanol (7) (B).

Results

In a first part of this section a solid-liquid extraction procedure to recover the pigments from *Ulva rigida* is proposed. Additionally, an effective and easy to scale-up liquid-liquid extraction is suggested to separate the chlorophylls and xanthophylls present on the extract.

Solid-liquid extraction

A screening of several organic solvents was performed to optimize the solid-liquid extraction of pigments and, particularly, chlorophylls from *Ulva rigida*. Included in the list of organic solvents tested are methanol, ethanol, hexane, dimethyl sulfoxide, acetonitrile, cyclohexane, dodecane, acetone, and heptane. These solvents were selected due to their different polarities. The Log_{Kow} was used as a hydrophobicity parameter of the screened solvents, in which K_{ow} = octanol/water partition coefficient.[132,133]

Despite the interest in both classes of pigments, carotenoids and chlorophylls, the latest class was focused considering the solid-liquid extraction. Thus, the first step in this work was the application of the various organic solvents in the extraction of chlorophylls from the fresh biomass, being the yields of extraction depicted in Figure 3.1.1. UV-Vis spectra of the extracts obtained in the screening of organic solvents are depicted in Figure B.1 in Appendix B. Concentration of chlorophylls and respective yields of extraction are presented in Table B1 in Appendix B.

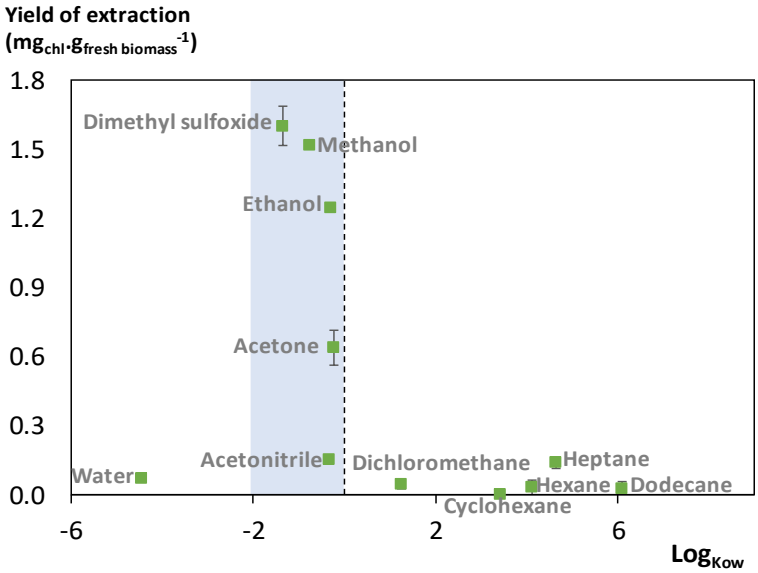


Figure 3.1.1. Yield of extraction ($mg_{chl}.g_{fresh\ biomass}^{-1}$) of chlorophyll obtained by the application of different organic solvents by the Log_{Kow} value of the respective pure solvents (depicted in literature).[132,133] K_{ow} of water was theoretically estimated.

According to the results obtained, the solvents with very high and very low values of Log_{Kow} (*i.e.* highly hydrophobic and hydrophilic solvents, respectively) have provided the lowest yields of extraction. In the other hand, solvents with Log_{Kow} between -2 and 0, performed the best results in terms of yields of extraction. These solvents are amphiphilic compounds that can interact with both chlorophylls and xanthophylls, that are not completely hydrophobic due to the presence of polar functionalities as mentioned above. Moreover, the enhancement in the yields of extraction with the polar solvents can also be associated to their increased capacity to disrupt the algae cells allowing the release of the target compounds. Indeed, solvents such as acetone, methanol, and ethanol are known for dissolving cell wall membranes, a mechanism that can strongly favour the yield of extraction.[134,135]

The set of the four solvents identified as the most efficient was used in further studies. Firstly, consecutive extractions were conducted to investigate the eventual solvent saturation (Figure 3.1.2). In this context, after the first solid-liquid extraction, the remaining biomass was recovered, homogenized and reused in a new cycle of extraction, with the results presented in Figure 3.1.2.

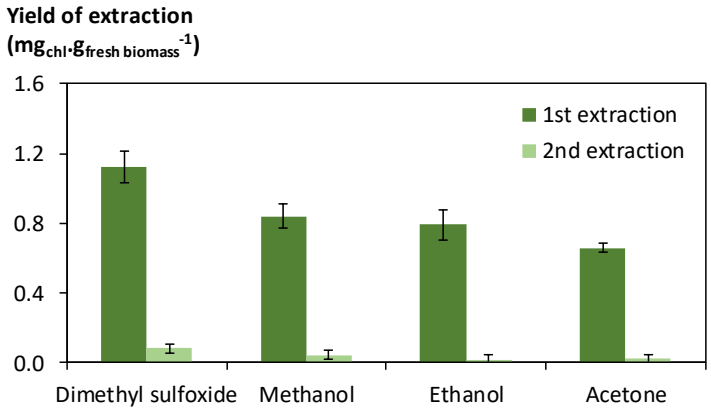


Figure 3.1.2. Yield of extraction of chlorophyll considering two consecutive extractions done for the same biomass using different solvents.

For all solvents, only a residual amount of chlorophylls was extracted in the second cycle. Thus, the solvent saturation does not seem to be happening in the first extraction, being the second extraction step not significant. However, it is important to notice that not all chlorophylls are being extracted, indicating the need for further optimization of the operational conditions in order to remove the highest possible chlorophylls content in a single extraction step. At the same time, some organic solvents are known for their toxic character and their safety issues at an industrial scale. For instance, the exceptionally high boiling temperature of dimethyl sulfoxide (189 °C)[136] makes it tricky to remove. Given these concerns and the insignificant difference among the yields of extraction provided by methanol and ethanol, ethanol was selected for further process optimization due to its green and sustainable nature.[2] In this sense, the SLR effect was studied between 0.05 and 0.015 $g_{fresh\ biomass}.mL_{solvent}^{-1}$, being the results presented in Figure 3.1.3. The yield of extraction increased with the SLR until 0.01 $g_{fresh\ biomass}.mL_{solvent}^{-1}$. Higher SLR do not seem to provide better results, being this parameter fixed at 0.01 $g_{fresh\ biomass}.mL_{solvent}^{-1}$.

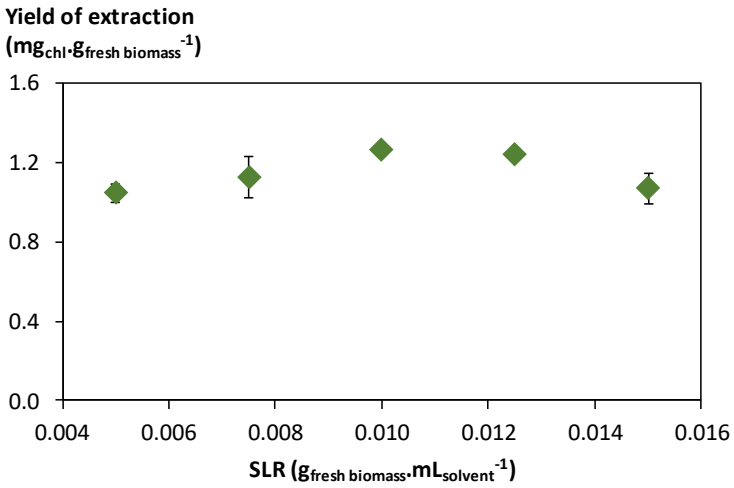


Figure 3.1.3. SLR effect on the yield of extraction of chlorophyll.

As described in the experimental part, in the standard methodology, liquid nitrogen was used to freeze the macroalgae samples, to facilitate the biomass milling and the cell wall breakage. Nevertheless, the associated costs with the use of liquid nitrogen, especially at industrial scale, makes this process less attractive.

Microwave- and ultrasound-assisted extractions were performed using intact biomass (which did not suffer any milling or maceration, and without the use of liquid nitrogen) and under the best conditions previously found for the appropriate solvent (ethanol) and SLR (0.01 $g_{fresh\ biomass}.mL_{solvent}^{-1}$) - Figure 3.1.4. UV-Vis spectra of the extracts are depicted in Figure B.2 in Appendix B. Concentration of chlorophyll and respective yields of extraction are presented in Table B2 in Appendix B.

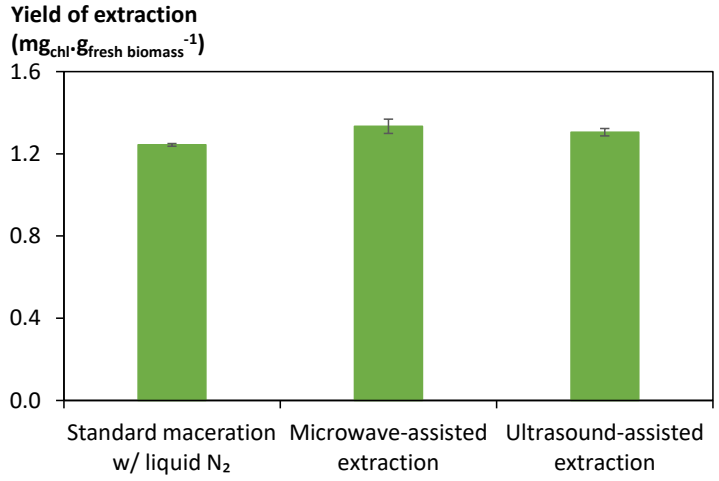


Figure 3.1.4. Yield of extraction of chlorophyll considering the optimized standard methodology using biomass previously grounded with liquid nitrogen and without

mechanical treatment, in comparison with the microwave- and ultrasound-assisted extractions using intact biomass.

Similar yields of extraction were achieved for all procedures, indicating that radiation treatments might also be promoting cell wall breakage, allowing the complete solubilization of chlorophyll in the solvent. It should be highlighted that according to the procedures used, the time of extraction went from 30 min with the conventional extraction, down to 10 and to 1 min with ultrasound and microwave treatments, respectively, being these approaches more appropriate to be applied from an industrial point of view. However, microwave-assisted extraction lead to an increase in temperature, which can compromise the viability of the pigments. In this sense, ultrasonic-assisted extraction using ethanol as solvent was preferred.

After the complete optimization of the solvent and process conditions, the ultrasonic-assisted ethanolic extract rich in pigments (particularly chlorophyll) was characterized by UHPLC-MS. The identification was based on a direct comparison of their retention times, UV–Vis spectra, and mass spectra data with reference standards and data reported in literature. The data and molecular structures of the proposed compounds obtained are depicted in Table 3.1.1 and Figure B.3 in Appendix B, respectively.

Table 3.1.1. Compounds present in the ultrasonic-assisted ethanolic extract from *Ulva rigida* and their molecular ions species (*m/z*) data.

Compound	**Retention time (min)**	**UV-Vis (nm)**	**Mass (*m/z*)**	**Molecular structure**
Chlorophyll *a*	12.02	416, 430, 663	$[M + H]^+$ 893	Figure B.3 (i)
Xanthophyll	12.57	422, 444, 472	569	---
Chlorophyll *b*	18.46	450, 650	$[M + CH_3OH + H]^+$ 939	Figure B.3 (ii)
Chlorophyll *b* derivative	19.45	460, 651	$[M + H]^+$ 923	Figure B.3 (iii)
Chlorophyll *b*	20.60	452, 552, 586, 635	$[M + HCO_2H + H]^+$ 953	Figure B.3 (ii)
Chlorophyll *b* derivative	21.99	453, 636	$[M + 2Na]^+$ 967	Figure B.3 (iv)

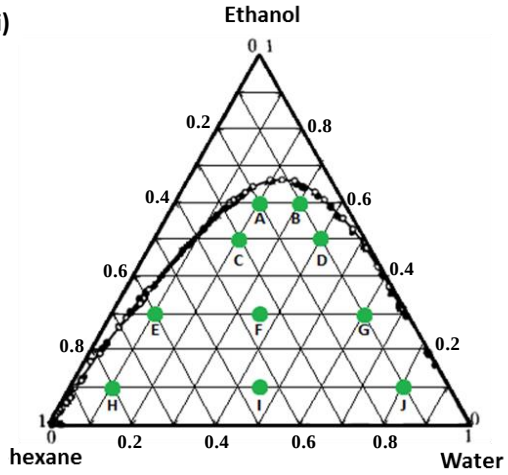
Different compounds were found in the raw extract, mainly chlorophyll *b*, xanthophyll, chlorophyll *b* derivatives, and chlorophyll *a* contributing with abundances of 52, 24, 18, and 6 %, respectively. The ionic species are a result of the UHPLC-MS analysis conditions, which are in positive mode so ions such as $[M + H]^+$, $[M + Na]^+$ and $[M + K]^+$ are usually the detected species. The chlorophyll derivatives can be formed during the extraction process due to the solvent, light and/or oxygen exposure. Since MS^2 was not performed at this stage, xanthophylls is not precisely identified, but considering the results found in literature,[137] this should be lutein or zeaxanthin.

Pigments separation by liquid-liquid extraction

Taking into account the UHPLC-MS results shown in Table 3.1.1, it is clear the presence of different classes of pigments, namely chlorophylls and xanthophylls and some of their derivatives on the extract, which demands the development of a separation step in order to separate chlorophylls from xanthophylls.

Aiming to design a process of extraction and purification of pigments, a liquid-liquid extraction system was applied, considering the pigment-based ethanolic extract rich in pigments as the basis. Systems combining the pigment-based ethanolic extract, hexane and water were experimentally prepared. Ten different mixture points were tested (Figure 3.1.5). The biphasic region was studied, and for all systems the two-phase formation was confirmed, as shown in Figure 3.1.5(iii).

(i)



(ii)

System	Water (%)	*n*-hexane (%)	Ethanolic extract (%)
A	20.0	20.0	60.0
B	30.0	10.0	60.0
C	20.0	30.0	50.0
D	40.0	10.0	50.0
E	10.0	60.0	30.0
F	35.0	35.0	30.0
G	60.0	10.0	30.0
H	10.0	80.0	10.0
I	45.0	45.0	10.0
J	80.0	10.0	10.0

(iii)

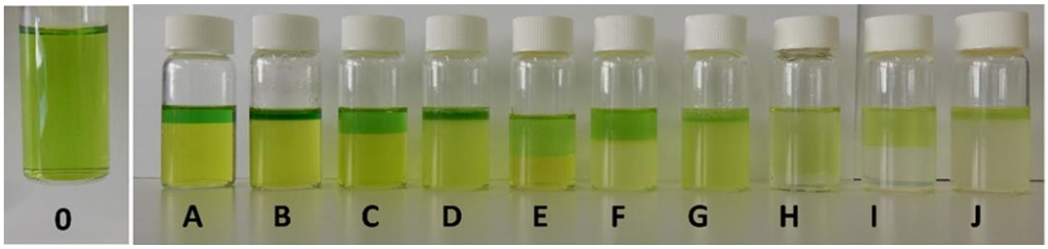


Figure 3.1.5. (i) Phase diagram and (ii) mixture points tested for pigments fractionation based on the phase diagram (adapted from Moriyoshi et al)[131] of the mixture water + ethanol + hexane. (iii) Photograph of the liquid-liquid extraction systems tested (from A to J) prepared with the pigment-based ethanolic extract (0) obtained from the ultrasound-assisted extraction.

All systems studied differ essentially in the volume ratio and pigmentation content, being the top phases preferably greenish denoting, the presence of chlorophylls and the yellow colour of the bottom phases representing the presence of xanthophylls (Figure 5). After the phase separation, both the top and bottom phases were recovered and analysed by UHPLC-MS. The data is shown in Table 3.1.2 and Figure 3.1.6 and structures are identified in Figure B.3 in Appendix B.

Table 3.1.2. Characterization of both top and bottom phases obtained after the fractionation of pigments, their molecular ions species and fragments (*m/z*) data. Green background means systems with complete separation of chlorophylls and xanthophylls, and red background means systems not completely pure.

System	Fraction	Compound	Retention time (min)	UV-Vis (nm)	Mass (*m/z*)	Molecular structure
A	Top	Chlorophyll *a*	12.85	431, 471, 663	$[M + Na]^+$ 915	Figure B.3 (i)
		Chlorophyll *b*	19.26	420, 439, 463, 653	$[M + K]^+$ 945	Figure B.3 (ii)
		Chlorophyll *b* derivative	20.09	420, 439, 463, 653	$[M + Na]^+$ 959	Figure B.3 (v)
		Chlorophyll *b* derivative	20.43	439, 483, 654	$[M + Na]^+$ 959	Figure B.3 (v)
		Chlorophyll *b*	22.09	463, 600, 653	$[M + Na]^+$ 929	Figure B.3 (ii)
	Bottom	Xanthophyll	12.58	440, 472	568	---
B	Top	Chlorophyll *a*	12.59	431, 471, 663	$[M + Na]^+$ 915	Figure B.3 (i)
		Chlorophyll *b*	21.71	463, 649	$[M + Na]^+$ 929	Figure B.3 (ii)
		Chlorophyll *b* derivative	26.50	428, 672	$[M + Na]^+$ 931	Figure B.3 (vii)

	Bottom	Xanthophyll	12.57	444, 473	658	---
C	Top	Chlorophyll *a*	12.68	469, 664	$[M + Na]^+$ 915	Figure B.3 (i)
		Chlorophyll *b*	14.20	462, 647	$[M + Na]^+$ 929	Figure B.3 (ii)
		Chlorophyll *a*	15.85	430, 663	$[M + H]^+$ 893	Figure B.3 (i)
		Chlorophyll *b* derivative	24.07	463, 649	$[M + H_2O + 2K]^+$ 725	Figure B.3 (viii)
	Bottom	Xanthophyll	8.61	460, 472	568	---
		Xanthophyll	12.53	440, 470	551	---
D	Top	Chlorophyll *a* derivative	12.63	442, 472, 663	$[M + HCO_2H + K]^+$ 993	Figure B.3 (vi)
		Chlorophyll *b*	18.98	462, 649	$[M + K]^+$ 945	Figure B.3 (ii)
		Chlorophyll *b*	21.71	463, 648	$[M + Na]^+$ 929	Figure B.3 (ii)
		Chlorophyll *b* derivative	26.50	429, 663	$[M + Na]^+$ 931	Figure B.3 (vii)
	Bottom	Xanthophyll	12.56	460, 472	568	---
E	Top	Chlorophyll *a*	11.80	429, 663	$[M + Na]^+$	Figure B.3 (i)

					915	
		Chlorophyll *b*	19.29	463, 647	$[M + K]^+$ 945	Figure B.3 (ii)
		Chlorophyll *b* derivative	20.11	462, 649	$[M + Na]^+$ 959	Figure B.3 (v)
		Chlorophyll *b* derivative	20.44	460, 662	$[M + Na]^+$ 959	Figure B.3 (v)
		Chlorophyll *b* derivative	27.04	460, 662	$[M + Na]^+$ 931	Figure B.3 (vii)
		Chlorophyll *b*	28.91	410, 422, 662	$[M + K]^+$ 945	Figure B.3 (ii)
	Bottom	Xanthophyll	12.93	420, 442, 472	568	---
		Xanthophyll	22.52	419, 438, 455	585	---
F	Top	Xanthophyll	12.86	443, 471	551	---
		Chlorophyll *b*	19.28	461, 648	$[M + K]^+$ 945	Figure B.3 (ii)
		Chlorophyll *b* derivative	20.10	462, 649	$[M + Na]^+$ 959	Figure B.3 (v)
		Chlorophyll *b* derivative	20.43	457, 645	$[M + Na]^+$ 959	Figure B.3 (v)
		Chlorophyll *b* derivative	27.04	414, 429, 663	$[M + Na]^+$ 931	Figure B.3 (vii)

	Bottom	Chlorophyll *a*	12.19	430, 663	$[M+HCO_2H+H]^+$ 940	Figure B.3 (i)
		Chlorophyll *a*	17.16	429, 480, 663	$[M + H]^+$ 893	Figure B.3 (i)
G	Top	Xanthophyll	12.86	424, 442, 471	551	---
		Chlorophyll *b*	19.27	415, 462, 646	[M + K]+ 945	Figure B.3 (ii)
		Chlorophyll *b*	22.09	462, 649	[M + Na]+ 929	Figure B.3 (ii)
		Chlorophyll *b*	22.31	463, 649	[M + Na]+ 929	Figure B.3 (ii)
		Chlorophyll *b* derivative	27.03	463, 652	[M + Na]+ 931	Figure B.3 (vii)

Due to the complexity of Table 3.1.2, the results were organized by mixture point (from A to G), considering the representation of their pigments composition and respective abundancies, for both top and bottom phases (Figure 3.1.6). To help the analysis, the green bars were defined as representing chlorophylls *a*, *b* and derivatives and in yellow are represented the xanthophylls.

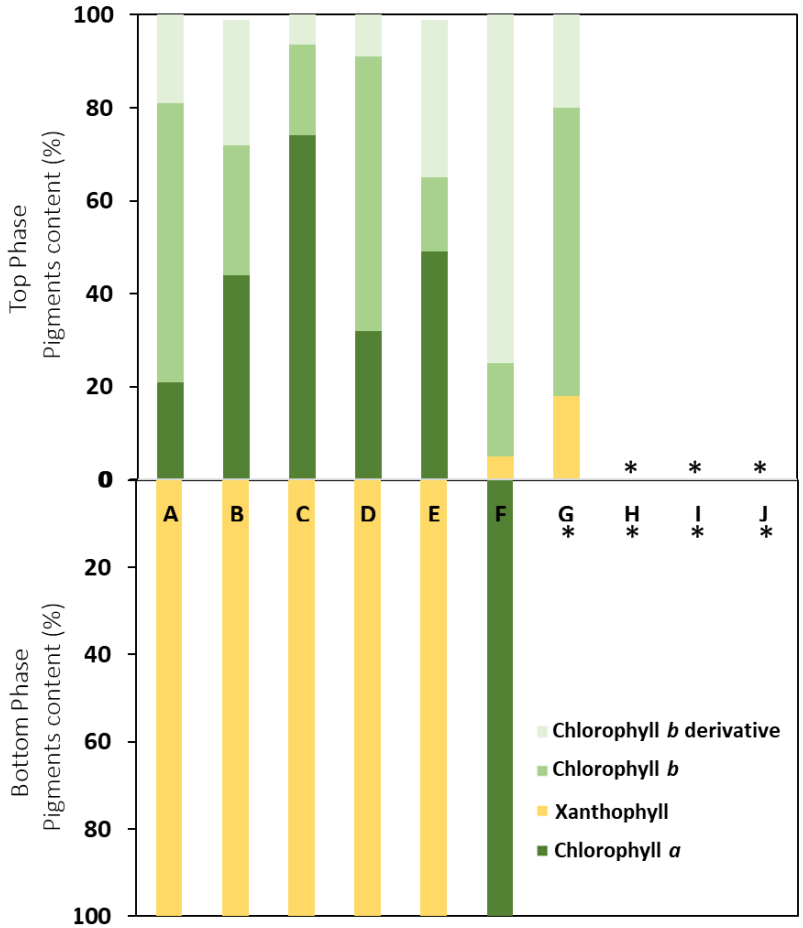


Figure 3.1.6. Total content of each pigment and derivatives identified for both top and bottom phases obtained after the application of the different liquid-liquid extraction systems. Presented results based on surface area peaks.

Note: Fractions identified with * were not analysed by UHPLC-MS due to their very low concentration in pigments, making these systems not particularly interesting for further application.

As already identified in the ultrasonic-assisted extract, the fractions obtained after the application of liquid-liquid extraction systems are essentially composed of xanthophyll, chlorophylls *a* and *b* and chlorophyll-derivatives. The results suggest the preferential partition of chlorophylls to the top phase, the hexane-rich phase, while the xanthophylls

stay preferentially in the ethanol-rich (bottom) phase, as pointed out in Figure 3.1.6. This behaviour does not follow the trend reported by Wall and Kelley[129] due to some significant differences. In their patent, the chlorophylls partition to the phase miscible in water due to their saponification. Chlorophylls, after alkaline saponification in which the ester groups are hydrolyzed and converted into salts, have then a higher affinity to the most hydrophilic phases. At the same time, the alkaline pH of this phase is forcing the partition of carotenoids to the most hydrophobic phase.[129] The process presented in this work is much simpler as it does not require a saponification. This fractionation can be explained by the more hydrophobic nature of chlorophylls allowing them to partition preferentially to the less polar phase while xanthophylls, which are less hydrophobic, partition to ethanol/water phase, the layer with higher polarity.

Moreover, analysing the systems A to E carefully, it is possible to identify the complete separation of chlorophylls and xanthophylls to distinct phases, purifying each class of pigments. Besides, in some cases (i.e. systems B and D) it is possible to concentrate the chlorophyll content in very small fractions without compromising their separation performance. Despite the efficient separation of chlorophylls and xanthophylls achieved by some liquid-liquid extraction systems, in some cases, some chlorophyll derivatives were formed. Chlorophylls have a chemically unstable molecule in the presence of oxygen, light, temperature, and/or type of solvent. Systems A, C, and D have less than 20 % of chlorophyll-derivatives. However, the best system seems to be system C composed of 50 % of ethanolic extract + 30 % of hexane + 20 % of water, in which only a tiny percentage of chlorophyll derivative was identified (≈ 6 %), meaning that the chlorophyll structures are pure and chemically intact.

Final downstream process

Considering the study on the optimization of both extraction and purification steps to obtain pure chlorophylls and xanthophylls, a final conceptual downstream process was designed, as depicted in Figure 3.1.7. It was achieved by the integration of three main tasks, starting by the (i) ultrasound-assisted solid-liquid extraction of pigments using ethanol (SLR of 0.01 $g_{fresh\ biomass}.mL_{solvent}^{-1}$), followed by the (ii) fractionation of chlorophylls and xanthophylls by applying a liquid-liquid extraction system composed of 50 % of ethanolic extract + 30 % of hexane + 20 % of water, and ending with the (iii)

solvents recycle and reuse by using a vacuum dryer with low pressures and temperatures, being this last step a proposal of what can be done in large scales. With the process envisaged, pure fractions in xanthophylls and chlorophylls could be obtained at the end. In the polishing step of pigments from both top and bottom phases, evaporation units were not considered in this process, due to the high sensitivity of chlorophylls[138] and xanthophylls[139] to the range of temperatures required to evaporate hexane (68 °C)[140], ethanol (around 78 °C)[141], and water (100 °C). Instead, it was considered the application of a vacuum dryer chamber with low pressure and temperature (≈ 35 °C), allowing the recovery of the pigments as powder without compromising their stability.

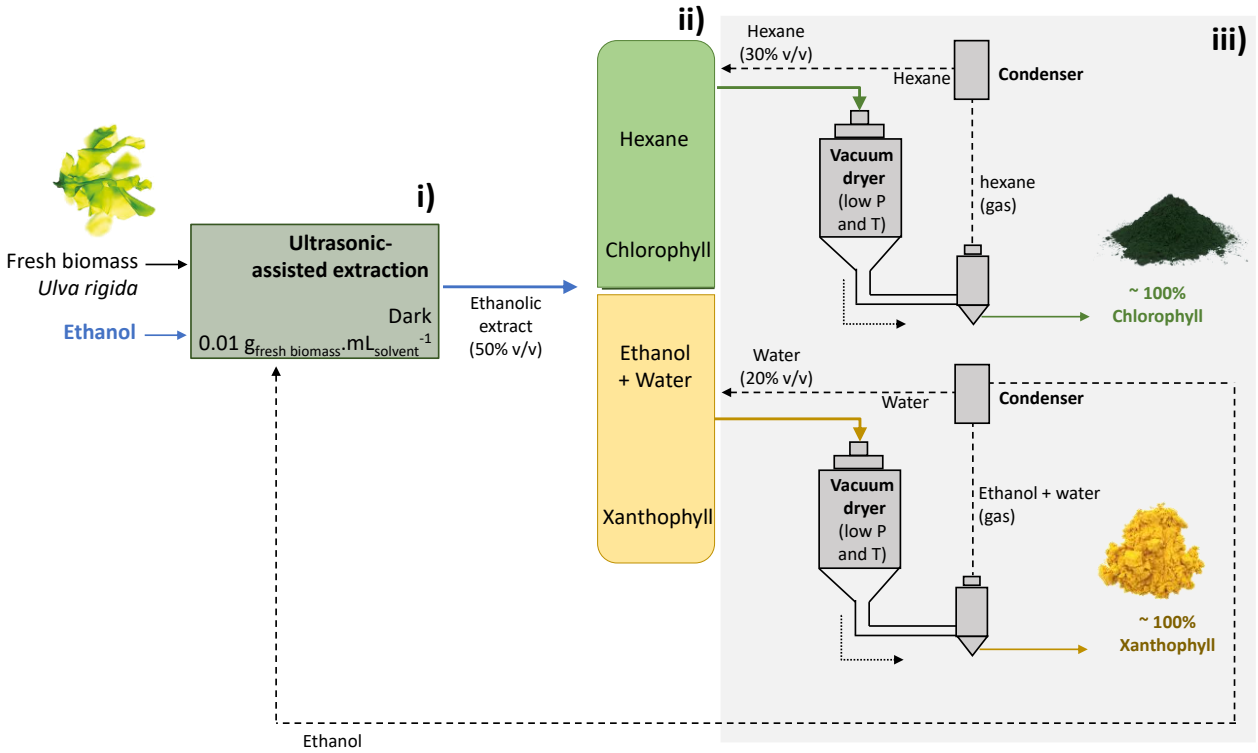


Figure 3.1.7. Downstream process diagram comprising the (i) solid-liquid extraction of pigments by ultrasound-assisted extraction with ethanol, (ii) pigments purification by applying liquid-liquid extraction, and (iii) pigments polishing and solvents recycle by vacuum drying using low pressure and temperature to avoid pigment degradation. The polishing of pigments and solvents (grey area in the figure) is just a proposal of what can be done industrially, not being tested in this work.

The ethanol/water mixture could be also separated by evaporation and each solvent reintroduced correctly in the process but only if demanded by the final application of each class of pigments. Both the vacuum dryer and evaporation are not only efficient in promoting the polishing of the pigments and reuse of the solvents, but they are also fast and feasible processes at both bench and industrial scales. This is a very simple, fast, and easy to scale-up process. Additionally, it can be used to extract and fractionate xanthophylls and chlorophylls from any natural source or raw material with similar pigment contents.

Conclusions

Pigments like chlorophylls and xanthophylls have an endless number of applications, for which a high purity level is demanded. In this work, an integrated downstream process was designed by the integration of three main tasks, starting by the (i) ultrasound-assisted solid-liquid extraction of pigments using ethanol (10 min, SLR of 0.01 $g_{fresh\ biomass}.mL_{solvent}^{-1}$), followed by the (ii) separation of chlorophylls and xanthophylls by applying liquid-liquid extraction system composed of 50 % (v:v) of ethanolic extract + 30 % (v:v) of hexane + 20 % (v:v) of water, and ending with the (iii) pigments polishing and solvents recycle and reuse by spray drying the phases. In the end and with the process envisaged, pure fractions in xanthophylls and chlorophylls were obtained. In summary, this work allowed the optimization and implementation of a simple, fast and efficient downstream process to separate hydrophobic classes of pigments, with industrial application and that could also be applied to the fractionation of other extracts with similar compositions from other natural sources and raw materials, like brown macroalgae, microalgae, and cyanobacteria.

3.2 Recovery of chlorophyll *a* derivative from *Spirulina maxima*, its purification and photosensitizing potential

This chapter is based on the published manuscript

Margarida Martins, Cristiana M. Albuquerque, Cátia F. Pereira, João A.P. Coutinho, M. Graça P.M.S. Neves, Diana C.G.A. Pinto, M. Amparo F. Faustino, Sónia P.M. Ventura,* "Recovery of chlorophyll *a* derivative from *Spirulina maxima*, its purification and photosensitizing potential", ACS Sustainable Chemistry & Engineering, 2021, DOI: 10.1021/acssuschemeng.0c07880.

*Contributions: M.M., C.M.A., and C.F.P. acquired the experimental data. M.M. performed the data analysis. M.A.F.F., M.G.P.M.S.N., and D.C.G.A.P. assessed the analysis of UHPLC-MS data while M.A.F.F. assessed the photodynamic potential assays. M.M. wrote the manuscript with substantial contributions from the remaining authors.

Abstract

Spirulina sp. is a cyanobacterium rich in the essential amino acids and pigments such as chlorophyll *a*, xanthophylls, and phycocyanin. Besides many other applications, chlorophyll *a* and its derivatives are being studied as photosensitizers in photodynamic therapy for cancer treatment. In this work, two methodologies of solid-liquid extraction were developed, and their performance compared, one using conventional organic solvents and the other using aqueous solutions of ionic liquids (ILs) and surfactants. It was found that an aqueous solution of an ammonium-based ionic liquid was able to increase the yield of extraction of chlorophyll a from Spirulina maxima in 25 % when compared to the conventional methodology using methanol. Besides, the proposed alternative methodology allows the separation of chlorophyll a from xanthophylls using a simple liquid-liquid extraction. The IL can be recovered by back-extraction using ethyl acetate, while the chlorophyll-derivative is shown to retain its ability to generate oxygen singlets, which is essential to its potential application as photosensitizer in photodynamic therapy.

Keywords: *Spirulina* sp., pigments, ionic liquids, extraction, fractionation, photodynamic therapy.

Introduction

Spirulina is a prokaryotic and photosynthetic cyanobacterium,[142] which has been consumed by humans for centuries. Besides its small size, fast and easy growth,[143] particular attention has been paid to its high nutritional value.[144] It is composed of high protein content, with all essential amino acids present,[145] high concentration of vitamins, essential fatty acids and minerals,[146] being thus recognized as a "superfood".[145] *Spirulina* also has an interesting pigment composition namely in chlorophyll *a*, xanthophylls, and phycocyanin. Regarding its content in chlorophyll, and contrarily to other plants/algae, it only has chlorophyll *a* on its composition.[147]

Chlorophyll *a* is one of the most abundant pigments in natural systems with an important role for sunlight-absorption, energy-transference, and electron-transport during the photosynthetic process.[93] Due to its photophysical and photochemical properties, chlorophyll *a* and its derivatives have been used in different fields, namely as colourants for food, as optically active centers to be applied on luminescent solar concentrators,[23,101] and more recently, as photosensitizers in photodynamic therapy.[122,148,149]

Photodynamic therapy is a technique used to kill malignant cells by apoptosis and/or necrosis.[150] It is based on the administration of a molecule known as the photosensitizer (not toxic in the absence of light), followed by the simultaneous incidence of harmless visible light that, combined with molecular dioxygen, will allow the formation of cytotoxic reactive oxygen species leading to the tumoral cell death.[151] Despite the increased progress of this field, the photodynamic therapy potential is still not being fully explored. It is recognized that an important issue in this approach is the structural features of the photosensitizer and its efficacy to generate reactive oxygen species (e.g. singlet oxygen $^1O_2$). Another important issue is related with the photosensitizer accessibility and the exploration of chlorophylls used on their own or after further derivatization is attracting the interest of the scientific community. So, some natural dyes, namely chlorophyll *a* and its derivatives have been applied on this therapeutic technique.[122,148,149] Since *Spirulina* has high amounts of chlorophyll *a* and this is the only being produced, it becomes a good natural source for the production of this compound. However, high purity levels are demanded for this compound and, in this sense, an effective and economically viable process of extraction and purification is needed. The

processes reported so far on literature[152,153] seem to fail in the sustainability criteria, regarding the need for mild conditions while maintaining high yields of extraction, and an economically viable approach to the purification of these green pigments.[38,154] The conventional processes are usually associated to low yields of extraction with very low selectivities.[104,105]

To enhance the sustainability of extraction and purification processes, some authors have been investigating the use of aqueous solutions. However, one of the major difficulties in the development of aqueous processes to extract chlorophylls is their well-recognized hydrophobic nature and, consequently, their insolubility in water.[155] To overcome this issue, recent studies have attempted the extraction of hydrophobic pigments from plants and macroalgae by using aqueous solutions of common surfactants and tensioactive ionic liquids (ILs).[57,101,104] ILs are recognized as designer solvents since their tunability can be achieved by varying the functional groups or alkyl chain length. This feature allows the design of the most appropriate ILs to a specific application, or in this case, to the selective extraction of a biomolecule.[65] Besides, their varied solubility in water and solvent capacity towards hydrophobic and hydrophilic compounds, makes them good candidates to be used as solvents even in the recovery of low water-soluble compounds.[58,101] This is not only due to the solubility of the target compound in the solvent, but also due to the solvent ability to disrupt the cell membranes.[57,68]

The recovery of chlorophyll *a* from *Spirulina maxima* cells was performed using aqueous solutions of tensioactive compounds to organic solvents. The organic solvent-based extractions were used to compare the performance of the conventional and alternative methods under study. The type and concentration of the tensioactive compounds and the extraction time were also investigated. The extracts rich in chlorophyll *a* obtained after a back-extraction were analysed and the photostability and the efficacy of chlorophyll *a* extracts to generate singlet oxygen were evaluated thinking on their future application in photodynamic therapy studies as photosensitizers.

Experimental

Biomass

Distinct batches of *Spirulina maxima* distributed by Ely Martins from São Paulo (Brazil) were used in this work.

Chemicals

Several organic solvents were used to test their ability to extract chlorophyll *a* from the biomass. Ethanol (CAS 64-17-5), hexane (CAS 110-54-3), dimethyl sulfoxide (CAS 67-68-5), acetonitrile (CAS 75-05-08), acetone (CAS 67-64-1), and methanol (CAS 67-56-1) were acquired from Fisher Scientific. Cyclohexane (CAS 110-82-7) and octanol (CAS 111-87-5) were purchased from Sigma-Aldrich. All described organic solvents are HPLC grade.

The series of 1-alkyl-3-methylimidazolium chloride-based ILs [$C_nC_1$im]Cl, including the 1-ethyl-3-methylimidazolium chloride ([C_2C_1im]Cl, 98 wt%, CAS 65039-09-0), 1-butyl-3-methylimidazolium chloride ([C_4C_1im]Cl, 99 wt%, CAS 79917-90-1), 1-hexyl-3-methylimidazolium chloride ([C_6C_1im]Cl, 98 wt%, CAS 171058-17-6), 1-methyl-3-octylimidazolium chloride ([C_8C_1im]Cl, 99 wt%, CAS 64697-40-1), 1-decyl-3-methylimidazolium chloride ([$C_{10}C_1$im]Cl, 98 wt%, CAS 171058-18-7), 1-dodecyl-3-methylimidazolium chloride ([$C_{12}C_1$im]Cl, > 98 wt%, CAS 171058-18-7), 1-methyl-3-tetradecylimidazolium chloride ([$C_{14}C_1$im]Cl, 98 wt%, CAS 171058-21-2), 1-hexadecyl-3-methylimidazolium chloride ([$C_{16}C_1$im]Cl, 98 wt%, CAS 61546-01-8) as well as tetrabutylphosphonium chloride ([$P_{4,4,4,4}$]Cl, 99 wt%, CAS 2304-30-5) and tributyltetradecylphosphonium chloride ([$P_{4,4,4,14}$]Cl, 95 wt%, CAS 81741-28-8) were acquired from IoLiTec (Ionic Liquids Technology, Germany). The hexyltrimethylammonium bromide ([$N_{1,1,1,6}$]Br, 98 wt%, CAS 2650-53-5) and octyltrimethylammonium bromide ([$N_{1,1,1,8}$]Br, 98 wt%, CAS 2083-68-3) were purchased from Alfa Aesar and Tokyo Chemical Industry, respectively. Other ILs and surfactants such as tetradecyltrimethylammonium bromide ([$N_{1,1,1,14}$]Br, 99 wt%, CAS 1119-97-7), sodium dodecyl sulfate (SDS, 99 wt%, CAS 151-21-3), polyoxyethylene(8) octylphenyl ether (Triton X-114, 100 wt%, CAS 9002-93-1), cholinium chloride ([$N_{1,1,1,2(OH)}$]Cl, 98 wt%, CAS 67-48-1) were provided by Acros Organics while dodecyltrimethylammonium bromide ([$N_{1,1,1,12}$]Br, > 98 wt%, CAS 1119-94-4), tetrabutylammonium chloride ([$N_{4,4,4,4}$]Cl, 97 wt%, CAS 1112-67-0), hexadecylpyridinium chloride ([C_{16}py]Cl, 99 wt%),

polyethylene glycol dodecyl ether (Brij L4, 99 wt%, CAS 9002-92-0), polyethylene glycol sorbitan monolaurate (Tween 20, purity information not available, CAS 9005-64-5), and polyethylene glycol sorbitan monooleate (Tween 80, purity information not available, CAS 9005-65-6) were supplied by Sigma-Aldrich. All molecular structures of ILs and common surfactants used in this work are presented in Figure C.1 in Appendix C.

Solid-liquid extraction

Two different approaches were established to extract chlorophyll *a* (the unique chlorophyll produced by the *Spirulina* cells) from the dry cells of *Spirulina maxima*: a conventional methodology, in which the solvents used were volatile organic solvents, and an alternative method using as solvents aqueous solutions of ILs and surfactants. Initially, both conventional and alternative extractions were performed with the same initial operational conditions: a solid-liquid ratio (SLR) of 0.025 $g_{dry\ biomass}.mL_{solvent}^{-1}$, at room temperature (20–25 ºC) and constant stirring (50 rpm) during 30 min in an orbital mixer. Regarding the solvent concentration, the extractions were performed using aqueous solutions of the alternative solvents at 250 mM,[156] while in the case of the organic solvents they were used at their pure state. Although these conditions were applied in an initial screening, both approaches were optimized in operational conditions. To optimize the operational conditions, individual extractions (in triplicate) were done considering each condition under analysis. Firstly, fresh solutions of IL at different concentrations were used and after, for the optimum concentration of IL in water, different times of extraction were tested. At the end of the extractions, the cells' suspensions were centrifuged at 14000 *g* for 10 min in a VWR microstar 17 centrifuge, and the pellet containing the cellular debris was discarded. The supernatant absorption spectra of all organic and aqueous extracts were determined between 200-700 nm in a microplate reader (Synergy HT microplate reader – BioTek). The chlorophyll quantification was done according to a calibration curve previously established for 667 nm (calibration curves are depicted in Figure C.2 in Appendix C) in the same UV-Vis equipment. All the assays were carried out in triplicate.

Response Surface Methodology using methanol as solvent

The time of extraction and SLR were the conditions tested simultaneously using a Response Surface Methodology. Using this methodology, it is possible to study different conditions simultaneously and find the relation between the independent and dependent variables, meaning between the operational conditions and the yield of extraction of chlorophyll *a*, respectively.

The optimization of the process was done by applying a central composite rotatable design (CCRD, 2^2) totalizing 11 extractions, including four extractions for factorial points, four extractions for axial points, and three repetitions of the central point, according to Table C.1 in Appendix C. To guarantee the accuracy of the data, the results were statistically analysed considering a confidence level of 95 %. The adequacy of the model was determined. The statistical analysis and preparation of the response surface and contour plots were done using the Statsoft Statistica 8.0© software.

Chlorophyll purification by liquid-liquid extraction

To allow the purification of chlorophyll *a*, after the solid-liquid extraction, a liquid-liquid extraction system was applied. For the most performant system of extraction, ethyl acetate was used. The system was composed of 1:1 (v:v) of ethyl acetate and the aqueous solution of the alternative solvent selected, and after the phase equilibrium, both organic and aqueous phases were separated. The chlorophyll *a* content was recovered in the ethyl acetate (top) phase and this process was repeated three times until no more pigments moved from the aqueous layer (bottom phase) to the organic phase. After the phase separation, the recovered fractions were analysed by UHPLC-MS and the purity level of the chlorophyll *a*-based product was also evaluated.

IL quantification

The $[N_{1,1,1,14}]Br$ was measured by using an ion-selective electrode (Metrohm) able to detect the bromide anion (electrode reference: 6.0502.100) in comparison with a calibration curve previously prepared (R^2 = 0.998).

Ultra-performance liquid chromatography coupled mass spectrometer (UHPLC-MS) analysis

The UHPLC-MS analysis was performed in a Thermo Scientific LC-MS Ultimate 3000RSLC. The separation of the compounds was carried out with a gradient elution program at a flow rate of 0.3 $mL.min^{-1}$, at 30 °C, using a Hypersil Gold C18 column (150 x 2.1 mm; 5 µm, Thermo Fisher). The injection volume in the UHPLC system was 3 µL and the mobile phase consisted of formic acid 0.1 % (A) and acetonitrile (3):methanol (7) (B).

Photostability assays

The extracts were irradiated in a quartz cuvette of 1 cm path length with red light (λ = 630 ± 20 nm) delivered by a homemade 5 x 5 light emitting diode (LED) array at an irradiance of 9 $mW.cm^{-2}$. The irradiation was run in dimethylformamide at room temperature under gentle magnetic stirring agitation. The irradiance was measured with an energy meter Coherent FieldMax-II-Top combined with a Coherent PowerSens PS19Q energy sensor. The Soret band absorption (~ 412 nm) was registered at 0, 5, 15, 30 and 60 min in a SHIMADZU UV-Vis spectrometer UV-2501 PC. The photostability was expressed as the ratio between the intensity of the Soret band at a given time of irradiation (I_t) and its intensity before irradiation (I_0), in percentage.

Singlet oxygen generation

The efficacy of the extracts to generate singlet oxygen was assessed using 9,10-dimethylantracene as a scavenger of this reactive oxygen species, following a previously reported procedure.[157,158] The extract samples in dimethylformamide and in the presence of 9,10-dimethylantracene were irradiated with blue light (412 ± 2 nm), in a 1 cm path length quartz cell. The photooxidation rate was assessed in 60 s intervals by following the decrease of 9,10-dimethylantracene absorbance at 378 nm. The Zn(II)chlorin-e6 dimethyl ester was used as a reference since it is a known $^1O_2$ producer.[148] A 9,10-dimethylanthracene solution was also irradiated in the absence of the extract to confirm that the photooxidation of 9,10-dimethylantracene is due to the production of $^1O_2$ by the extract and not due to a photodegradation process. All the assays were performed in triplicate.

Results

The efficiency of the conventional approaches to extract chlorophyll *a* from *Spirulina maxima* cells using different organic solvents is summarized in Figure 3.2.1. To facilitate the discussion/comparison of the results, an optimization on the organic solvent-based extraction conditions to extract chlorophyll *a* was firstly carried out (Figure 3.2.1).

Conventional method: extraction of chlorophyll a using organic solvents

An initial screening was carried out using a large set of organic solvents to evaluate their ability to extract chlorophyll *a* from *Spirulina* cells (Figure 3.2.1). In the same figure, the logarithmic function of octanol-water partition coefficient (Log_{Kow}) was used as a way of analysing the hydrophobicity/hydrophilicity of the screened solvents.

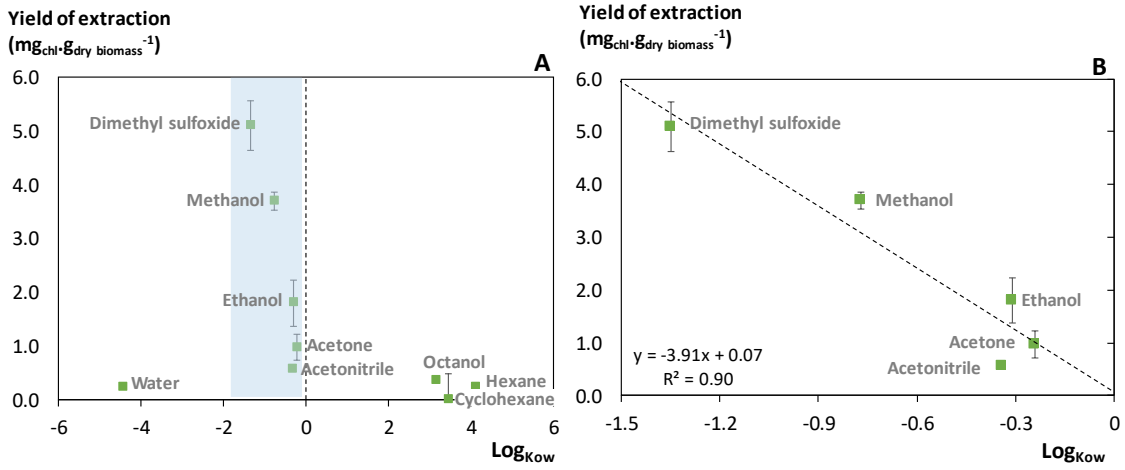


Figure 3.2.1. Yield of extraction of chlorophyll *a* from *Spirulina maxima* using (A) different solvents (pure organic solvents and water) and (B) only amphiphilic solvents as function of their Log_{Kow} (the results were adopted from literature).[132,133] K_{ow} of water was theoretically estimated.

According to Figure 3.2.1 A, highly hydrophilic and hydrophobic solvents do not provide high yields of extraction of chlorophyll *a*. In some cases, a slight amount of chlorophyll is extracted for these extreme conditions of polarity, as it happens with water since this macrocycle is not entirely hydrophobic due to the presence of ester and carbonyl polar functional groups in its structure. It is also clear that the best results of yield of extraction of chlorophyll are achieved when solvents with Log_{Kow} ranging between -2 and 0 are used, which is in agreement with the results obtained in one of our previous works regarding the extraction of chlorophyll from a green macroalga. Indeed, a good

correlation (R^2 = 0.86) between the yield of extraction of chlorophyll and the Log_{Kow}, considering the same solvents, was obtained (see Figure C.3 in Appendix C). Like the chlorophyll, these solvents are amphiphilic compounds (having both hydrophilic and lipophilic properties). Focusing on the amphiphilic solvents (Figure 3.2.1 B), a linear increase (R^2 = 0.90) in the yield of extraction while augmenting the solvent hydrophilicity (*i.e.* lower values of log K_{ow}[132,133]) is observed. Regarding the phenomena of cell disruption and the consequent release of the intracellular material, high concentrations of ethanol and other short-chain alcohols (such in the case of this study) can promote serious damages in cells, by solubilizing cell membranes and by changing the tertiary structure of the membrane proteins, thus causing the instant cell destruction.[134] In a similar way, dimethyl sulfoxide also acts in the phospholipid membrane by inducing transient water pores, and, at higher concentrations, the complete disintegration of the bilayer structure.[159] Given that, for dimethyl sulfoxide and methanol, both mechanisms of cell disruption and chlorophyll increased solubility should be originating the higher yields of extraction of chlorophyll. Considering the high boiling temperature of dimethyl sulfoxide (189 °C at atmospheric pressure)[136] and the difficulty to remove it at an industrial scale (even at low pressures in procedures of vacuum dryers), methanol (boiling temperature ~65 °C at atmospheric pressure)[160] was the solvent selected for further studies.

The combined effect of solid-liquid ratio (SRL) and time of extraction was studied at room temperature (20–25 °C) to avoid the pigment degradation and additional solvent evaporation using methanol as the best organic solvent to extract chlorophyll *a*. The optimization of the process was done by applying a central composite rotatable design (CCDR, 2^2), allowing the simultaneous analysis of different parameters and the determination of the relationship between the yield of extraction of chlorophyll (dependent response) and the operational conditions, namely time of extraction and SLR (independent variables). A total of eleven extractions (with three repetitions of the central point, four extractions at the factorial points, and four extractions at the axial points) were performed (Table C.1 in Appendix C) and the results were analysed using Statistica®.

The model was fitted using pure error with a confidence level fixed at 95 %, in order to guarantee that it is a highly predictive model. The accuracy and the precision of the

model equations were validated by statistical analysis using ANOVA (analysis of variance). It was achieved a coefficient of determination (R^2) of 0.92283 with $F_{calculated}>F_{tabulated}$ being the model considered as predictive. The graph of the predictive *vs.* observed data shows high confidence, which guarantees the reproducibility of the process at a high-confidence level (Figure C.4 in Appendix C). The significance of the conditions studied (time of extraction and SLR) was also confirmed, which is supported by the Pareto Chart (Figure C.5 in Appendix C).

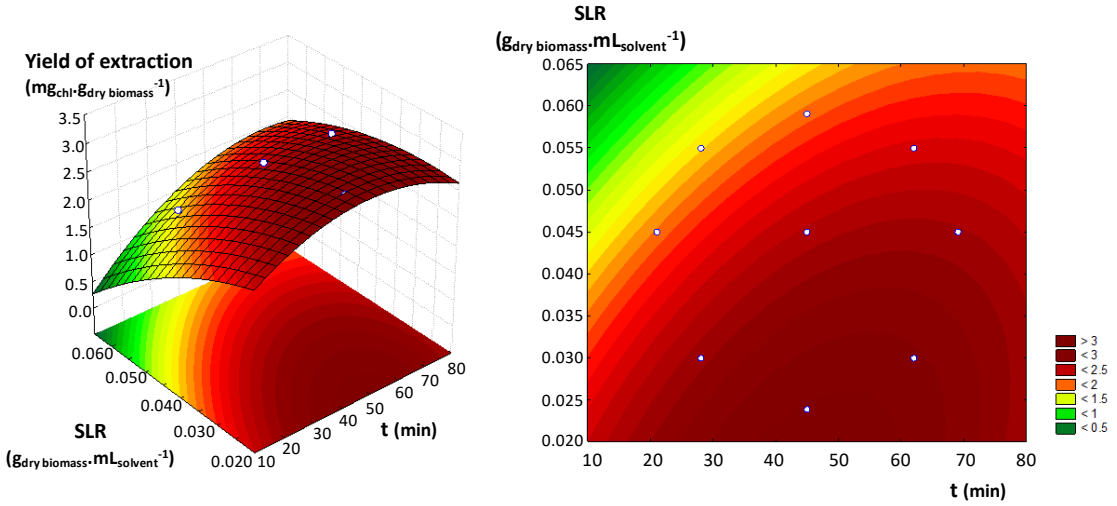


Figure 3.2.2. Response surface plot (left) and contour (right) of the CCRD (2^2) obtained for the combined effect of SLR and time of extraction optimization using methanol as solvent.

In the response surface plot (Figure 3.2.2), it is possible to observe a parabolic shape in which a theoretical maximum yield of extraction was reached (3.1 $mg_{chl}.g_{dry\ biomass}^{-1}$). The assay suggests a SLR of 0.024 $g_{dry\ biomass}.mL_{solvent}^{-1}$ and a time of extraction of 43.9 min as optimal conditions. These conditions were experimentally tested in triplicate, and the maximum value of extraction was confirmed as being 3.50 ± 0.19 $mg_{chl}.g_{dry\ biomass}^{-1}$, which is in agreement with the values previously published regarding the extraction of chlorophyll from *Spirulina* sp.[153,161–163]

Chlorophyll a *extraction using aqueous solutions of ILs and surfactants*

After the optimization of the conventional extraction using methanol as solvent, a screening of aqueous solutions of ILs and surfactants was carried out, aiming at the development of an alternative extraction. Compounds with different cations, anions,

and alkyl side chain lengths were tested (structures depicted in Figure C.1 in Appendix C). The main results are depicted in Figure 3.2.3.

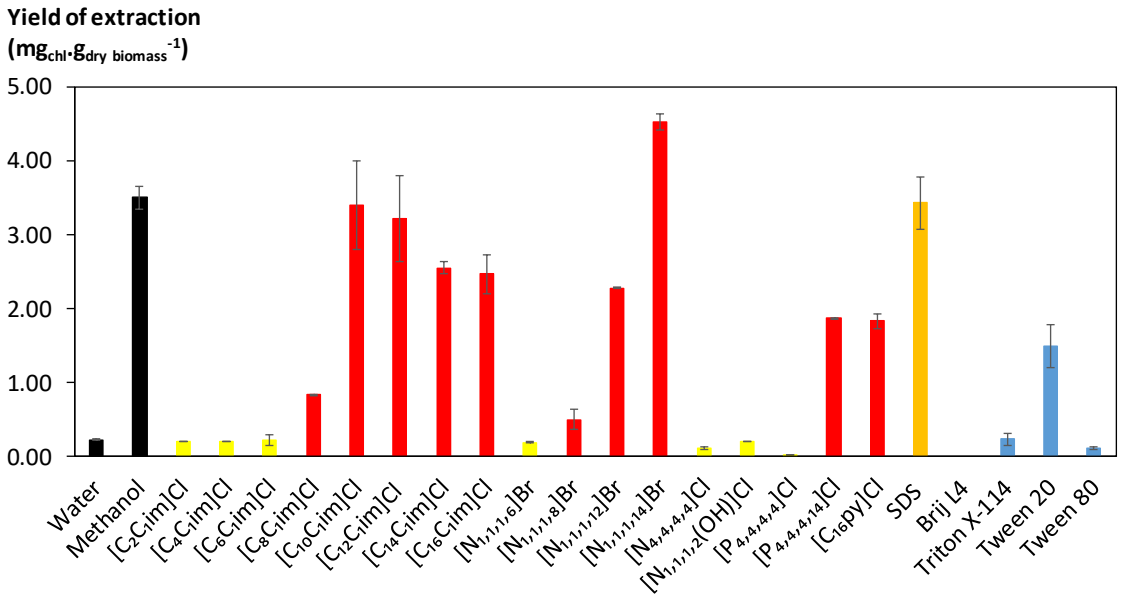


Figure 3.2.3. Yield of extraction (mg$_{chl}$.g$_{dry\ biomass}$$^{-1}$) of chlorophyll *a* from *Spirulina maxima* using aqueous solutions of different ILs and surfactants at 250 mM: non-tensioactive (yellow bars), cationic (red bars), anionic (orange bars), and non-ionic (blue bars). Methanol maximum yield of extraction and water is also depicted as a comparative term (black bars).

According to the results, in general, the cationic tensioactive compounds represented by the red bars were more efficient to extract chlorophyll *a* than the non-tensioactive (yellow bars) and non-ionic tensioactive compounds (blue bars). *Spirulina* sp. as a cyanobacteria, has in its composition a cell wall composed of an outer membrane, a peptidoglycan layer and a cell membrane. The last two referred membranes are essentially bilayers composed of phospholipids with hydrophobic tails and negatively charged hydrophilic heads. This cell wall composition is very similar to the gram negative bacteria such as *Escherichia coli*, thus explaining the high similarity between the results obtained for the disruption of *Spirulina* and *E. coli* when using aqueous solutions of ILs and surfactants as alternative solvents.[68] Moreover, the cationic tensioactive compounds can interact electrostatically with the negatively charged heads of the phospholipids of the outer and cell membranes. Additionally, the similarity in length of some tensioactive compounds and the lipidic part of the phospholipids of the

membranes may promote cell changes such as their expansion and permeabilisation leading to cell disruption and releasing of intracellular material.[164–166] Furthermore, and contrarily to what happens with non-tensioactive compounds, the tensioactive solvents can form micelles above the critical micelle concentration (CMC), a condition assured for all tensioactive compounds tested at 250 mM (CMC data detailed in Table C.2 in Appendix C). This means that a more suitable environment for more hydrophobic molecules such as chlorophyll can be provided using these compounds, enhancing even more the yields of extraction. These results agree with previous studies on the selectivity of IL to a certain target compound only by playing with its alkyl chain length.[57] Taking into account that the aqueous solutions of $[N_{1,1,1,14}]Br$ was the most efficient solvent extracting chlorophyll *a* from the *Spirulina* cells (Figure 3), this IL was thus selected for further studies.

Operational conditions optimization using aqueous solutions of $[N_{1,1,1,14}]Br$

In the optimization of the conditions using the tensioactive $[N_{1,1,1,14}]Br$ to extract chlorophyll *a* concentrations of IL between 50 and 500 mM (Figure 3.2.4 A) and extraction times between 2.5 and 30 min (Figure 3.2.4 B) were studied. All concentrations used were above the CMC.

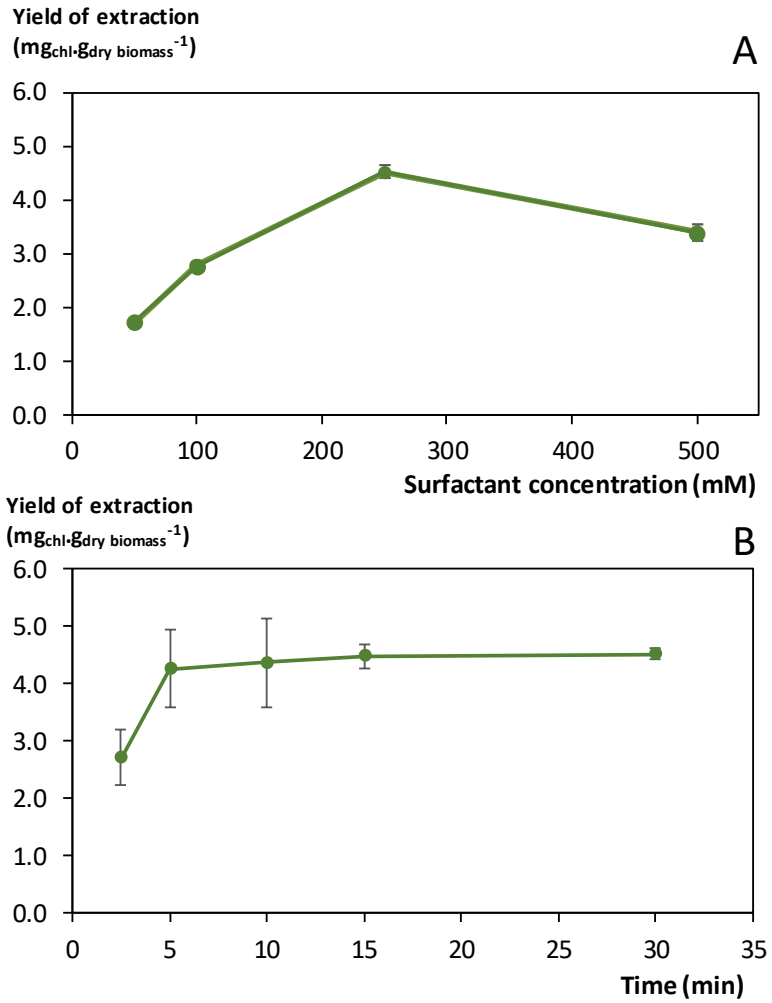


Figure 3.2.4. Yield of extraction ($mg_{chl}.g_{dry\ biomass}^{-1}$) of chlorophyll *a* from *Spirulina maxima* cells using aqueous solutions of $[N_{1,1,1,14}]Br$ considering (A) concentration of IL in water and (B) time of extraction.

The results presented in Figure 3.2.4 A suggest an almost linear increase in the yield of extraction with the IL concentration in water until 250 mM, where the maximum yield of extraction is achieved, in a 30 min extraction. Moreover, higher tensioactive concentrations did not provide any increase in the yield of extraction, being thus the 250 mM adopted for the subsequent studies.

The time of extraction was also studied for the tensioactive selected, $[N_{1,1,1,14}]Br$, at 250 mM (Figure 3.2.4B). The results show an increase in the yield of chlorophyll *a* extraction during the first 15 min of extraction using $[N_{1,1,1,14}]Br$. After this period, the yield of extraction remains practically constant. The time of extraction was thus fixed in 15 min to guarantee that the maximum extraction was achieved.

A comparison of the solid-liquid extraction best results obtained for both the conventional (using methanol) and the alternative method, using aqueous solutions of $[N_{1,1,1,14}]Br$ are presented in Table 3.2.1.

Table 3.2.1. Comparison of operational conditions and yield of extraction of chlorophyll *a* obtained for the conventional and alternative methods of extraction.

Method	**Conventional**	**Alternative**
Solvent	Methanol	$[N_{1,1,1,14}]Br$
Solvent concentration	Pure (100 %)	250 mM in water
Time of extraction (min)	44	15
SLR ($g_{dry\ biomass}.mL_{solvent}^{-1}$)	0.024	0.025
Yield of extraction ($mg_{chl}.g_{dry\ biomass}^{-1}$)	3.50 ± 0.19	4.36 ± 0.78

The results presented in Table 3.2.1, show that the alternative methodology using an aqueous solution of $[N_{1,1,1,14}]Br$ has a better performance. Actually, besides the potential health risk and environmental impact of the use of methanol, the alternative method based on aqueous solution of IL at 250 mM was able to extract more 25 % of chlorophyll *a* in only 15 min instead of the 44 min required by the conventional method with pure methanol.

Polishing and purification of chlorophyll a

After selecting the best solvent to extract chlorophyll *a* and optimize the extraction conditions, the purification of the chlorophyll content from other pigments and from the IL used was performed. This purification was carried out by applying a back-extraction using ethyl acetate, which is not miscible with the IL aqueous solution. This procedure was repeated three times until no more pigments are extracted to the organic phase, as described in the methodology section.

After the adequate separation of the phases each phase was analysed by UHPLC-MS to determine the purity of the pigments obtained. Additionally, an ion-selective electrode for the quantification of the bromide anion was used to establish the concentration of

the IL in the purified samples. In what respects to the extract from the alternative methodology, the fractions obtained from the back-extraction were analysed separately and compared with the methanol extract from the conventional method. The results can be seen in Table 3.2.2 and Figures C.6 to C.11 in Appendix C, where the UHPLC chromatograms and UV-Vis spectra of the indicated peaks are depicted. The molecular structure of the compounds identified is represented in Figure 3.2.5.

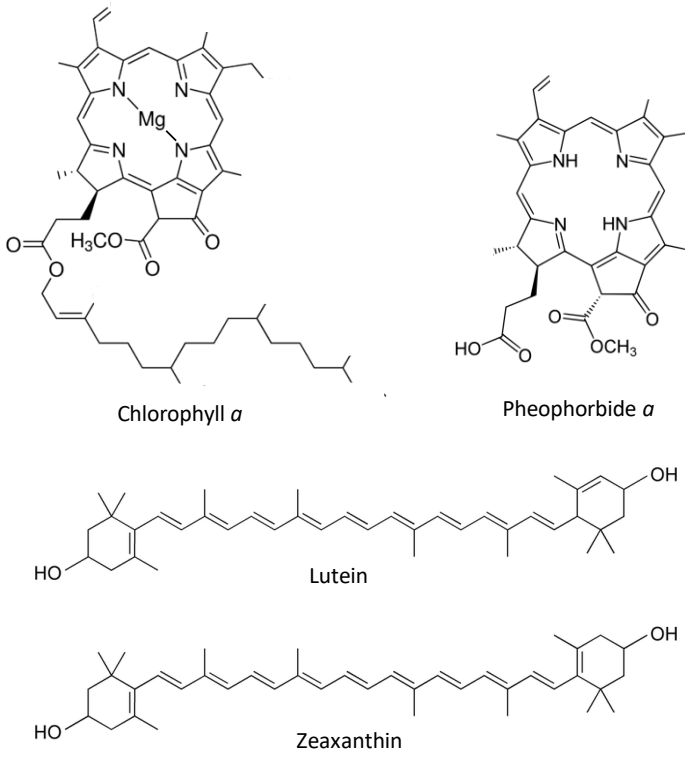


Figure 3.2.5. Molecular structure of the proposed compounds.

In the conventional extract, chlorophyll *a* and pheophorbide *a* (chlorophyll *a* without Mg^{2+} and the phytol chain) and xanthophylls were identified. A different situation was found in the analysis of the 1st and 2nd fractions obtained in the chlorophyll back-extraction from the alternative extract, where pheophorbide *a* was found as the main chlorophyll derivative present. This chlorophyll derivative is obtained by the loss of both the Mg^{2+} and the phytol chain, which may be related to some pH changes that have facilitated the hydrolysis of the ester chain and the removal of the metal from the inner core of the macrocycle (see Figure 3.2.5). Contrarily, the 3rd fraction was already free of chlorophyll, having on its composition only xanthophylls, namely lutein or zeaxanthin. The results from the back-extraction suggest that this can be a simple and efficient technique to fractionate and purify different pigments from a crude extract. The

bromide content was checked using an ion-selective electrode and the three organic fractions recovered from the liquid-liquid extractions showed a low IL concentration (~ 16 mM in each fraction). At the end of the process, the aqueous solution of IL, already free of pigments, has a final concentration in IL of *circa* 200 mM, which corresponds to 80 % of the initial IL used in the solid-liquid extraction. Summing up, a diagram of the integrated process of extraction and purification of both chlorophyll *a* and xanthophylls is depicted in Figure 3.2.6. Solid-liquid extraction, liquid-liquid extraction, and the solvents recycling and polishing of pigments were the tasks considered. A vacuum dryer, using low pressure and temperature (~ 35 ºC), was proposed for the last step of recovery of ethyl acetate and polishing of pigments.

Although an economic and environmental analysis were not performed in this work, there are several indications of the environmentally-friendly nature and economic viability of this process, namely the reuse of the solvents in new cycles of extraction,[152,161] the multi-product scenario represented by this process and the fact that this process employs an aqueous solution of an ammonium-based IL (representing the cheapest family[103]) instead of a pure organic solvent.[156]

Table 3.2.2. Extracts from conventional and alternative optimized methodologies composition and their molecular ions species and fragments (*m/z*) data.

Extract origin	Compound	Retention time (min)	UV-Vis (nm)	Mass (*m/z*)	Compounds abundance (%)
Methanol-based extract	Pheophorbide *a*	11.27*	408, 471, 503, 534, 608, 664	593	37
	Xanthophyll			663	
	Chlorophyll *a*	12.95	410, 471, 537, 609, 665	$[M + Na + K]^+$ 954	63
Alternative optimized methodology + Back Extraction: 1st and 2nd fractions	Pheophorbide *a*	10.83	408, 473, 505, 534, 607, 664	593	100
Alternative optimized methodology + Back Extraction: 3rd fraction	Xanthophyll (Lutein)	10.62	449, 475, 502	567	14
	Xanthophyll (Zeaxanthin)	12.65	450, 474	568	86

*Large peak corresponding to two different compounds.

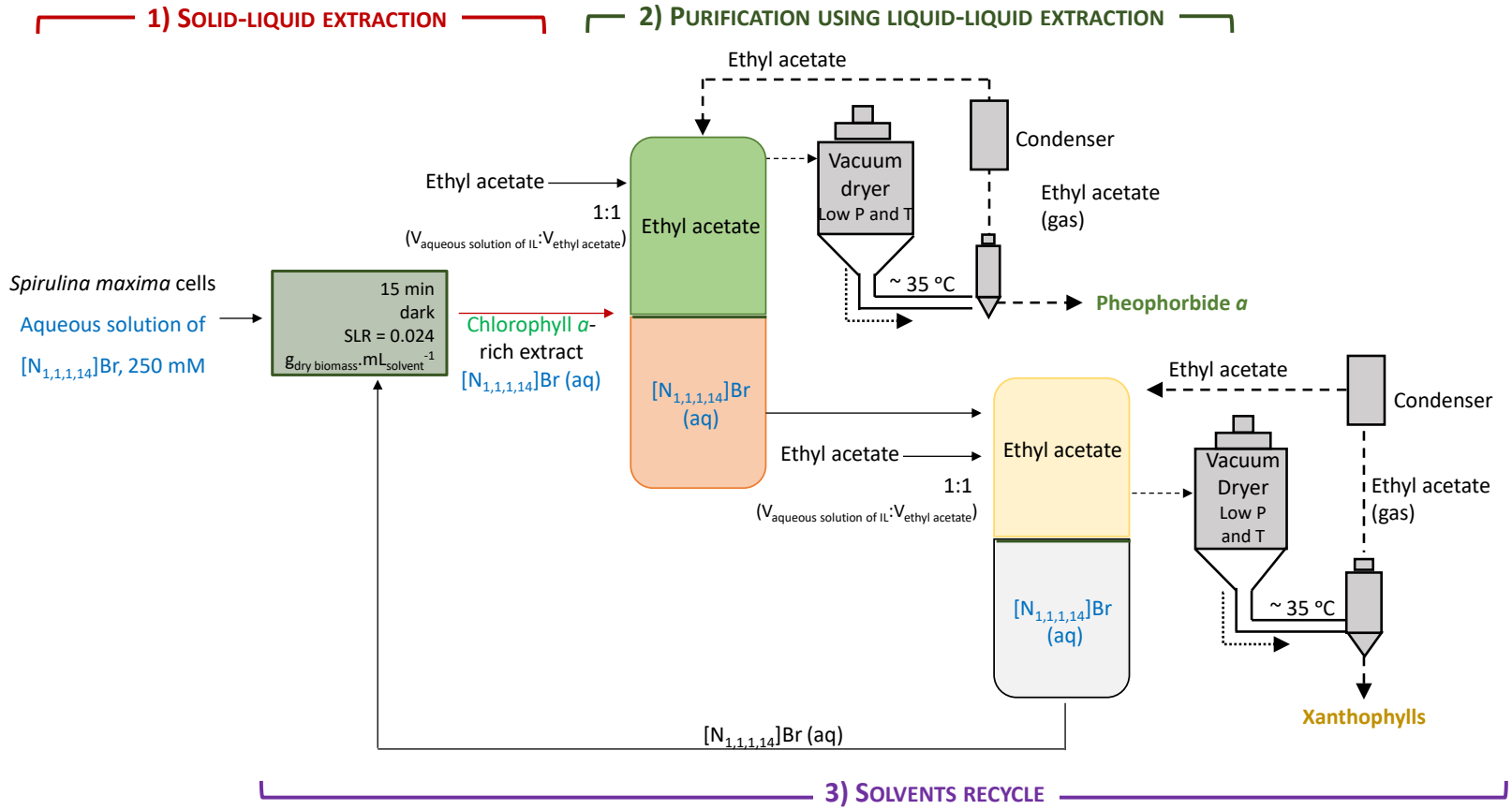


Figure 3.2.6. Diagram of the integrated process of extraction and purification of both pheophorbide *a* and xanthophylls. Although not experimentally tested, the dashed lines represent the proposal of what can be done to close the process from the point of view of industrial implementation.

Photosensitizing potential of the extracts

Considering the potential of the extracts for use in photodynamic therapy applications, it was decided to evaluate their efficacy to produce singlet oxygen and also their photostability (Figure 3.2.7). In these assays the extracts obtained using the conventional and alternative procedures were considered. It is important to remember that, according to Table 3.2.2, the conventional extract is composed of chlorophyll *a*, pheophorbide *a*, and xanthophyll. In contrast, the alternative extract after the back-extraction step (first and second fraction) is composed of only pheophorbide *a*, a very well-known photosensitizer that can induce significant antitumoral effects.[167,168] According to the results depicted in Figure 3.2.7 A, the photodecomposition of 9,10-dimethyanthracene used as singlet oxygen scavenger is similar for both extracts, although their efficiency to generate $^1O_2$ is minor than the one observed with Zn(II)chlorin e_6 dimethyl ester used as reference (Φ_Δ = 0.53). It is important to highlight that, in the absence of these photoactive extracts, no anthracene decomposition was observed. The similar ability to generate singlet oxygen is not surprising considering that all the chromophores present in both extracts (conventional extraction and alternative extraction) are good singlet oxygen generators which explains the behaviour found.[169] Regarding the photostability of the extracts (Figure 3.2.7 B), both proved to be quite photostable during the irradiation period (UV-Vis spectra of the extracts along the irradiation period are depicted in Figure C.12 in Appendix C). More than 50 % of the chromophores (conventional extract 55 %, alternative extract 58 %) present in the extracts remained unchanged after 60 min of the red-light irradiation. This is an adequate irradiation period for photodynamic therapy treatments of tumors and eradication of microorganisms. This balance between photostability and photodegradation is important. It is required the chromophores being present in an adequate amount to generate the desired amount of singlet oxygen to eradicate tumoral cells or microorganisms, albeit, after the photodynamic action its photodegradation is beneficial considering their fast elimination from the body or environment. Besides, no significant deviations were observed between conventional and alternative extracts, proving that both are suitable to be used in photodynamic therapy. However, it is important to note that deeper polishing is required to eliminate

the residual amount of IL present in the extracts before any practical use in photodynamic therapy.

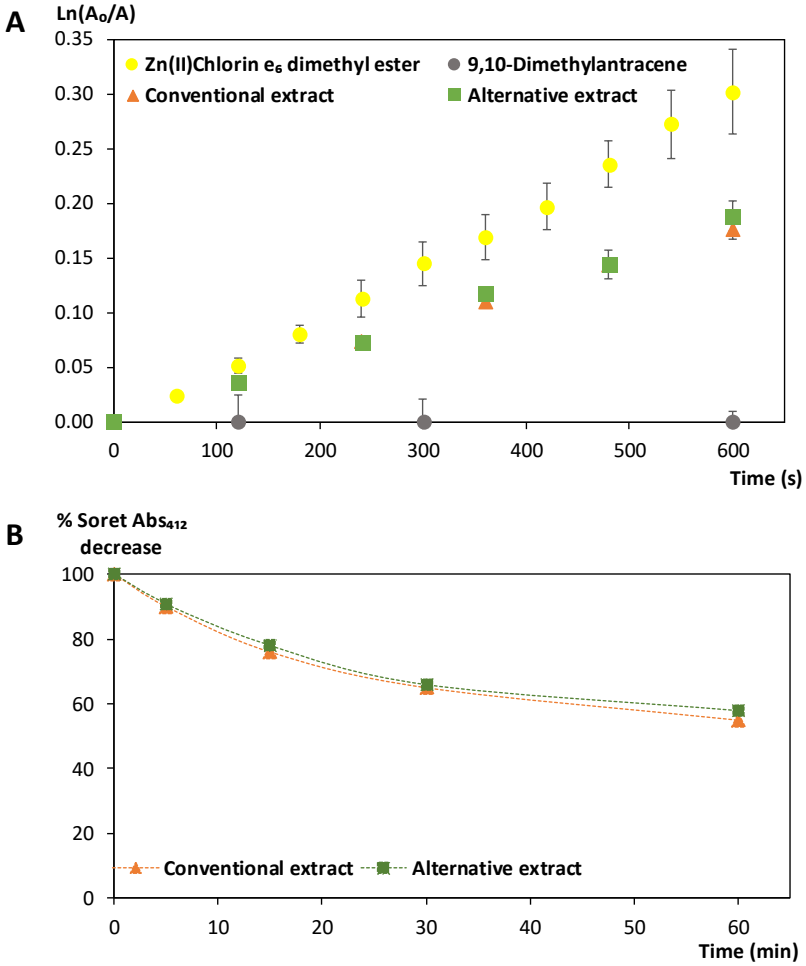


Figure 3.2.7. (A) Singlet oxygen production and (B) photostability of conventional and alternative extracts in dimethylformamide.

Conclusions

In this work, two methodologies were developed to extract chlorophyll *a* from the cyanobacteria *Spirulina maxima*, one using organic solvents and another using aqueous solutions of IL and surfactants. After the screening of solvents and the study of several operational conditions, it was found that one process using an aqueous solution of $[N_{1,1,1,14}]Br$ at 250 mM during 15 min can increase the yield of extraction in chlorophyll in *circa* of 25 % over the conventional methodology using pure methanol in a 44 min extraction. A chlorophyll back-extraction from the alternative extract was performed

using ethyl acetate, allowing to purify the extract and to recover the aqueous solvent, which can be further reused for extraction. During the back-extraction process, fractions with different compounds' compositions were obtained, namely pheophorbide *a* in the 1st and 2nd fractions and xanthophylls in the 3rd fraction, being 80.6 % of the IL removed from the pigments extract. Lastly, both extracts showed efficacy to generate oxygen singlet and an adequate photostability to be used in photodynamic therapy applications. Summing up, the results obtained for the alternative downstream process allow an efficient fractionation of different pigments and the recovery of the solvent in a simple separation process, maintaining the efficacy of pigments to be used as photosensitizers.

3.3 Extraction and fractionation of pigments from *Saccharina latissima* (Linnaeus, 2006) using an ionic liquid+oil+water system

This chapter is based on the manuscript

Margarida Martins, Leonardo M. de Souza Mesquita, Bárbara M.C. Vaz, Ana C.R.V. Dias, Mario A. Torres-Acosta, Benoit Quéguineur, João A.P. Coutinho, Sónia P.M. Ventura,* "Extraction and fractionation of pigments from *Saccharina latissima* (Linnaeus, 2006) using an ionic liquid+oil+water system", ACS Sustainable Chemistry & Engineering, 2021, DOI: 10.1021/acssuschemeng.0c0911.

*Contributions: M.M. and B.M.C.V acquired the experimental data. M.M. performed the data analysis. L.M.S.M. performed the response surface methodology and statistical analysis. A.C.R.V.D. and M.A.T.-A. assessed the environmental and economic impact, respectively. M.M. wrote the manuscript with substantial contributions from the remaining authors.

Abstract

There is a strong interest in the development of greener and more sustainable processes based on the use of renewable resources, and a biorefinery based on marine resources, such as macroalgae, stands as a major opportunity towards that end. In this work, *Saccharina latissima* (Linnaeus), a brown macroalga, was used as source of pigments to develop an integrated platform able to promote the extraction and fractionation of chlorophyll and fucoxanthin in one single step. The process was studied and its operational conditions optimized with yields of extraction of chlorophyll and fucoxanthin of 4.93 ± 0.22 $mg_{chl}.g_{dry\ biomass}^{-1}$ and 1956 ± 84 $\mu g_{fuco}.g_{dry\ biomass}^{-1}$, respectively. These results were achieved with extraction systems composed of 84 % of an aqueous solution of a tensioactive phosphonium-based ionic liquid (IL) at 350 mM + 16 % of sunflower oil, during 40 min, using a solid-liquid ratio of 0.017 $g_{dry\ biomass}.mL_{solvent}^{-1}$. After the separation of both aqueous IL-rich and oil-rich phases, the IL content in both phases was investigated, being the oil phase free of IL. Envisioning the industrial potential of the process developed in this work, the recovery of the IL from the aqueous IL-rich phase of the initial system was attempted by a back-extraction using organic solvents immiscible in water, being shown that 82 % of the IL can be recovered and reused in new cycles of extraction. The environmental and economic impacts of the final process proposed for the extraction and fractionation of chlorophyll and fucoxanthin were

evaluated. Different scenarios were considered, but summing up the main results, the solvents' recycling allowed better results, proving the economic and environmental viability of the overall process.

Keywords: *Saccharina latissima* (Linnaeus), pigments, one-pot, liquid-liquid extraction, ionic liquid, vegetable oil.

Introduction

Consumers are changing and their demands for natural products are increasing, pushing for the development of new processes and the use of renewable resources.[1–3] This is also encouraged by the economic plan of the European Commission for a cleaner and more competitive Europe, based on a bioeconomy action, craving high-quality, functional, and safer products and processes, which are efficient and approachable.[170] Thus, the use of natural raw materials and biomasses to manufacture consumer goods is an excellent alternative for a more sustainable society.

Algae are a good example of a natural, renewable resource on which a bioeconomy can be built on.[64] Besides its fast growth rates, lack of freshwater and pesticides requirement, and possibility of cultivation even on non-arable areas, algae are a very interesting raw material, not only from the point of view of composition, but also considering the flexibility of application of their compounds as source of various food ingredients, e.g. colourants, phycocolloids, thickening formulants, and gelling agents. Moreover, they have been shown to be a rich source of different bioactive compounds with commercial interest in different sectors of activity.[11,18,19] Pigments are a very good example to highlight due to the large range of potential applications. They can be used as food ingredients (as colourants and/or antioxidants),[171] but also in photodynamic therapy, imaging, solar energy conversion, and hydrogen production.[172,173] Pigments also stand out due to their biological activities. Chlorophyll and derivatives have been reported as having antimutagenic, chemo-preventive, antioxidant, anti-inflammatory, and gut microbiota regulator activities,[25] while carotenoids are known for their antioxidant function (preventing oxidative stress), immune response stimulation, and pro-vitamin A activity, allowing them to act in the prevention of tumours and other

diseases,[27,28] which significantly increases their potential in high-end applications, and thus the interest in their recovery from natural sources.

Despite the high diversity of macroalgae species, most have been poorly explored so far. The brown macroalgae *Saccharina latissima*, also known as sugar kelp or Kombu royal, are one of the two algae species studied on the framework of the European project GENIALG (GENetic diversity exploitation for Innovative macro-ALGal biorefinery). It has a high biomass yield and, simultaneously, a high farming expansion potential, already validated in Europe.[87] As previously identified in literature, and further demonstrated during the development of the GENIALG project, a large number of bioactive compounds can be obtained from *Saccharina latissima*. Contrarily to what was observed for *Ulva* species, abundant in chlorophylls,[156] this brown alga (besides chlorophyll *a* and chlorophyll *c*)[174] also produces high amounts of carotenoids, namely the xanthophylls, in particular the fucoxanthin, being the most abundant. Fucoxanthin has a number of properties that are worth exploring from a commercial point of view.[26,175] However, its recovery is still not straightforward or carried out in large scale for the lack of cost-efficient processes, with low environmental footprint and good scalable potential.[176] Also, since carotenoids and chlorophylls have similar polarity and are usually present into the same cellular site (chromoplasts, chloroplasts, leucoplasts, and fat globules),[177] their simultaneous extraction is very common,[178] impairing the selectivity of the extraction process and, consequently, the purification of the compounds.

The use of aqueous solutions of ILs in the extraction of biomolecules from different biomass matrices is not new.[58,179] ILs have been recognized as powerful task-specific solvents for this purpose. The mechanisms behind their success are normally assigned to an increased solubility of the target molecule in the IL media, their improved ability to disrupt the cell membranes, or the combination of both.[68,156] Following this rationale, and considering the properties of carotenoids and chlorophylls, aqueous solutions of tensioactive ILs have been successfully employed.[68,100,156,180] Nevertheless, pure extracts are difficult to obtain when molecules with similar structures and/or polarities are present in the same cellular site (e.g. chlorophylls and carotenoids),[177] and thus, additional steps of purification are required, involving higher costs, energy consumption, and specific equipment and/or material.[181] The use of liquid-liquid extraction techniques is well known in purification processes. It has many advantages in

comparison with other purification techniques, such as conventional chromatography and saponification,[30,32] due to its simplicity and familiarity, being easy to implement and scale-up. There are many variations, ranging from the use volatile organic solvents, aqueous systems, and oil systems.[182–184] Vegetable oils are a food ingredient very well accepted in many industries,[171] that can work not only in the extraction of compounds from biomass such as pigments, namely chlorophylls and carotenoids, but also in the formation of liquid-liquid extraction systems.[171,185–187] In addition, edible oils are non-volatile, cost-effective, and excellent solvents to be applied in the food sector, since they may be directly used in food-formulations,[171] without the need of performing the separation of the pigments from the solvent.[176,186]

In this work, a new process able to extract and separate, in a single step, both chlorophylls and fucoxanthin from *Saccharina latissima*, by an integrated solid-liquid and liquid-liquid extraction process, using aqueous solutions of a tensioactive IL and a common vegetable oil, was designed as an alternative to the conventional extraction processes. After a first screening of different ILs as solvents to extract the pigments from the biomass, the best solvent was selected, and the principal operational conditions optimized. Envisioning the industrial potential of the process developed, the IL recovery was tested, enabling thus, the analysis of the environmental and economic impacts of the final process.

Experimental

Biomass

The biomass used in this work was kindly provided by one of the industrial partners of project GENIALG, Algaia SA (Saint-Lô, France) and the Station Biologique de Roscoff, CNRS. *Saccharina latissima* was collected in Roscoff, France (48°43'54"N, 3°59'23"W). The fresh biomass was harvested in February 2019, washed, frozen with liquid nitrogen and ground in a coffee grinder, freeze dried, and sifted to achieve a particle size < 1 mm afterwards. The biomass was kept at -20 ºC until needed.

Chemicals

The series of 1-alkyl-3-methylimidazolium chloride-based ILs [$C_nC_1$im]Cl, as 1-hexyl-3-methylimidazolium chloride ([C_6C_1im]Cl, 98 wt%, CAS 171058-17-6), 1-dodecyl-3-

methylimidazolium chloride ($[C_{12}C_1im]Cl$, > 98 wt%, CAS 171058-18-7), 1-methyl-3-tetradecylimidazolium chloride ($[C_{14}C_1im]Cl$, 98 wt%, CAS 171058-21-2), were acquired from Iolitec. The decyltrimethylammonium chloride ($[N_{1,1,1,10}]Cl$, 98 wt%, CAS 10108-87-9), and the decyltrimethylammonium bromide ($[N_{1,1,1,10}]Br$, 99 wt%, CAS 2082-84-0) were from Tokyo Chemical Industry (TCI). The dodecyltrimethylammonium bromide ($[N_{1,1,1,12}]Br$, 99 wt%, CAS 1119-94-4) and tetradecyltrimethylammonium bromide ($[N_{1,1,1,14}]Br$, 98 wt%, CAS 1119-97-7) were purchased from Alfa Aesar. The tributyltetradecylphosphonium chloride ($[P_{4,4,4,14}]Cl$, 95 wt%, CAS 81741-28-8) was purchased from Iolitec. All molecular structures of the ILs used in the screening of solvents are depicted in Figure D.1 in Appendix D.

Refined sunflower oil (brand Auchan) purchased at Auchan supermarket (Aveiro, Portugal) was used on the pigment extraction. Standard fucoxanthin (≥ 95%, CAS 3351-86-8) was acquired from Sigma-Aldrich. The organic solvents used in the screening of solvents and back-extraction step, namely ethanol (HPLC grade, CAS 64-17-5), toluene (HPLC grade, CAS 108-88-3) and ethyl acetate (HPLC grade, CAS 141-78-6) were purchased from Fisher Scientific, while diethyl ether (99.8 %, CAS 60-29-7) was acquired from Panreac.

Screening of solvents

The extractions were performed at room temperature (20–25 ºC) under a constant vertical rotation of 80 rpm in a shaker IKA TRAYSTER digital, during 30 min. The ILs were screened at 250 mM in aqueous solution, being the list of ILs screened and initial concentration chosen according to previous works.[156,180,188] Water, sunflower oil, and ethanol were tested as control systems. A solid-liquid ratio (SLR) of 0.017 $g_{dry\ biomass}.mL_{solvent}^{-1}$ was used, meaning 0.2 g of dry biomass and 12 mL of the respective solvent. All extractions were done in triplicate. In order to separate the cell debris from the supernatant, a centrifugation step was carried out in a Thermo Scientific Heraeus Megafuge 16R centrifuge at 4700 *g* for 15 min at 10 ºC, and the supernatant recovered and analysed.

Pigments fractionation: from a two-step to a single-step approach

After a first step of selection of the best solvent to extract the pigments, a second step consisted in a liquid-liquid extraction system, obtained by adding and mixing sunflower oil to the pigments-based aqueous IL extract in the proportion (in volume) of 60 % of the aqueous solution of IL ($\%_{IL}$) to 40 % of sunflower oil. The two phases were formed in a Thermo Scientific Heraeus Megafuge 16R centrifuge at 4700 *g* for 15 min at 10 °C, and both phases analysed.

In the single step approach, the dry biomass (0.2 g) was mixed with a fresh aqueous solution of IL (instead of the crude extract) and with oil, in a system using the same volume ratio than before (60 % IL aqueous solution + 40 % sunflower oil). Temperature, agitation, time of extraction, and concentration of IL in aqueous solution was kept as described for the screening of solvents. In order to separate the various phases, a centrifugation was done in a Thermo Scientific Heraeus Megafuge 16R centrifuge at 4700 *g* for 15 min at 10 °C, and both phases were analysed.

Optimization of the process conditions by a response surface methodology

Each system was composed of biomass, an aqueous solution of the best ILs screened, and sunflower oil. The optimization of the process was done by applying a central composite rotatable design (CCRD, 2^4 plus axial) totalizing 28 extractions with four replicates at the central point. The independent variables optimized were the time of extraction (t in min), the concentration of IL in water (C_{IL} in mM), the volume of aqueous solution of IL towards the oil volume ($\%_{IL}$ in %), and the solid-liquid ratio (SLR in $g_{dry\ biomass}.mL_{solvent}^{-1}$) considering as solvent both aqueous and organic fractions. Temperature and agitation were kept constant as described for the screening of solvents, *i.e.* room temperature (20–25 °C) and 80 rpm, respectively. The conditions are presented in Table D.1 and Table D.2 in Appendix D. All the experimental planning analysis was performed following the theory exposed by Dean, Voss & Dragulic and Rodrigues & Lemma.[189,190] The obtained results were analysed using the Statista 12.0 and statistically verified for a confidence level of 95 %.

Pigments quantification

The absorption spectra of the aqueous phases were measured between 300 and 700 nm using a UV-Vis microplate reader (Synergy HT microplate reader – BioTek). The chlorophyll and fucoxanthin contents were evaluated according to calibration curves previously determined and depicted in Figure D.2 in Appendix D [R^2 = 0.9389, R^2 = 0.9805, and R^2 = 0.9986, respectively for chlorophyll in aqueous solutions (at 667 nm), chlorophyll in ethanol (at 665 nm), and fucoxanthin in aqueous solution (at 457 nm)]. The absorption spectra of the oil phases were analysed between 350 and 750 nm using a UV-Vis spectrophotometer (SHIMADZU UV-1700 PharmaSpec Spectrometer). In this case, the chlorophyll content was quantified following an equation that allows the determination of chlorophyll in vegetable oils adopted from Pokorný et al.[191] The results are expressed in terms of yield of extraction ($mg_{chl}.g_{dry\ biomass}^{-1}$ and $\mu g_{fuco}.g_{dry\ biomass}^{-1}$).

Statistical analysis

The analysis of variance (ANOVA) followed by Bonferroni post-hoc was performed using the BIOESTAT 5.3 to compare the significance of the obtained extraction yields of fucoxanthin and chlorophyll using a degree of significance of 95 % ($p < 0.05$, $n = 3$).

IL recovery and quantification

The pigments were separated from the IL using a back-extraction with organic solvents with low miscibility in water. Systems composed of an aqueous extract of IL (rich in fucoxanthin) and different organic solvents (toluene, ethyl acetate, and diethyl ether) were tested in a ratio of 3:2 (v:v). The mixtures were centrifuged in a Thermo Scientific Heraeus Megafuge 16R centrifuge at 3300 *g* for 30 min at 25 °C and a two-phase system was obtained. In order to quantify the recovery of IL in the extracts, an ion-selective electrode (Metrohm) able to detect the chloride anion (electrode reference: 6.0502.120) was used, after preparing the respective calibration curve (R^2 = 0.9999).

Environmental analysis by life cycle assessment

Life Cycle Assessment was applied following the ISO 14040 standard[192] to determine the environmental impacts of the $[P_{4,4,4,14}]Cl$-based process proposed for the extraction and fractionation of chlorophyll and fucoxanthin from *Saccharina latissima* (Linnaeus). Two

scenarios were analysed, one where $[P_{4,4,4,14}]Cl$ is not reused and the other where the $[P_{4,4,4,14}]Cl$ remaining in the aqueous phase (82% of the initial amount) is reused. The impacts derived from the production of electricity, $[P_{4,4,4,14}]Cl$, toluene, sunflower oil, and distilled water were calculated based on the amounts consumed during the experimental procedure (Table D.3 in Appendix D) multiplied by the respective impact factors [e.g. mass of greenhouse gas emissions expressed as carbon dioxide equivalent (CO_2 eq) per mass of toluene]. These impact factors were taken from the World Food LCA Database 3.5[193] for sunflower oil and Ecoinvent 3.5 database[194] for the other inputs.

Economic analysis

The economic analysis done in this work is based on two equations published before.[156,195] Briefly, Eq. 1 calculates the production cost of the materials employed *per* unit of mass of product obtained.[156] Eq. 2 was used to calculate the potential return given that the products generated in this process can be sold for a profit.[103]

$$\frac{Production\ cost\ of\ materials}{Unit\ mass\ of\ pigment} = \frac{\sum_{i=1}^{n} \frac{Use\ of\ material_i}{Batch} \times \frac{Price\ of\ material_i}{Unit\ of\ material_i}}{\frac{Amount\ of\ pigment}{Unit\ of\ dry\ biomass} \times Mass\ of\ dry\ biomass\ used} \qquad \textbf{Eq. 1}$$

$$\text{Return}\ (\text{EU}.\,g^{-1}_{\text{dry biomass}}) = \left[C_{\text{prod}} \times \$_{\text{prod}}\right] - \$_{\text{biom}} - [\alpha \times \text{Production cost } per \text{ g of biomass}] \qquad \textbf{Eq. 2}$$

Using Eq. 1 it is possible to obtain the production cost *per* unit of mass of any product, but it only considers materials employed for its production. Given the nature of this work, this is ideal as there is no real information on how a potential scaled-up version of the process will look or behave. It is possible to use published data to make projections to have the complete cost in theory, which includes the capital contribution, materials/consumables, labour, and others (waste disposal, insurance, and utilities). First, as there is no data for this process, the capital contribution was decided to be fixed at 50 % of the total cost. From literature, labour was established at 15 % and others at 4 %.[196] This allows for materials/consumables to take up the remaining 31 % of the total projected cost. Using these proportions, it was possible to have a complete production cost *per* mg of pigment (or Cost of Goods *per* mg of pigment, CoG.mg^{-1}).

Eq. 2 requires the input of five variables, which can vary to enhance the potential of the analysis, thus containing a comprehensive collection of possibilities. C_{prod} considers the concentration of product *per* unit of mass of dry biomass processed (yield of extraction for each pigment in the best operational conditions) and $\$_{prod}$ is the market price of each product (pigment) based on suppliers. It is important to note that commercial prices might be higher than the possible actual selling price of the product developed here, and for this reason, this analysis considered prices 10 and 100-fold lower as well. $\$_{biom}$ is the variable to capture the cost of obtaining the biomass, in this study this cost was fixed at 0 $EU.g_{dry\ biomass}^{-1}$. The production cost *per* dry biomass is obtained by multiplying the complete production cost *per* mg of pigment ($CoG.mg^{-1}$) and the yield of extraction ($mg_{pigment}.g_{dry\ biomass}^{-1}$). The term α is used to express a multiplier of the production costs. This term allowed to analyse the impact of having a higher or lower production cost in practice. This study analysed an α of 0.1, 1 and 10, representing an increase and decrease by 10-fold, besides the base scenario.

To calculate the production cost in Eq. 1 and the subsequent complete costs, the materials used in this work and their respective costs are included in Appendix D (Table D.4). Additionally, one of the aims of the work is to provide an insight on the economic aspects of including the recycling of the IL on the step of extraction and the toluene recycling on the final polishing (and back-extraction) step. To capture this, several scenarios were included after the base calculation was completed. These scenarios include (i) no recycling (benchmark), (ii) recycling of only IL, (iii) recycling of IL and the water where it is contained, (iv) recycling only toluene and (v) recycling everything (IL, water, and toluene). Moreover, each of these scenarios (except for when no recycling is included) were evaluated for different recycling scenarios, namely 20 %, 40 %, 60 %, 80 %, and 100 %. By doing all these combinations, it is possible to have a comprehensive collection of data to include potential real-life scenarios and set-up a benchmark for future developments.

Through Eq. 2, the potential profit possible to be achieved from these two products (chlorophyll and fucoxanthin) is determined. First, the Return of each scenario was analysed individually as a benchmark to determine potential areas of improvement. Then, a combined Return (Total Return) was calculated through Eq. 3, which is an

updated version of Eq. 2. This can reflect the more realistic scenario as both products are generated in the same process and with an overall production cost.

$$\text{Total Return } (\text{EU}.\text{g}^{-1}_{\text{dry biomass}}) = \left[\text{C}_{\text{prod chl}} \times \$_{\text{prod}} + \text{C}_{\text{prod fuco}} \times \$_{\text{prod}}\right] - \$_{\text{biom}} - [\alpha \times \text{Production cost } \textit{per} \text{ g of biomass}] \quad \textbf{Eq. 3}$$

Results

Screening of ILs as extraction solvents

The results of a screening of aqueous solutions of different ILs (at 250 mM), with the extracts quantified in terms of yield of extraction of chlorophylls ($mg_{chl}.g_{dry\ biomass}^{-1}$), are presented in Figure 3.3.1. Water, ethanol, and a vegetable oil were used at the same conditions of extraction as control solvents. Photographs and the UV-Vis spectra of the extracts are depicted in Figure D.3 in Appendix D.

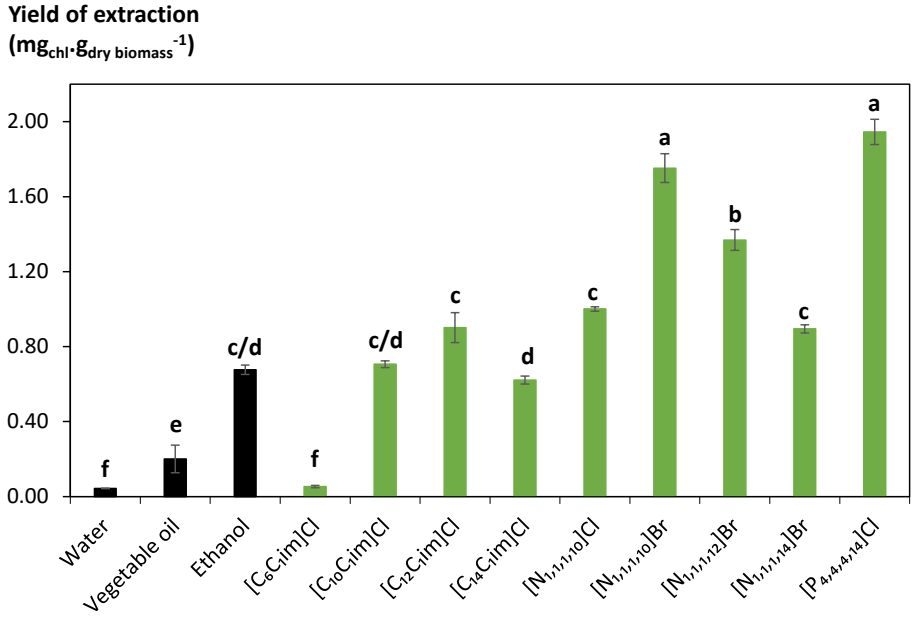


Figure 3.3.1. Screening of different solvents in the extraction of pigments from *Saccharina latissima* (Linnaeus) in terms of yield of extraction of chlorophyll ($mg_{chl}.g_{dry\ biomass}^{-1}$). Black bars are solvents tested as controls. Different letters represent statistically different values ($p < 0.05$).

As it can be seen in Figure 3.3.1, and as expected due to the pigments hydrophobicity, water and aqueous solutions of $[C_6C_1im]Cl$, are not efficient on the extraction of the pigment, which may be explained by the low capacity of these hydrophilic solvents to

interact with the membrane phospholipids and, consequently, to disrupt the cells. A similar behaviour was obtained with the use of vegetable oil, also showing a low performance in the extraction. This could be explained by its high viscosity that hinders mass transfer of pigments from biomass to the oil, and by the low capacity of the oil components to interact with the cells structure, without any mechanical help, as reported elsewhere.[186]

In the other hand, ethanol, a well-known solvent in pigment extraction and membrane solubilization, and aqueous solutions of (cationic) tensioactive compounds are able to extract chlorophylls (and carotenoids) as already shown in previous works.[100,156] As previously discussed in literature, the tensioactive ILs are able to form micelles in aqueous solution above certain concentrations, named as critical micelle concentration (CMC), as presented in Table D.5 from Appendix D. The ability of these ILs to extract these hydrophobic pigments can be explained by two different phenomena: (i) the creation of a perfect environment for the solubilization of the pigments within the micelles, since all ILs were tested at concentrations above their CMC, and (ii) the ability of these cationic ILs to disrupt the phospholipidic cell membranes and the thylakoid membranes that protect the pigments involved in the photosynthesis. This disruption happens due to the formation of electrostatic interactions between the cationic ILs with the negatively charged head of the phospholipids. Moreover, hydrophobic interactions have also an important role on this process as supported by the similarity in length of the alkyl chain of the IL and the lipidic part of the phospholipids that can lead to cell disruption and release of intracellular material, by mechanisms of expansion and permeabilisation.[164,165]

Among the ILs screened, aqueous solutions of $[N_{1,1,1,10}]Br$ and $[P_{4,4,4,14}]Cl$ stand out as the most efficient in the extraction of pigments, in accordance with previous works.[156,180,197]

Pigments fractionation

Given the good performance of aqueous solutions of $[P_{4,4,4,14}]Cl$ and $[N_{1,1,1,10}]Br$, both were considered in further experiments. The extracts obtained using the aqueous solutions of the two ILs and a low-cost vegetable oil (sunflower oil) were combined to form a liquid-liquid extraction system (resulting from the immiscibility between the oil

and the water). The two-phase system formed is composed of a top phase rich in oil, presenting a green colour (rich in chlorophylls), and a bottom phase rich in the IL aqueous phase, with a yellow colour (rich in fucoxanthin), as shown in Figure 3.3.2.

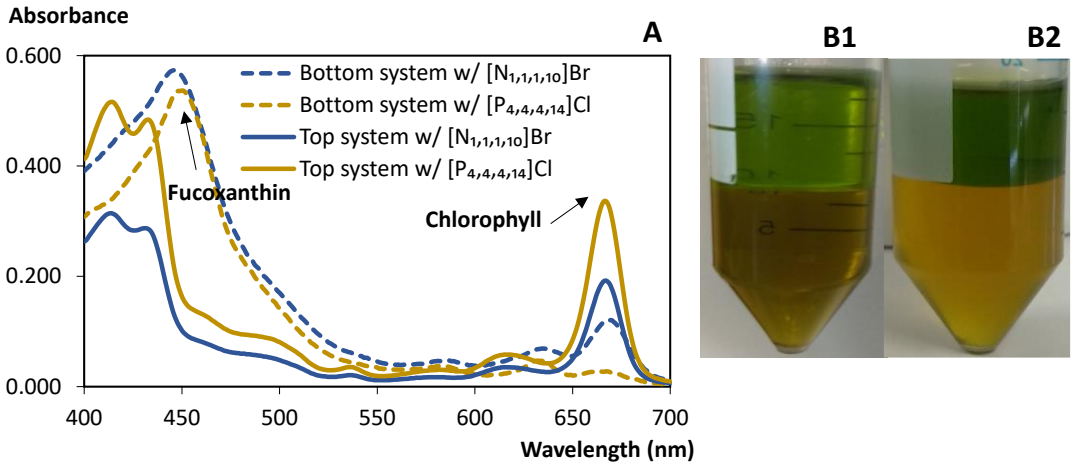


Figure 3.3.2. Liquid-liquid extraction systems composed of the extract obtained using the aqueous solutions of $[N_{1,1,1,10}]Br$ and $[P_{4,4,4,14}]Cl$ and the vegetable oil: (A) UV-Vis spectra of top and bottom phases of both systems; and Photographs of the system with (B1) $[N_{1,1,1,10}]Br$ and (B2) $[P_{4,4,4,14}]Cl$. Blue lines represent systems with $[N_{1,1,1,10}]Br$ while dashed lines represent the systems' bottom phases.

Note: Top and bottom phases were analysed in different spectrophotometers as described in experimental section and by applying different dilutions.

From the results depicted in Figure 3.3.2, it is possible to conclude that, besides the chlorophyll, a significant amount of fucoxanthin was also extracted from the biomass by using the ILs aqueous solutions. Chlorophylls present higher hydrophobicity than xanthophylls (miLogP around 9.8, whereas for fucoxanthin is around 8.5),[178] which explains the partition of the chlorophyll to the oil phase, highly hydrophobic, while fucoxanthin remains in the aqueous phase. Although the separation of phases starts to occur just a few minutes after homogenization, the centrifugation step allowed a faster and complete phase separation and, consequent fractionation of pigments. Yields of extraction of fucoxanthin of 1397 ± 3 and 1376 ± 79 $\mu g_{fuco}.g_{dry\ biomass}^{-1}$ and chlorophyll of 1.65 ± 0.02 and 2.9 ± 0.1 $mg_{chl}.g_{dry\ biomass}^{-1}$ were obtained for $[N_{1,1,1,10}]Br$ and $[P_{4,4,4,14}]Cl$, respectively. Interestingly, the system with $[P_{4,4,4,14}]Cl$ is not only providing higher yields of extraction of chlorophyll but also higher selectivity when compared to the system

based on $[N_{1,1,1,10}]Br$. As can be seen in Figure 3.3.2, by the UV-Vis absorption spectra and the photographs, there is a higher contamination of chlorophyll in the fucoxanthin rich-phase in the systems with $[N_{1,1,1,10}]Br$, showing the less efficient separation of the pigments. In the other hand, the bottom phase of the system with $[P_{4,4,4,14}]Cl$ (fucoxanthin rich-phase) is almost free of chlorophyll (3.9 ± 0.2 mg.L^{-1}, which corresponds to 2.11 % of the initial amount of chlorophyll).

Aiming at simplifying the methodology, the previous assays were replicated, but replacing the two-step procedure by a combined approach of extraction and purification in a single-step. Briefly, fresh (i) aqueous solutions of $[N_{1,1,1,10}]Br$ or $[P_{4,4,4,14}]Cl$, (ii) vegetable oil, and (iii) biomass were mixed together, under the same conditions of agitation and IL:oil ratio previously used in the two-step approach. After the extraction, a centrifugation step was carried out, and, as previously, a liquid-liquid extraction system was obtained, but with the biomass deposited as a solid pellet in the bottom of the vial. Subsequently, the quantification of chlorophylls and fucoxanthin released from the biomass and partitioned between the phases, was carried out with the results presented in Table 3.3.1.

Table 3.3.1. Comparison between the *single-step* and the *two-step approaches* in terms of yield of extraction of fucoxanthin and chlorophyll for the systems based on $[N_{1,1,1,10}]Br$ and $[P_{4,4,4,14}]Cl$. Different letters represent statistically different values ($p < 0.05$). The analysis was performed considering a comparison of significance in the yield of extraction of each pigment, in separate, using the two different procedures proposed for the same IL.

		Yield of extraction of fucoxanthin ($\mu g_{fuco}.g_{dry\ biomass}^{-1}$)	**Yield of extraction of chlorophyll ($mg_{chl}.g_{dry\ biomass}^{-1}$)**
$[N_{1,1,1,10}]Br$	Two-step	1397.4 ± 3.2^{a}	1.649 ± 0.023^{b}
	Single-step	**1289 ± 18^{a}**	**4.70 ± 0.23^{a}**
$[P_{4,4,4,14}]Cl$	Two-step	1376 ± 79^{a}	2.88 ± 0.11^{b}
	Single-step	**1226 ± 91^{b}**	**4.04 ± 0.54^{a}**

Table 3.3.1 shows that the yield of extraction of fucoxanthin using the $[P_{4,4,4,14}]Cl$-based system decreased when the single-step approach was applied, while no significative changes were observed using the $[N_{1,1,1,10}]Br$-based system. In the other hand, the yield

of extraction of chlorophyll was enhanced for the $[N_{1,1,1,10}]$Br- and $[P_{4,4,4,14}]$Cl-based systems using the single-step approach. Even though the two-step procedure showed the best results extracting fucoxanthin when systems with $[P_{4,4,4,14}]$Cl were used, considering the simplicity of the process and the lower energy spent, the single-step approach turned out to be most promising.

The aqueous solution of IL has allowed to decrease the viscosity of the system (in comparison with oil alone), allowing to demonstrate the advantages of combining solvents to enhance the yield of extraction of chlorophyll (in comparison with IL aqueous solution alone) and allowing the simultaneous separation of two different classes of pigments, which have no precedent in the scientific literature. Although this process was designed with sunflower oil due to its low cost, tests were carried out using other vegetable oils with similar results [data not shown in this work].

Optimization of the process operational conditions

The optimization of the process conditions was performed based on the following independent variables: (X1) volume fraction of aqueous solution of IL in the system ($\%_{IL}$ in %), (X2) the concentration of IL in water (C_{IL} in mM), (X3) the solid-liquid ratio (SLR in $g_{dry\ biomass}.mL_{solvent}^{-1}$), and (X4) the time of extraction (t in min) using an experimental design based on a CCDR (2^4). Liquid-liquid extraction systems were obtained for all tested conditions for both the $[N_{1,1,1,10}]$Br (Table D.1 in Appendix D) and $[P_{4,4,4,14}]$Cl (Table D.2 in Appendix D). The same behaviour was again observed; a yellowish bottom phase (IL-rich phase) and a greenish top phase (oil-rich phase), and the biomass was recovered as a solid pellet at the bottom of the vial.

The optimization was planned considering the *single-step approach*, being the yield of extraction of fucoxanthin and chlorophylls (expressed in $\mu g_{fuco}.g_{dry\ biomass}^{-1}$ and $mg_{chl}.g_{dry\ biomass}^{-1}$, respectively) the dependent responses used on the predictive model. The yields of extraction of the pigments experimentally determined are shown in Table D.1 and Table D.2 in Appendix D, along with the respective conditions of extraction. The model was fitted using pure error with a confidence level fixed at 95 %, in order to guarantee its high predictivity. The parameters not statistically significant were incorporated into the lack of fit for calculation of the R^2 and $F_{calculated}$ and $F_{tabulated}$

difference. In each assay, the optimum conditions were chosen by the interpretation of the respective response surfaces.

Regarding the $[N_{1,1,1,10}]Br$-based systems, the yield of extraction of fucoxanthin ranged between 133.4 and 1687.4 $\mu g_{fuco}.g_{dry\ biomass}^{-1}$. As depicted in Eq. 4 and demonstrated in the predicted *vs.* observed graph and Pareto Chart (Figures D.4 and D.5 in Appendix D), three variables were significant considering the extraction efficiency of carotenoids, namely $\%_{IL}$ (X1), C_{IL} (X2), SLR (X3), and the interaction between C_{IL} and SLR.

$$\text{Yield of extraction}\left(\mu g_{\text{fuco}}.\, g_{\text{dry biomass}}^{-1}\right) = -542 + 13.1(\text{X1}) + 9.2(\text{X2}) - 36769.1(\text{X3}) + 71.6(\text{X2}.\,\text{X3}) \quad \textbf{Eq. 4}$$

In these assays, a R^2 = 0.98 was achieved with *F* calculated-value at 649 (~ 216-fold higher than the tabulated *F*), showing a high-predictive model at 95 % of confidence level. The results depicted in Figure 3.3.3 evidence that the aqueous solution of $[N_{1,1,1,10}]Br$ at 400 mM, in a system composed of 84 % of aqueous solution of IL (and 16 % of oil), with homogenization fixed in 30 min, and SLR at 0.017, provides the highest yield of extraction (1836 ± 36 $\mu g_{fuco}.gr_{dry\ biomass}^{-1}$). The accuracy and precision of the model were carried out by a validation experimental test at the optimum operational conditions, with a low deviation (2.21 %) compared to the predicted results (Table D.6 in Appendix D). Also, the predictive *vs.* experimental data demonstrates a high confidence of the obtained results, guaranteeing the reproducibility of the process in a high-confidence level (Figure D.4 and Table D.6, respectively, in Appendix D).

The second response that was evaluated was the yield of extraction of chlorophyll. The main effects and interactions were estimated for the yield of extraction of chlorophyll, resulting in Eq. 5. A R^2 value of 0.78 was obtained showing that the predictive model could be achieved. The *F* value was approximately 70-fold higher than the respective tabulated *F*. The pure error was acceptable to generate a predictive model for the yield of extraction chlorophylls using aqueous solution of $[N_{1,1,1,10}]Br$, being the response surfaces plotted in Figure 3.3.4.

$$\text{Yield of extraction}\left(\text{mg}_{\text{chl}}.\, g_{dry\ biomass}^{-1}\right) = -2.95 + 0.00003(\text{X1})^2 + 0.3146(\text{X2}) - 0.00005(\text{X2})^2 + 0.12391(\text{X4}) - 0.00172(\text{X4})^2 \quad \textbf{Eq. 5}$$

By the analysis of the Pareto Chart (Figure D.7 in Appendix D), the SLR is not a significant variable in the extraction of chlorophylls, contrary to what was seen in the extraction of fucoxanthin, where the combination of the SLR with the other independent variables positively influences the response. Figure 3.3.4 also shows that when the C_{IL} ranges between 250–450 mM at a time of extraction of 30–40 min, the ratio of volumes IL and oil ($\%_{IL}$) is almost indifferent, *i.e.* more or less oil can be used allowing the manipulation of this parameter according to the application of the extracted pigment.

In conclusion, based on the data provided in Figures 3.3.3 and 3.3.4, the best operational conditions to be further applied were the $\%_{IL}$ of $[N_{1,1,1,10}]Br$ (aq.) = 84 % (consequently 16 % of oil), C_{IL} = 400 mM, SLR = 0.017 $g_{dry\ biomass}.mL_{solvent}^{-1}$, and t up to 30 min. Considering these operational extraction conditions, the predictive model was validated with a low deviation (3.44 %, Table D.7 in Appendix D). These data are in agreement with the predicted *vs.* observed graph and Pareto Chart (Figure D.6 and Figure D.7 in Appendix D, respectively), which showed the main influence of the C_{IL} and the lack of influence of the parameter SLR in the response, showing that the whole process was optimized under the expected predictions.

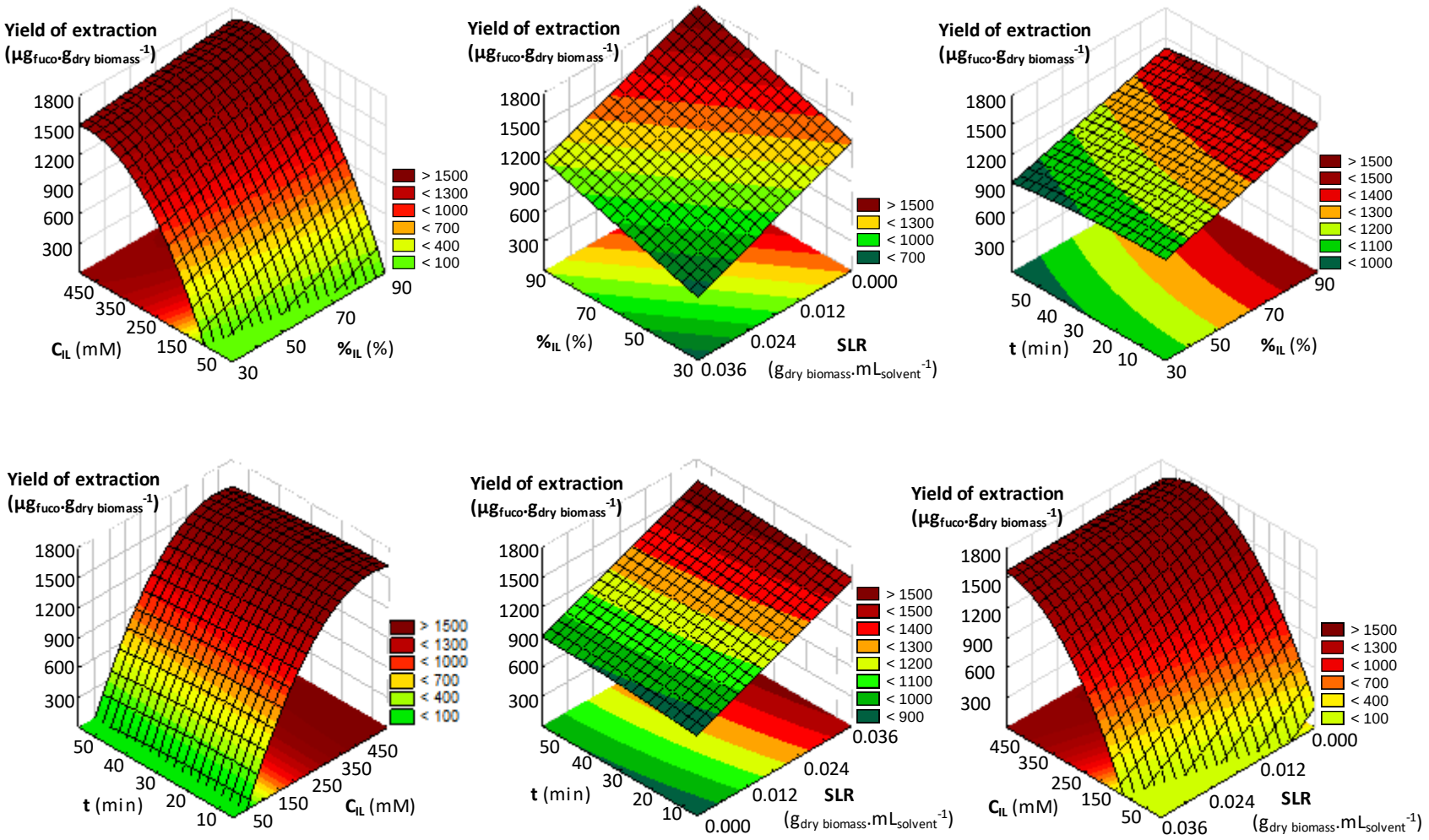


Figure 3.3.3. Response surface plots obtained for the CCRD (2^4) using a system with $[N_{1,1,1,10}]Br$ regarding: content of aqueous solution of IL ($\%_{IL}$ in %), time (t in min), IL concentration (C_{IL} in mM), and solid-liquid ratio (SLR in $g_{dry\ biomass}.mL_{solvent}^{-1}$) in terms of yield of extraction of fucoxanthin ($\mu g_{fuco}.g_{dry\ biomass}^{-1}$).

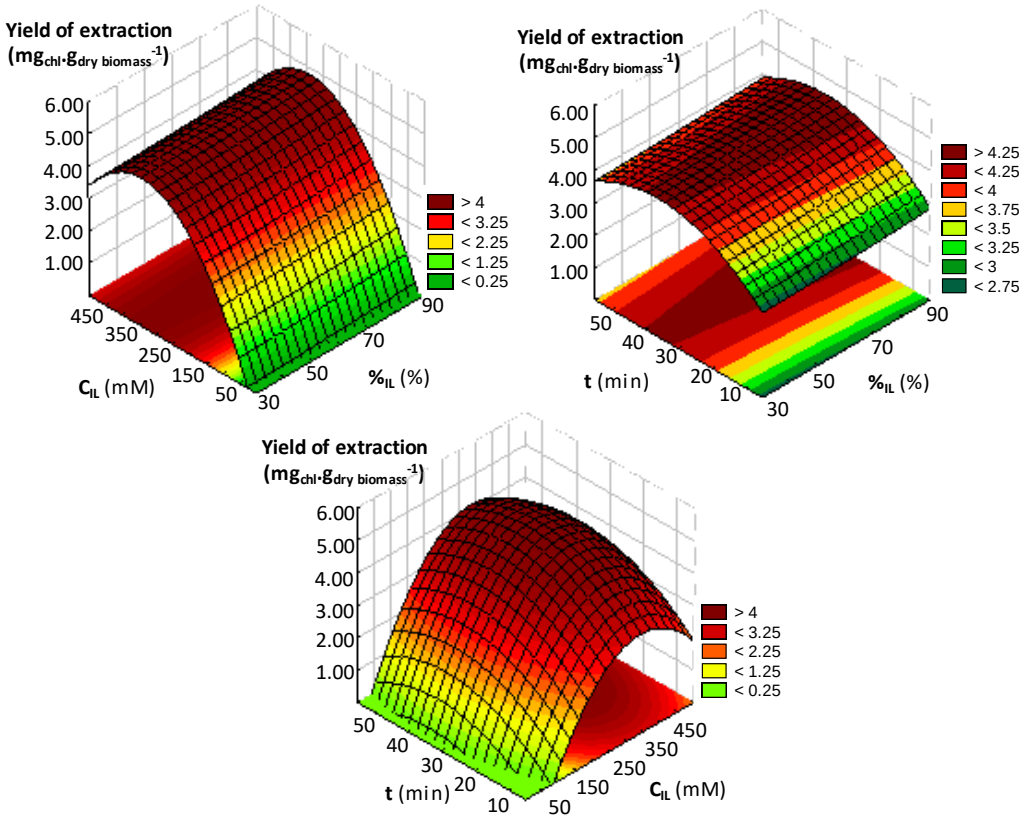


Figure 3.3.4. Response surface plots obtained for the CCRD (2^4) using the system based on an aqueous solution of $[N_{1,1,1,10}]Br$ regarding: content of aqueous solution of IL ($\%_{IL}$ in %), time (t in min), and IL concentration (C_{IL} in mM) in terms of yield of extraction of chlorophyll ($mg_{chl}.g_{dry\ biomass}^{-1}$). Graphs regarding SLR are not depicted since this condition is not significant in this context.

The single-step approach using $[P_{4,4,4,14}]Cl$ was also optimized. A coefficient of determination (R^2) of 0.86 indicated a good agreement of the model (Eq. 6) with the experimental results (Figure D.8 in Appendix D). The influence exerted by three independent variables on the extraction yield of fucoxanthin during the assays are displayed in the Pareto Chart presented Figure D.9 in Appendix D. At 95 % confidence level of significance, the linear effect of C_{IL} was the most significant, followed by the negative quadratic effect of C_{IL}, linear effect of the time, and linear effect of $\%_{IL}$. These effects could be observed in the response surface plots (Figure 3.3.5), which clearly illustrate the combined interaction of the extraction operational conditions optimized. As depicted in Figure 3.3.5, the best response on the extraction of fucoxanthin was obtained by using 40 min of homogenization, C_{IL} of 350 mM, and the highest $\%_{IL}$ (84 %).

This model has a high predictive accuracy since the optimal conditions of fucoxanthin extraction were validated by a low relative deviation (1.61 %, Table D.8 in Appendix D).

$$\text{Yield of extraction } \left(\mu g_{\text{fuco}} \cdot g^{-1}_{\text{dry biomass}}\right) = -714.284 + 6.513(X1) + 7.934(X2) - 0.011(X2)^2 + 8.527(X4) \quad \textbf{Eq. 6}$$

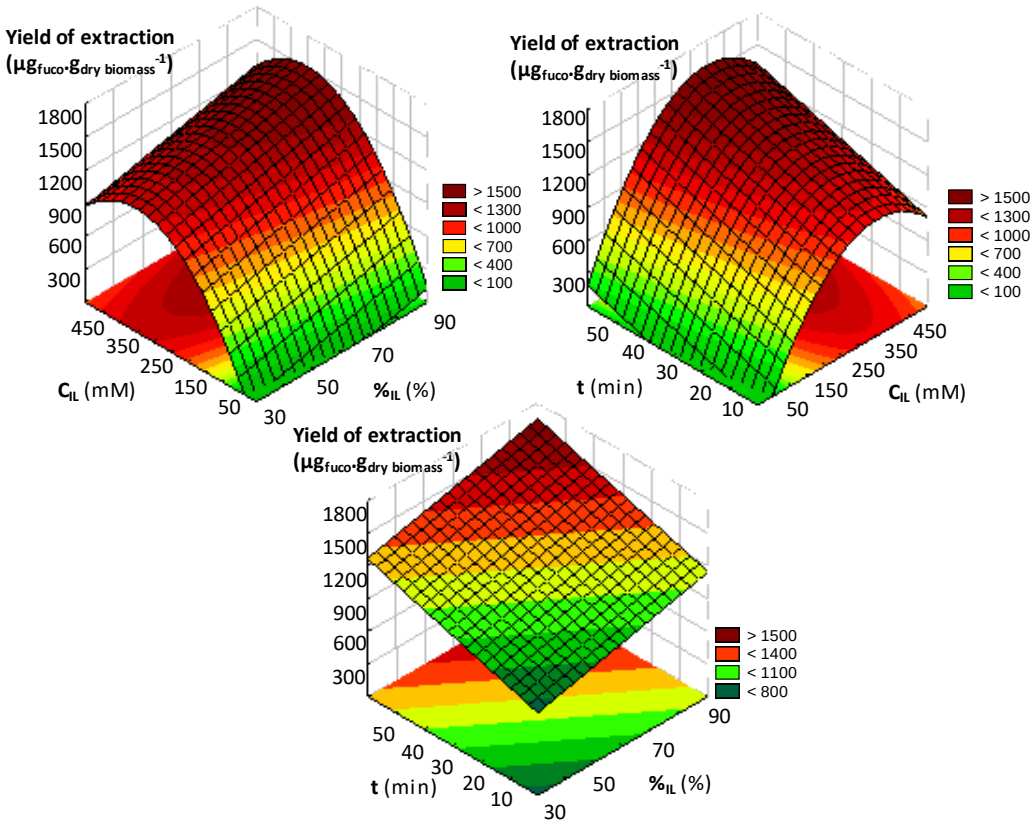


Figure 3.3.5. Response surface plots obtained for the CCRD (2^4) using a system with $[P_{4,4,4,14}]Cl$ regarding: content of aqueous solution of IL ($\%_{IL}$ in %), time (t in min), and IL concentration (C_{IL} in mM) in terms of yield of extraction of fucoxanthin ($\mu g_{fuco}.g_{dry\ biomass}^{-1}$). Graphs regarding solid-liquid ratio are not depicted since the condition is not significant in this context.

Regarding the extraction of chlorophyll using the $[P_{4,4,4,14}]Cl$, the model was not considered as predictive. This means that any change in the studied operational conditions is not statistically significant to improve the yield of extraction of chlorophyll. However, at the optimum conditions to extract fucoxanthin, the biomass residues at the end of the process are almost colourless, suggesting a complete extraction of the pigments, including the chlorophylls (Figure D.10 in Appendix D). After a careful analysis,

the best operational conditions for each system, as well as the results obtained are summarized in Table 3.3.2 and Figure D.11 in Appendix D.

Table 3.3.2. Optimized operational conditions for the systems composed of aqueous solutions of $[N_{1,1,1,10}]Br$ and $[P_{4,4,4,14}]Cl$ and oil, plus the respective results in terms of yields of extraction of fucoxanthin and chlorophyll. Different letters represent statistically different values ($p < 0.05$). The analyses were carried out separately for each result, to allow the comparison of systems based on different ILs.

		$[N_{1,1,1,10}]Br$	**$[P_{4,4,4,14}]Cl$**
Operational conditions	$\%_{IL}$ (%)	84	84
	C_{IL} (mM)	400	350
	SLR ($g_{dry\ biomass}.mL_{solvent}^{-1}$)	0.017	0.017
	t (min)	30	40
Results	Yield of extraction of fucoxanthin ($\mu g_{fuco}.g_{dry\ biomass}^{-1}$)	1836 ± 54[a]	1956 ± 84[a]
	Yield of extraction of chlorophyll in ($mg_{chl}.g_{dry\ biomass}^{-1}$)	4.528 ± 0.079[b]	4.93 ± 0.22[a]
	Contamination of chlorophyll in the IL-rich phase ($mg.L^{-1}$)	10.48 ± 0.40[b]	8.76 ± 0.42[a]

Most works focusing on the extraction of bioactive molecules from algae use multiple operations. As example in a recent work using *Spirulina* sp.,[153] supercritical CO_2 was used to recover in separate steps carotenoids, chlorophylls, and phycocyanin. The process here proposed, while using a simple approach allows the simultaneous extraction and purification of the two main pigments present in the alga studied, both of high interest and commercial value. In addition to the stability of carotenoids provided by ILs (e.g. fucoxanthin,[198] all-isomers of lycopene,[199] and all-isomers of carotene[200]), the oil fraction rich in hydrophobic compounds is usually more thermally stable than aqueous- and ethanolic-extracts. This guarantees the pigment stability, allowing its higher shelf time, and thus increasing the range of possibilities for new products (e.g. as emulsifiers, supplements) or even loaded in the formulation of new bio-materials.[180,201] Considering

the purity of the fractions, in comparison to the initial screening (Figure D.3 of Appendix D) the spectra depicted in Figure D.11 of Appendix D clearly show the purification of both pigments during the process, presenting spectra very similar to the pure pigments, as can be checked in literature.[202,203] In the end, considering not only the final results obtained after the optimization, but also the higher selectivity, the time saved, and the scalable potential, the single-step approach based on $[P_{4,4,4,14}]Cl$ was selected for complementary studies envisioning the design of a complete process.

IL recovery

Aiming to decrease the environmental and economic impacts of the process, it is imperative to define a strategy to recover and reuse the IL, that is the costliest solvent used. The aqueous IL-rich (bottom) phase was separated from the oil-rich (top) phase. Then, the IL content was measured in the IL-rich phase and in the respective bottom phase of the control, represented by the system where no biomass was used. The same amount of IL initially added to the system (oil+IL+water) was quantified in the IL-rich phase, meaning that the oil-rich phase is free of IL, which consequently indicates that the chlorophyll-based extract is also free of IL.

Regarding the aqueous IL-rich phase, a back-extraction was applied to remove the fucoxanthin from the aqueous phase of $[P_{4,4,4,14}]Cl$, thus allowing the IL recovery. Toluene, ethyl acetate, and diethyl ether were chosen due to their immiscibility with water and approved industrial application despite the need for explosive atmosphere certified facilities and subsequent capital expenditure (CAPEX) involved. Each organic solvent was individually added to the aqueous phase of IL, being these mixtures homogenized and centrifuged to allow the phase separation and the pigment partition. The results are presented in Table 3.3.3.

Table 3.3.3. Pigment partition to the organic phase and the % of the IL recovered after back-extraction for each organic solvent tested.

Organic solvent	Complete pigment extraction	% IL remaining in the aqueous phase
Diethyl ether	X	-
Ethyl acetate	✓	30 %
Toluene	✓	82 %

All systems were able to form two immiscible phases. In the case of diethyl ether, the pigment partitioned equally between the two phases, meaning that the organic phase (diethyl ether-rich phase) was not able to recover the total content of pigment. In the other hand, in the systems composed of ethyl acetate and toluene, all pigment content has partitioned to the organic phase, leaving the aqueous phase completely clear. For the systems with the complete partition of pigments for the organic phase, *i.e.* ethyl acetate and toluene, the presence of IL was identified and further quantified by its content in chloride ion using an ion selective electrode. According to the results, 30 % (*i.e.* 105 mM) and 82 % (*i.e.* 287 mM) of the initial amount of IL within the system (350 mM) remained in the aqueous phase for systems composed of ethyl acetate and toluene, respectively. This means that when fucoxanthin partitions towards the ethyl acetate phase, around 70 % of the IL also partitions to the ethyl acetate phase. However, good results were obtained using toluene, which proved to be a good candidate to recover the IL content (more than 80 % of the initial amount of IL remain in the aqueous phase and can be reused).

Final conceptual process

In Figure 3.3.6 is represented the final conceptual process proposed to recover the pigments from *Saccharina latissima* based on the results obtained in this work. This final process is composed of a: (i) single-step approach to simultaneously extract and fractionate fucoxanthin and chlorophylls, being at this point the chlorophyll-rich phase (oil phase) ready for further use in food applications for example, since it is free of IL; (ii) a back-extraction using toluene allowing the IL recovery; and lastly (iii) a vacuum drier is proposed as a method to recover fucoxanthin[204] from the toluene phase at low pressures and temperatures (35 °C) to avoid the carotenoids degradation and allowing the recovery and reuse of the organic solvent, closing the recycle loops of the process. As example of pigments in oil for food application, different products are available in the market such as “liquid chlorophyll super concentrated” used as food supplements in glycerin. Following the same rationale, but with another pigment, a mayonnaise-like food using a rich carotenoid-oil obtained from an Amazonian fruit was developed with enhanced biological properties.[171] Additionally, the analysis of the fucoxanthin powder was not performed, however it may contain trace elements of IL and/or toluene, being

this analysis needed before application. The scale-up trials shall also include fresh material either from *Saccharina latissima* (Linnaeus) or other industrially relevant biomass, as a source of fucoxanthin and chlorophylls.

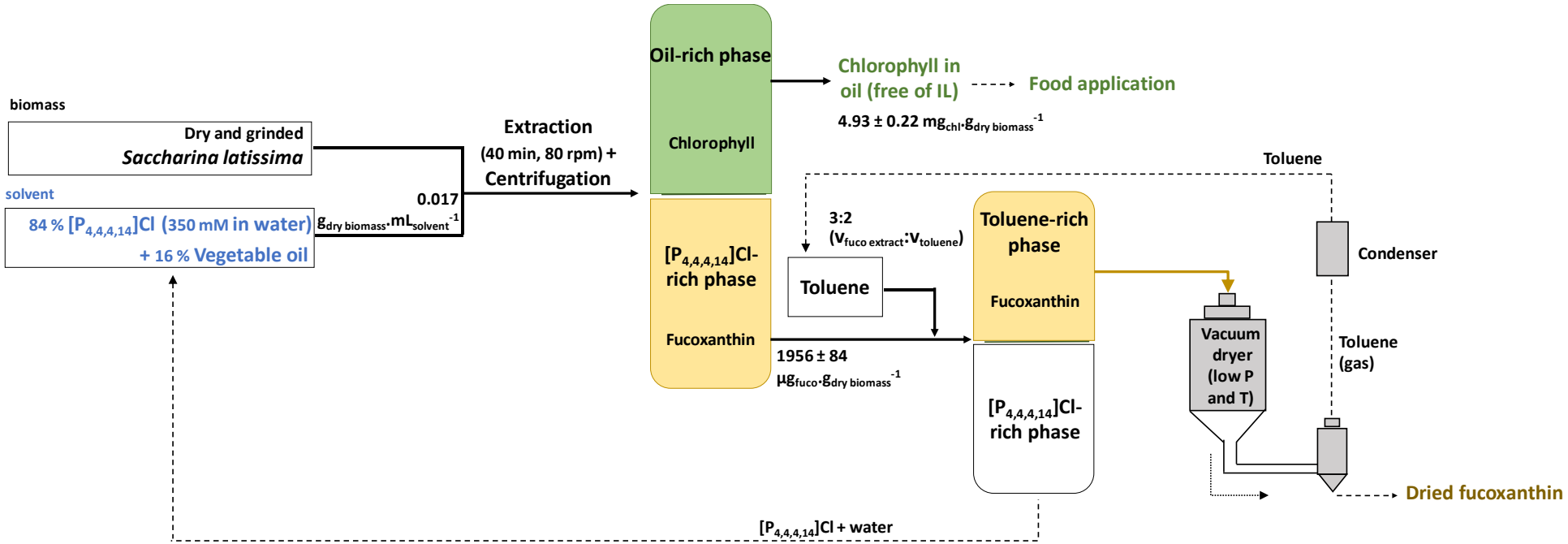


Figure 3.3.6. Conceptual process diagram proposed for the recovery of chlorophyll and fucoxanthin from *Saccharina latissima*. Dashed lines are just a proposal of what can be done, not having been tested in this work.

Environmental analysis by life cycle assessment

The results obtained using the ReCiPe 2016 Midpoint method at the Hierarchist perspective[205] to translate environmental emissions and resource extraction into environmental impacts are presented in Table 3.3.4 and Figure 3.3.7.

Table 3.3.4. Life cycle assessment results for the recovery of chlorophyll and fucoxanthin from 0.2 g of dry biomass of *Saccharina latissima*.

Impact category	**No IL reuse**	**IL reuse**
Global warming (g CO_2 eq)	35.5	31.4
Ozone formation, human health (g NO_x eq)	0.0924	0.0828
Terrestrial acidification (g SO_2 eq)	0.181	0.166
Fossil resource scarcity (g oil eq)	15.3	13.3

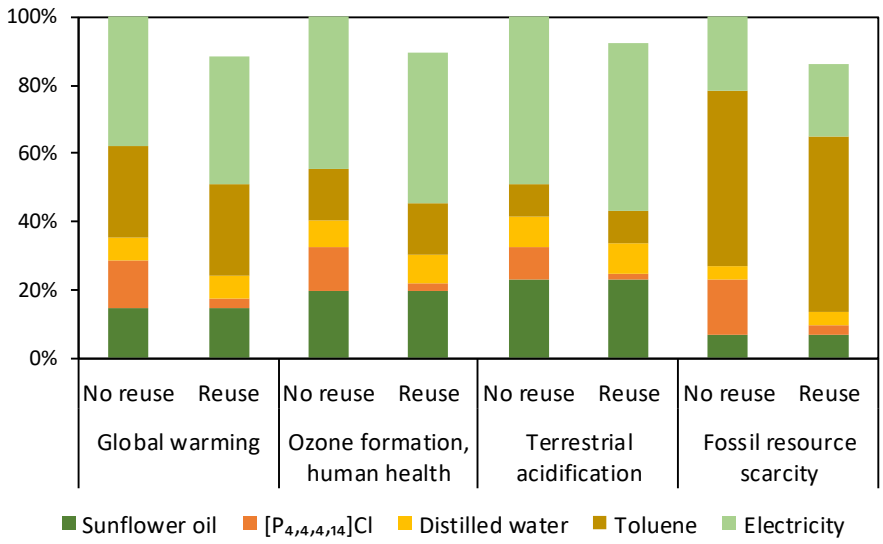


Figure 3.3.7. Relative contribution of the inputs for the life cycle assessment results.

The impact categories selected for analysis comprise the global warming (equivalent to the carbon footprint), photochemical ozone formation (effects on human health), terrestrial acidification and fossil resource scarcity. The main contribution to the impacts other than fossil resource scarcity is the electricity consumption by the equipment, which amounts to 38–49% of the total impacts. For fossil resource scarcity, toluene has the major role not only due to the use of fossil-based energy during its production but also because it is produced from naphtha. The reuse of the IL leads to a reduction of the environmental impacts in the order of 8–14% due to savings of fresh IL.

Economic analysis

After defined the environmental impact of the overall process proposed in this work, the economic footprint was evaluated, envisioning a more complete analysis for the potential scale-up of the process. The results from the calculation of production costs *per* mg of each pigment are depicted in Figure 3.3.8. The complete collection of results for all scenarios (production costs *per* mg of each pigment with and without recycling of solvents, with all recycling scenarios and percentages) are included in Table D.9 in Appendix D.

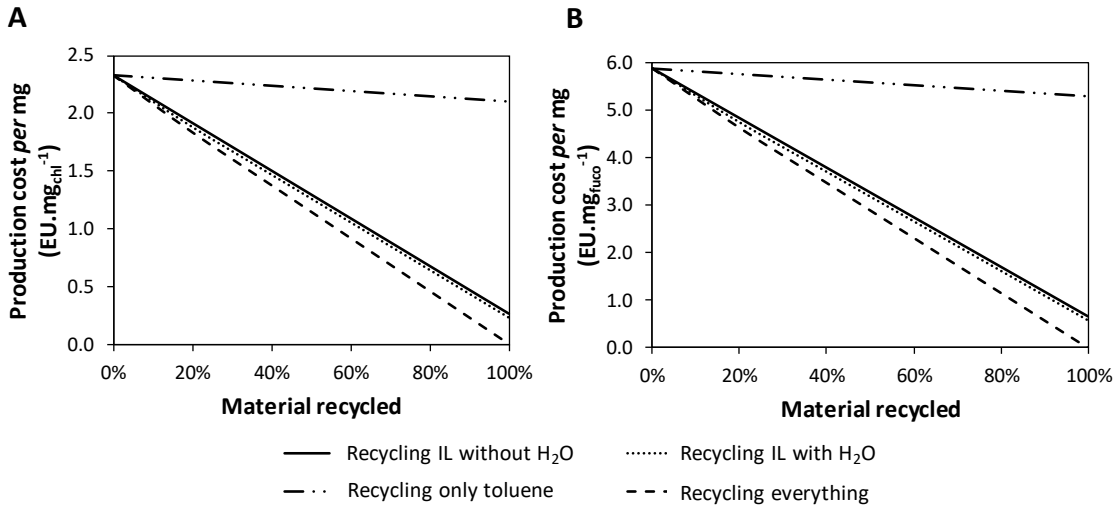


Figure 3.3.8. Economic evaluation of the production process for chlorophyll (A) and fucoxanthin (B).

Note: For both graphs, all lines start from a single data point (no recycling of any of the solvents) and spread across the graphs depending on the percentage of material recycled and the recycling scenario.

The base complete production cost (capital charge, materials/consumables, labour, and others) for the process depicted in Figure 3.3.8 is 2.33 and 5.86 EU *per* mg for chlorophyll and fucoxanthin, respectively. After including the recycling scenarios, the production costs decrease (Figure 3.3.8). Results for chlorophyll and fucoxanthin show that recycling toluene, even at 100 %, has the lowest impact on the production costs. For a deeper interpretation, even recycling only 20 % of the ILs grants a lower production cost. Preliminary recycling results presented in previous sections showed that it was possible to recover up to 82 % of IL. If it is possible to reuse this amount, this will provide a complete production cost lower than 0.64 EU *per* mg and 1.61 EU *per* mg for chlorophyll

and fucoxanthin, respectively. As a framework, recycling 100 % of IL, water, and toluene will further decrease the production costs providing the minimum cost for this process. For the Return, Eq. 2 and Eq. 3 provided a deep insight on the impact that the production costs can have on the profit. For this part of the analysis, the variables studied (not fixed values) were the percentage of recycled material (0 % to 100 %), the product market price (based price and 10- and 100-fold decrease), and the multiplier of production costs (10-fold increase and decrease). Full results are included in the Appendix D (Tables D.10 and D.11), while the most relevant results are depicted in Figure 3.3.9.

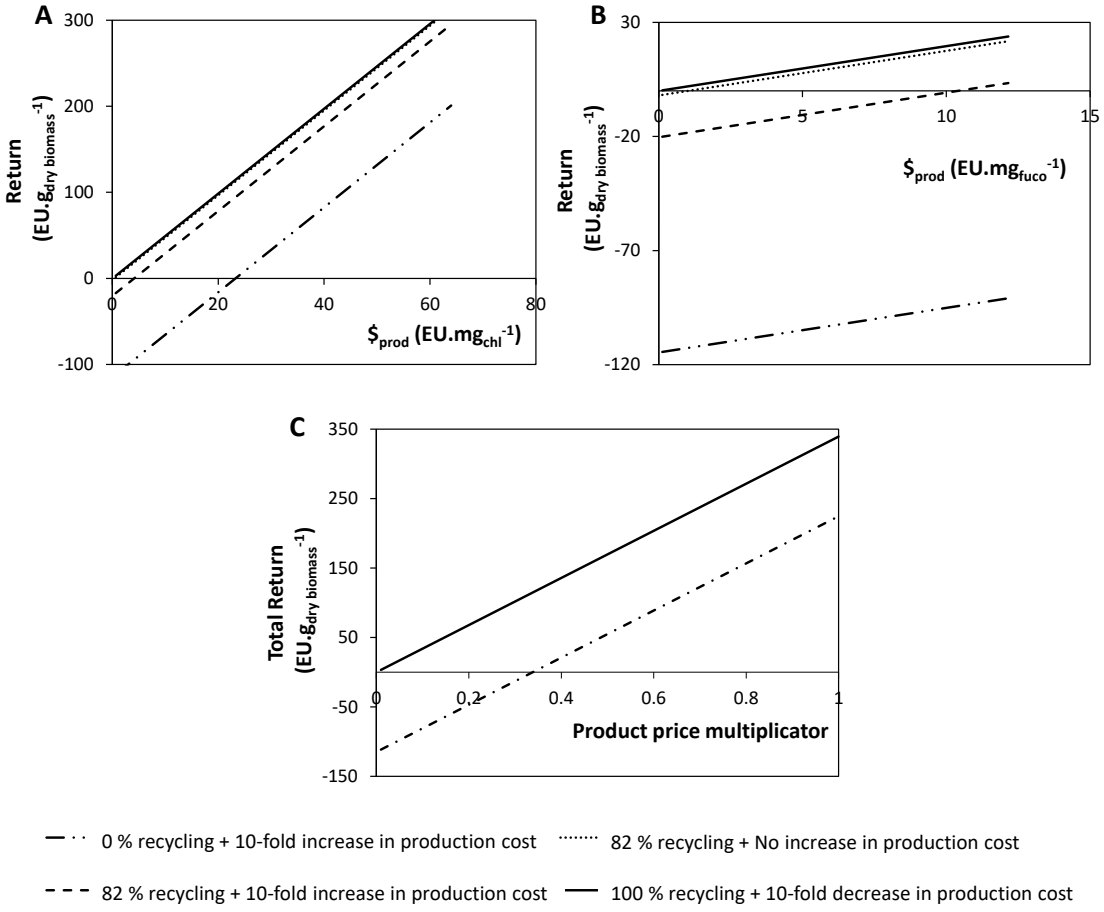


Figure 3.3.9. Return analysis of chlorophyll (A), fucoxanthin (B) and of both products (C). $\$_{prod}$ (market price) of each pigment is based on Sigma-Aldrich values. In (A) the solid line almost overlaps the dash line. In (C) x-axis was changed to the product price multiplier (0.01X, 0.1X, and 1X).

For the calculation of production costs, all operation units were considered for both products even if to get each product only a smaller set of unit operations was needed. This is because the design presented in Figure 3.3.6 generates chlorophyll and

fucoxanthin together, so both paths are going to be completed each batch. This indicates that both products have the same behaviours seen in Figure 3.3.8 A and Figure 3.3.8 B. Although they behave similarly, their absolute values are different. Contrarily, the results for Return consider different product prices, so the potential profit can be substantially different. This differential behaviour can be seen when contrasting Figures 3.3.9 A and 3.3.9 B, this is captured by the slopes of the lines depicted here. Moreover, the relevance of Figure 3.3.9 is that both lines show the boundaries of the analysis. For both graphs, the top line shows the best scenario, this is 100 % of material is recycled and production costs are 10-fold decreased, while the bottom line is the worst scenario, where no material is recycled, and costs increased 10 times. Both graphs show in the X-axis the product market prices with a 10- and 100-fold decrease. Additionally, Figure 3.3.9 C shows a potentially more real scenario. As both products are generated simultaneously, production costs are shared, so the profit will actually be higher overall, as shown in this graph.

With the help of the boundaries shown in Figure 3.3.9, it is possible to determine that most combinations of other scenarios will be found in between both lines. Figure 3.3.9 A shows that a chlorophyll market price above 20 EU *per* mg is required to have all scenarios with a positive Return. If this condition is met, recycling is not needed to have a positive outcome. As the chlorophyll price decreases, either the recycling percentage needs to increase or production costs to decrease (or a combination of both). For fucoxanthin the results seem to be different. There is a wide range of scenarios where it is not possible to have a positive outcome. Considering the base product price for fucoxanthin, a positive Return is possible even without recycling, but if production costs increase 10-fold, a recycling of 80 % of material is needed for a positive Return (1.16 EU.$g_{dry\ biomass}^{-1}$). Meanwhile, for analysing product price, if it decreases 10-fold, while production costs staying at the base level, then a recycling of 100 % will be required (0.23 EU.$g_{dry\ biomass}^{-1}$). If the worst cases for production costs (10-fold increase) and product price (10-fold decrease) are considered, then a 100 % of recycling is needed to have a positive outcome (0.19 EU.$g_{dry\ biomass}^{-1}$). As a contrast for the results for fucoxanthin, Figure 3.3.9 C shows that when both products are considered simultaneously, the low Return that fucoxanthin provides is greatly improved by the Return obtained from chlorophyll. Moreover, the combined effect (total return)

increased the range of possible scenarios that can grant a positive Return to the final process.

The experimental results showed that possibly 82 % of material can be recycled. This scenario is considered in both graphs (Figure 3.3.9 A and Figure 3.3.9 B) while maintaining the base production costs and product price. Overall, it is possible to obtain a positive Return for while maintaining realistic values for the rest of variables. This serves as a platform in which to based further developments in this area. Besides, it should also be considered that the remaining biomass has potential to be applied, either as a final product as feed or fertilizer, or as matrix to extract other biomolecules, which again will improve the value of the biomass in a biorefinery (multi-product) chain.

Conclusions

In this work a single-step approach to extract and fractionate chlorophyll from fucoxanthin was proposed. A mixture composed of an aqueous solution of a tensioactive IL and a vegetable oil was used together with dry biomass culminating in the preparation of a liquid-liquid extraction system, being the oily phase rich in chlorophyll and the IL-phase mainly composed of fucoxanthin. After selecting the best systems to extract the pigments from the brown algae ($[N_{1,1,1,10}]Br$ and $[P_{4,4,4,14}]Cl$), the operational process conditions were optimized, statistically analysed and validated. The best performance were achieved for systems with $[P_{4,4,4,14}]Cl$ with $\%_{IL}$ = 84 % ($\%_{oil}$ = 16 %); C_{IL} = 350 mM, SLR = 0.017 $g_{dry\ biomass}.mL_{solvent}^{-1}$, t = 40 min leading to yields of extraction of 4.93 ± 0.22 $mg_{chl}.g_{dry\ biomass}^{-1}$ and 1955.7 ± 84.4 $\mu g_{fuco}.g_{dry\ biomass}^{-1}$. Besides the recovery of two different pigments with high commercial value in a single- step approach, the recovery of up to 82% (= 287 mM) of the IL from the fucoxanthin phase was also achieved. In the end, this work provides an optimized, simple and efficient process to extract and purify the hydrophobic pigments from *Saccharina latissima* (Linnaeus). The scale-up of this process being straightforward, the industrial potential of this process is envisioned which is supported by both environmental and economic analysis.

3.4 Ionic liquids as eluents in solid-phase extraction to purify pigments recovered from *Isochrysis galbana*

This chapter is based on the submitted manuscript

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*Contributions: M.M. and B.M.C.V acquired the experimental data. M.M. performed the data analysis. L.M.S.M. performed the response surface methodology and statistical analysis. M.C.N. supervised the continuous process in column. M.G.P.M.S.N., and D.C.G.A.P. assessed the UHPLC-MS experimental data. M.M. wrote the manuscript with substantial contributions from the remaining authors.

Abstract

Purification processes are bottlenecks in the downstream processes. The need for handy and fast techniques to purify biomolecules to increase their stability and value is clear. Solid-phase extraction is a technique that allows the purification of a target compound by its adsorption from a liquid matrix. The AmberLite™ HPR900 OH is a resin that fractionate carotenoids and chlorophylls from extracts containing both pigments. An innovative procedure to elute the chlorophyll from AmberLite™ HPR900 OH, allowing to obtain it as a secondary product, is proposed in this work. Aqueous solutions of tensioactive ionic liquids were shown to elute chlorophylls from the AmberLite™ HPR900 OH resin successfully. The chemical structure of the obtained pigments was proposed. The operational conditions were optimized, showing that the resin can be reused for up to five cycles without losing its efficiency.

Keywords: AmberLite™ HPR900 OH, solid-phase extraction, pigments, resin reuse, ionic liquids as eluents.

Introduction

The demand for natural products is growing. This trend is associated with the increased awareness of human health and environmental concerns that may be addressed using compounds from natural sources instead of their synthetic alternatives.[2,3] The concerns

about climate change and the new policies to reduce the environmental impact of industrial processes and products make the use of natural products a highdemand,[206] particularly when integrated into a smart and low-waste chain of different products or within a circular economy approach.[1–3,5]

Natural pigments represent one of the most important families of compounds that can be obtained from natural sources. Their use can boost the visual appearance of a final product and act as health promoters due to their multiple biological activities, especially regarding their antioxidant and anti-inflammatory properties.[7,21,92] As examples are chlorophylls and xanthophylls (oxygenated carotenoids). These are well-known pigments present in all photosynthetic organisms, such as plants, microorganisms, and algae.[92]

Along with the sustainable use of natural resources, obtaining and processing them should also be a concern.[1,5] Several works have proposed alternative approaches to extract these pigments without compromise their stability,[156,200] and studied the economic[156] and environmental[180] viability of these processes. Aqueous solutions of tensioactive ionic liquids (ILs) and common surfactants have been proposed as solvents to extract different pigments, such as chlorophylls[156,207] and carotenoids[180,208] from different natural matrices. Tensioactive ILs with surfactant features are salts with low charge density that can be very efficient in extraction procedures due to their tunability.[209]

Although the extraction of these pigments is straightforward even using cheap and simple methodologies,[156] since chlorophylls and carotenoids have similar polarities and are usually present in the same cellular sites,[177] their co-extraction usually occurs at similar conditions. This means that an additional purification step is required to separate chlorophylls from carotenoids. The purity level is usually defined by the extract's final application, with high purity levels allowing the extract to be more stable. Additionally, the separation of chlorophylls from carotenoids allows a more correct quantification of these compounds by simple techniques such as UV-Vis spectroscopy, since carotenoids and chlorophylls present a maximum absorption at close wavelengths.[210] However, this purification step remains the bottleneck of the whole process.[181]

A simpler and reliable separation process for this purpose is the solid-phase extraction. It is based on the adsorption of chlorophylls on a resin due to intermolecular and

interionic interactions (e.g., dipole-dipole, ion-dipole, hydrogen bonding, ion-ion) between chlorophyll derivatives and the tetramethylammonium functional group of AmberLite™ HPR900 OH (previously known as *Ambersep 900 OH*).[211–213] AmberLite™ HPR900 OH is a strong basic anionic resin that allows an effective and fast adsorption of chlorophyll, while the carotenoids remain in solution.[211–213] Its efficiency was already demonstrated for various extracts from green vegetables such as beans, broccoli, spinach, lettuce, and peas, among others.[212,213] However, no previous work has successfully eluted the chlorophyll from the resin, allowing the recovery of the chlorophyll as a secondary product, and enhancing the resin's lifetime through more cycles of reuse.

In this work, a simple process to separate carotenoids and chlorophylls from an extract of microalgae *Isochrysis galbana* Parke 1949 using the resin AmberLite™ HPR900 OH is proposed. In particular, the elution of the chlorophylls was studied and optimized. The resin's reutilization was also carried up to five times in batch and in a continuous process envisioning its industrial application.

Experimental

Biomass

The microalgae used in this work is the Isochrysis galbana Parke 1949 and it was obtained in systems of photobioreactors (PhytoBloom). It was purchased at Necton S.A., a company located in Olhão (Portugal). The batch used in this work was produced in July 2018, being freeze dried and grinded in August 2018. The biomass was kept in a dry and dark environment until usage.

Chemicals

Ethanol (HPLC grade, CAS 64-17-5), methanol (HPLC grade, CAS 67-56-1), and acetone (HPLC grade, CAS 67-64-1) used on the extraction of pigments from the biomass were acquired from Fisher Scientific. AmberLite™ HPR900 OH (CAS 9017-79-2), a strong basic anionic resin, which is composed of approximately 35–55 % quaternary amine styrene-divinylbenzene copolymer of the OH form and 45–65 % water,[213] was purchased from Sigma-Aldrich. Several organic solvents were additionally used in the attempts to elute chlorophyll from the resin. Dichloromethane (99.9 wt% of purity, CAS 75-09-2), and

toluene (99.8 wt% of purity, CAS 108-88-3) were purchased from Fisher Scientific. Formic acid (99 wt% of purity, CAS 64-18-6), acetonitrile (99.99 wt% of purity, CAS 75-05-08), and petroleum ether (PA-ACS-ISO, CAS 8032-32-4) were purchased from Carlos Erba, Fisher Chemical, and Panreac, respectively. The sodium hydroxide (98.0 wt% of purity, CAS 1310-73-2) was supplied by Fisher. The ionic liquids (ILs) based on ammonium family such as dodecyltrimethylammonium bromide, $[N_{1,1,1,12}]Br$ (99 wt% of purity, CAS 1119-94-) and tetradecyltrimethylammonium bromide, $[N_{1,1,1,14}]Br$ (98 wt% of purity, CAS CAS 1119-97-7), were purchased from Alfa Aesar while the decyltrimethylammonium bromide, $[N_{1,1,1,10}]Br$ (99 wt% of purity, CAS 2082-84-0), was acquired from Tokyo Chemical Industry (TCI). The tributyltetradecylphosphonium chloride, $[P_{4,4,4,14}]Cl$ (95 wt% of purity), was supplied by Iolitec. The molecular structures of the ILs used in this work are depicted in Figure E.1 in Appendix E. The deuterium oxide (99.9 % of purity, CAS 7789-20-0) used for NMR spectroscopy was purchased from Aldrich.

Pigments extraction

The solid-liquid extraction step was performed using a methodology adapted from Martins et al.[156] Pure ethanol, acetone, and methanol were screened as extraction solvents. The dry biomass was homogenised with the solvent in a shaker IKA TRAYSTER digital under constant vertical rotation (80 rpm). The extraction was performed using 0.01 $g_{biomass}.mL_{solvent}^{-1}$, at room temperature (20–25 °C), during 30 min and protected from light exposure.[156] The samples were centrifuged in a Thermo Scientific Heraeus Megafuge 16R centrifuge at 4700 *g*, for 30 min at 4 °C. The supernatant (initial extract) was collected and the biomass debris were discarded.

Chlorophyll adsorption and carotenoids recovery

The commercial resin AmberLite™ HPR900 OH was washed with distilled water according to Larsen and Christensen (2005)[213] and dried in an oven at 50 °C for 15 min. The initial extract collected with the different solvents was further diluted in the same solvent 1:1 (v:v) to avoid the pigment saturation in the resin. Initially, 1 g of resin was put in contact with 10 mL of diluted extract under magnet stirring at room temperature (20–25 °C) for 30 min. The chlorophyll content was mostly adsorbed by

AmberLite™ HPR900 OH, while the carotenoid content remained in organic solution. The organic fraction was collected, being the carotenoid content analysed for each initial solvent.

Chlorophyll elution and resin regeneration

Various solutions, reported in Table 3.4.1, were screened in terms of their performance to elute chlorophylls from the resin, where the adsorption step used ethanolic extracts. Concentrations above the critical micellar concentration were always used for the aqueous solutions of ILs (CMC values available at Table E.1 in Appendix E). For each case, the resin in the presence of 10 mL of the regenerating eluent was maintained under a magnet stirring at room temperature (20–25 ºC) during 30 min. After this period, the chlorophyll content of the solution was analysed by UV-Vis spectroscopy. Three replicates were made in order to decrease the error associated with each assay.

Table 3.4.1. Screening of eluents used to recover chlorophyll.

Type	**Eluents**	**Concentration[Ref]**
Alkaline	Sodium hydroxide	4 % (w:v) in water[211]
Hot water	Distilled water at 80 ºC	100 %[214]
Organic solvents	Dichloromethane	100 %[215]
	Acetonitrile	100 %[216]
	Toluene	100 %[217]
	Petroleum ether	100 %[218]
Mixture of solvents	Formic acid, acetonitrile, and methanol	0.1 % : 69.93 % : 29.97 %[158]
Aqueous solutions of ILs	$[N_{1,1,1,10}]Br$	250 mM in water[156,180]
	$[N_{1,1,1,12}]Br$	250 mM in water[156,180]
	$[N_{1,1,1,14}]Br$	250 mM in water[156,180]
	$[P_{4,4,4,14}]Cl$	250 mM in water[156,180]

Optimization of the elution of chlorophylls from the resin

In order to optimize the elution of the chlorophylls from the resin, a central composite rotatable design (CCRD – 2^3 with 6 central points and axial points) was applied in a total of 20 assays. This assay was done separately for the two best eluents found. The results obtained were statistically analysed with a confidence level of 95 %, using pure error as standard. Three independent variables were studied, namely the solid-liquid ratio, *i.e.*

the ratio between the mass of resin and the volume of eluent used (SLR, $g_{resin}.mL_{eluent}^{-1}$), time of contact (t, min), and concentration of IL (C_{IL}, mM), being their performance analysed in terms of the chlorophyll recovery from the resin (%). Regarding the SLR study, the volume of extract (in adsorption step) and the volume of eluent (in the elution step) were kept constant, being 5 and 15 mL, respectively. The mass of resin was variable according to the SLR along the 20 runs, however, constant from the start to the end of each run individually, *i.e.* the initial mass of resin used in the adsorption step was the same used in the elution step for each run. All the codified and real values used in the CCRD are shown in Table 3.4.2. The results were analysed using the software Statistica® 12. After interpretation of the response surface methodology results, the optimum conditions to elute chlorophylls were determined, with further validation of the optimum conditions in triplicate by the means of relative deviation (%).

Table 3.4.2. Real and coded values of the CCRD (2^3).

Coded variable level	SLR ($g_{resin}.mL_{eluent}^{-1}$)	t (min)	C_{IL} (mM)
-1.68	0.016	11.5	48.5
-1	0.030	19.0	130.0
0	0.050	30.0	250.0
+1	0.070	41.0	370.0
+1.68	0.084	48.5	451.6

Pigments quantification

The UV-Visible spectra of the collected samples were measured between 200 and 700 nm in a UV-Vis microplate reader (Synergy HT microplate reader – BioTek). The mass percentage of chlorophyll was determined according to calibration curves previously obtained at 667 nm (R^2 = 0.9389 and R^2 = 0.9805 for aqueous and ethanolic extracts, respectively) and comparing the chlorophyll mass loaded in the resin (present in the organic extract) and the chlorophyll mass of each fraction collected (Eq. 1). Note that, during this work, it was used *chlorophyll recovery* instead of *eluted chlorophyll* since the chlorophyll content was analysed not only in the elution step but also in the collected fraction of carotenoids and fraction of NaOH used in the resin regeneration. Although *Isochrysis galbana* has different xanthophylls, such as diadinoxanthin,[219]

diatoxanthin,[220] and fucoxanthin[221,222] (being also the diadinoxanthin the biologic precursor of both diatoxanthin and fucoxanthin)[223], in this work the total xanthophylls content was directly related to the fucoxanthin content. The fucoxanthin quantification was done using a calibration curve in ethanol previously determined (R^2 = 0.998) at 450 nm, being its concentration ($mg_{fuco}.L_{ethanol\ extract}^{-1}$) calculated afterwards.

$$\text{Chlorophyll recovery (\%)} = \frac{\text{Chlorophyll in the collected fraction (mg)}}{\text{Chlorophyll in organic extract loaded into the resin (mg)}} \times 100$$

Eq. 1

Ultra-performance liquid chromatography coupled mass spectrometer (UHPLC-MS) analysis

The initial extract, the recovered fraction of carotenoids after the adsorption of the chlorophyll, and the eluted fraction of the chlorophylls after the polishing step were analysed by UHPLC-MS. The UHPLC-MS analysis was performed by Thermo Scientific Ultimate 3000RSLC (Dionex) equipped with a Dionex UltiMate 3000 RS diode array detector and coupled to a mass spectrometer. The separation of the compounds was carried out with a gradient elution program at a flow rate of 0.3 $mL.min^{-1}$, at 30 °C, using a Hypersil Gold C18 column (150 × 2.1 mm; 5 µm, Thermo Fisher). The injection volume in the UHPLC system was 3 µL, and the mobile phase consisted of formic acid 0.1 % in water (A) and acetonitrile (7):methanol (3) (B), both degassed and filtered before use. The solvent gradient was 85 % of solvent B in the first 3.9 min, followed by the increase up to 100 % during 2.2 min, and maintaining 100 % of solvent B for 18.9 min, returning to 85 % during 6 min, and equilibrating during 7 min. The injection volume was 2 µL. UV-Vis spectral data were gathered in a range of 200 to 700 nm.

Continuous process in column

The continuous process was performed using a solid-phase extraction cartridge and a peristaltic pump to ensure a constant flow (45 $\mu L.s^{-1}$). In this step, the best eluent and the optimized conditions in the response surface methodology for the batch assays were used. The time of contact adopted in the continuous process was not the same in comparison to the optimum values for the batch assays due to experimental limitations. The solid-phase extraction cartridge was prepared by packing 5.25 g of AmberLite™

HPR900 OH resin (previously washed with water and dried) between two frits into a 20 mL empty polypropylene cartridge (Bio-rad Econo-Pac), being the resin conditioned with 25 mL of sodium hydroxide aqueous solution (4 % w:v) afterwards. A volume of 25 mL of the ethanolic extract was passed through the cartridge, and subsequently, the adsorbed chlorophyll content was eluted with 75 mL of 370 mM aqueous solution of the $[N_{1,1,1,12}]Br$. To regenerate the OH^- groups of the resin, 25 mL of an aqueous solution of sodium hydroxide (4 % w:v) was passed through the column. The chlorophyll content of each solution collected from the solid-phase extraction cartridge was analysed by measuring the absorption at 667 nm and applying the correspondent calibration curve.

Chlorophyll polishing

The aqueous solution of IL containing eluted chlorophyll was freeze-dried to remove the water content of the sample. The powder obtained was dissolved in ethanol in the proportion of 10:3 ($V_{ethanol}$:$V_{initial\ aqueous\ solution}$). The ethanolic solution containing the chlorophyll content and IL was homogenized and kept at -80 °C, for three days. A viscous and colourless pellet was formed in the bottom of the flask and the content in IL of the ethanol was analysed. In order to quantify the content of $[N_{1,1,1,12}]Br$ in the ethanolic fraction, an ^1H NMR spectroscopy was performed. The ^1H NMR spectrum of pure IL and ethanolic fraction rich in chlorophylls was carried out using a Bruker AC 30 spectrometer (250 MHz) at room temperature with deuterium oxide (D_2O) as solvent.

Results

Carotenoids recovery and screening of solutions to elute chlorophyll

The use of the commercial resin AmberLite™ HPR900 OH to purify carotenoids by the adsorption of the chlorophyll content is not new. In the published works, initial extracts were obtained using acetone as solvent, extracts that are later loaded in the resin.[212,213] Although the scope of the present work was to develop a technique to elute the chlorophyll from the resin, the initial extracts were briefly studied. Three initial extracts were prepared from *Isochrysis galbana* using as solvents acetone, methanol, and ethanol. These solvents were chosen due to their ability to extract carotenoids and chlorophylls.[178,212] Extracts rich in these pigments were obtained for the three organic solvents. However, when loaded in the resin, the xanthophyll rich extract collected had

the lowest chlorophyll contamination when the initial ethanolic extract was used (1.8 $mg_{chl}.L^{-1}$) followed by the acetone and methanol initial extracts (5.9 and 8.0 $mg_{chl}.L^{-1}$, respectively). Based on these results, and taking into account the carbon footprint and environmental impact of the screened solvents,[194] ethanol was used to progress study.

The adsorption mechanism was first reported to be mediated by ion-ion interactions after the saponification of the chlorophyll and the release of phytol [213] and posteriorly to occur principally through hydrogen-bonds and dipole–dipole interactions between resin and chlorophylls polar units, leaving the carotenoid content in the ethanol.[212] In this work, a carotenoid rich-extract of orange colour was obtained after 30 min of contact, while the resin acquired a green colour due to the adsorption of the chlorophyll pigments (see Figure E.2 B in Appendix E).

According to the data sheet of the resin,[211] its regeneration should be done with an aqueous solution of NaOH (2–4 %) to re-establish the OH^- groups of the resin. Several cycles of reuse of the resin with new batches of initial extract were performed with and without a step of regeneration in order to evaluate the importance of this regeneration step (Figure 3.4.1).

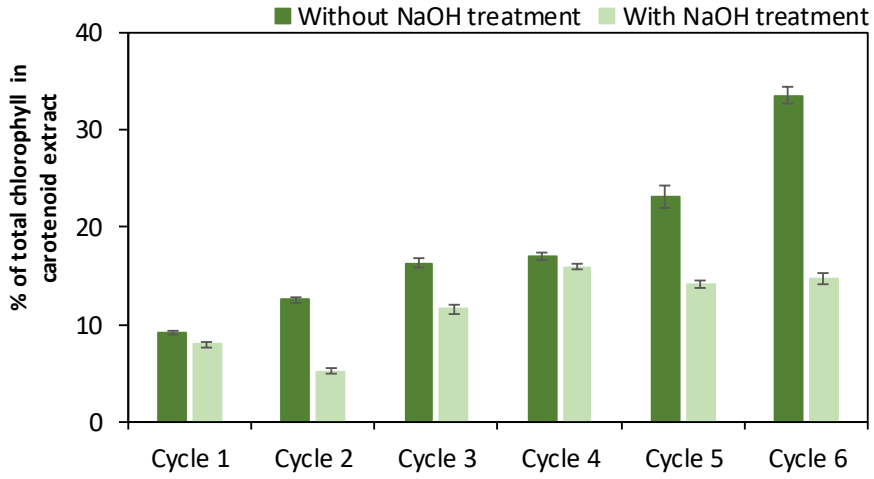

Figure 3.4.1. Percentage of the total chlorophyll in the carotenoid extracts in several cycles of reuse of the resin without and with NaOH treatment.

The results show that the contamination of chlorophyll in the carotenoid extract increases in each cycle, after addition of a new batch of ethanolic initial extract, when the NaOH regeneration solution was not previously used (9.1–34 %). In the other hand, the contamination of chlorophyll in the carotenoid extract is almost constant (5.2–16 %) even with the increase in the number of cycles of reuse, when the NaOH solution is

applied. This indicates the need of using NaOH to replace the OH^- groups within the resin in a so-called *regeneration process*, allowing a more efficient adsorption of the chlorophyll in the subsequent cycles.

Although it was shown that the resin can be regenerated for several cycles with solutions of NaOH without compromising the quality of the carotenoid extract, this does not mean that the chlorophyll can be efficiently recovered from the resin. Given the chlorophyll market value, and if possible, to extend the lifetime of the resin, the recovery of the chlorophyll pigments adsorbed in the resin is crucial. Therefore, the performance of different eluents for an efficient recovery of the chlorophyll pigments was studied being the main results shown in Figure 3.4.2.

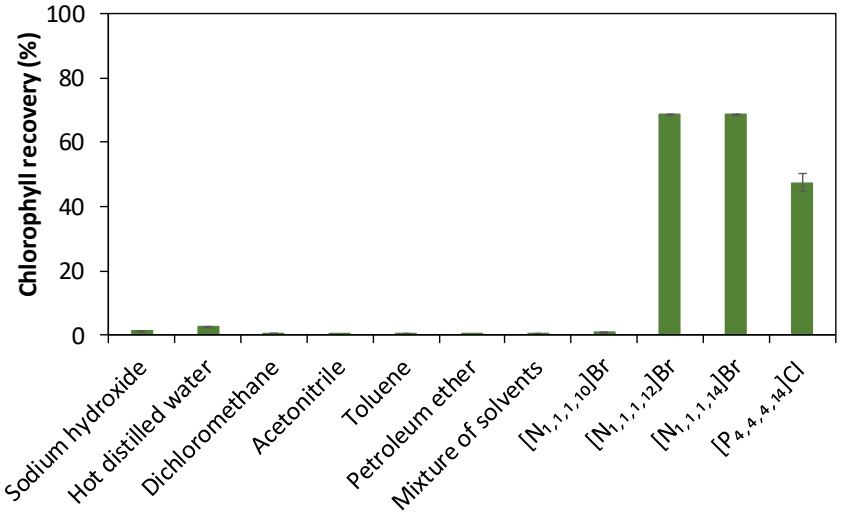

Figure 3.4.2. Screening of different solutions used to elute the chlorophyll from the resin. Aqueous solutions of IL were screened at 250 mM and aqueous solutions of NaOH at 4 % (w:v).

Although solutions of NaOH were essential to regenerate the resin and restore the terminal OH^- groups, they were not able to desorb chlorophylls from the resin. Several organic solvents that are used in chlorophyll extraction from biomass were tested, as well as mixtures of solvents used in the washing procedures of reverse-phase columns. None of them were efficient in the removal of the chlorophyll content (< 2.5 %) from the resin as can be observed in Figure 3.4.2.

More appealing results were achieved when aqueous solutions of various ILs (at 250 mM) were investigated as eluents (Figure 3.4.2, right-side). The screened solutions of ILs were used before with success in the extraction of chlorophyll and carotenoids

from biomass.[156,180] Unlike the other screened solutions, most of the aqueous solutions of ILs showed to be able to remove the adsorbed chlorophyll from the resin, namely the $[N_{1,1,1,12}]Br$, $[N_{1,1,1,14}]Br$ and $[P_{4,4,4,14}]Cl$. These aqueous solutions of ILs were previously identified as efficient solvents to extract pigments due to their ability to form micelles above a certain concentration (as it happens at 250 mM in all tested solvents) providing the perfect environment to hydrophobic molecules.[156] However, an additional interaction is behind their good performance as eluents. These cationic ILs have the same positively charged head as the one present in the functional group of the resin (Figure E.3 in Appendix E). This said, the type of interaction between the chlorophyll derivatives and the functional group of the resin can be replaced by similar interactions but now involving the cationic IL. In this work, the $[N_{1,1,1,12}]Br$ and the $[P_{4,4,4,14}]Cl$, were selected to further optimize the operational conditions.

Successive elutions of the resin, applying the same mass of resin and volume of eluent (fresh solutions of IL), were applied to achieve the maximum chlorophyll recovery from the resin. As conclusion, chlorophyll is successively extracted during three elution steps. Given that, and to keep the elution in a single step, the volume of eluent used is always 3 times higher than the volume used in the adsorption step (with ethanolic initial extract) and in the regeneration step (with aqueous solution of NaOH), in the following proportion 1:3:1 ($V_{initial\ extract}$:V_{eluent}:V_{NaOH}). However, a more detailed analysis on the SLR, namely by the manipulation of the resin mass used, was done by applying a response surface methodology for both aqueous solutions of $[N_{1,1,1,12}]Br$ and $[P_{4,4,4,14}]Cl$.

Optimization of the process conditions by a response surface methodology

For the central composite rotatable design (CCRD), three variables were studied to achieve a complete optimization of the best possible chlorophyll recovery (response), namely solid-liquid ratio (SLR in $g_{resin}.mL_{eluent}^{-1}$, X1), time of contact (t in min, X2), and concentration of IL in water (C_{IL} in mM, X3). A total of 20 runs was performed, including the three common levels (-1, 0, +1), axial (-1.68, and +1.68 levels), and six central points (level 0). According to the CCRD experiment using aqueous solutions of $[P_{4,4,4,14}]Cl$, the percentage of chlorophyll recovery from the resin varied from 26.8 % (assay 13) to 80.5 % (assay 14), both regarding the axial points from the variable C_{IL}, which

demonstrates its high influence on the response (Table E.2 in Appendix E). The predicted values were expressed by the model provided by Eq. 2.

$$\text{Chlorophyll recovery } (\%) = 15.13349 + 0.29636(\text{X2}) + 0.28748(\text{X3}) - 0.00042(\text{X3})^2 \quad \textbf{Eq. 2}$$

Using aqueous solutions of $[P_{4,4,4,14}]Cl$, the main effects responsible for the recovery of chlorophylls from the resin are the time of contact and the concentration of $[P_{4,4,4,14}]Cl$ in aqueous solution with no interaction between them, as reported in Table E.3 in Appendix E. The variables were fitted to a first-order model and examined in terms of goodness of fit. The ANOVA was used to evaluate the adequacy of the fitted model considering a 95 % confidence level, with $F_{calculated} > F_{tabulated}$, and $R^2 = 0.72275$. Additionally, the Pareto Chart and the graph of the predicted *vs.* observed values (Figure E.4 and E.5 in Appendix E) show additional information regarding the influence of the independent variables in the predictive model, demonstrating the high influence of the performance of the aqueous solution of $[P_{4,4,4,14}]Cl$ in the elution performance.

The model expressed by Eq. 2 was used to draw the response surfaces shown in Figure 3.4.3. As described in Eq. 2, the SLR has no influence on the response. The time of contact was positively significant in the percentage of the chlorophyll recovered ($p < 0.05$), being 48.5 min chosen as the optimum value. Even if the time of contact is not fully optimized, this variable does not cause a significant environmental and economic impact, since the homogenization method used in this work does not need an intense energy performance such as ultrasound- and microwave-assisted extractions. The concentration of IL was completely optimized, achieving an optimum value around 350–400 mM (Figure 3.4.3). Concentrations above 400 mM impair the performance of chlorophyll recovery, probably due a steric impediment for micelle formation, and consequently, chlorophyll recovery. Thus, the optimum operational conditions were set by using aqueous solutions of $[P_{4,4,4,14}]Cl$ at 370 mM and 48.5 min of contact at room temperature.

A model validation experiment using the optimized operational conditions was carried out in triplicate. The chlorophyll recovery from the resin obtained was around 80 ± 2 %, which corresponds to a relative deviation of 2.7 % (Table E.4 in Appendix E), a very good

result that suggests the high confidence and accuracy of the predictive model designed by the CCRD (2^3).

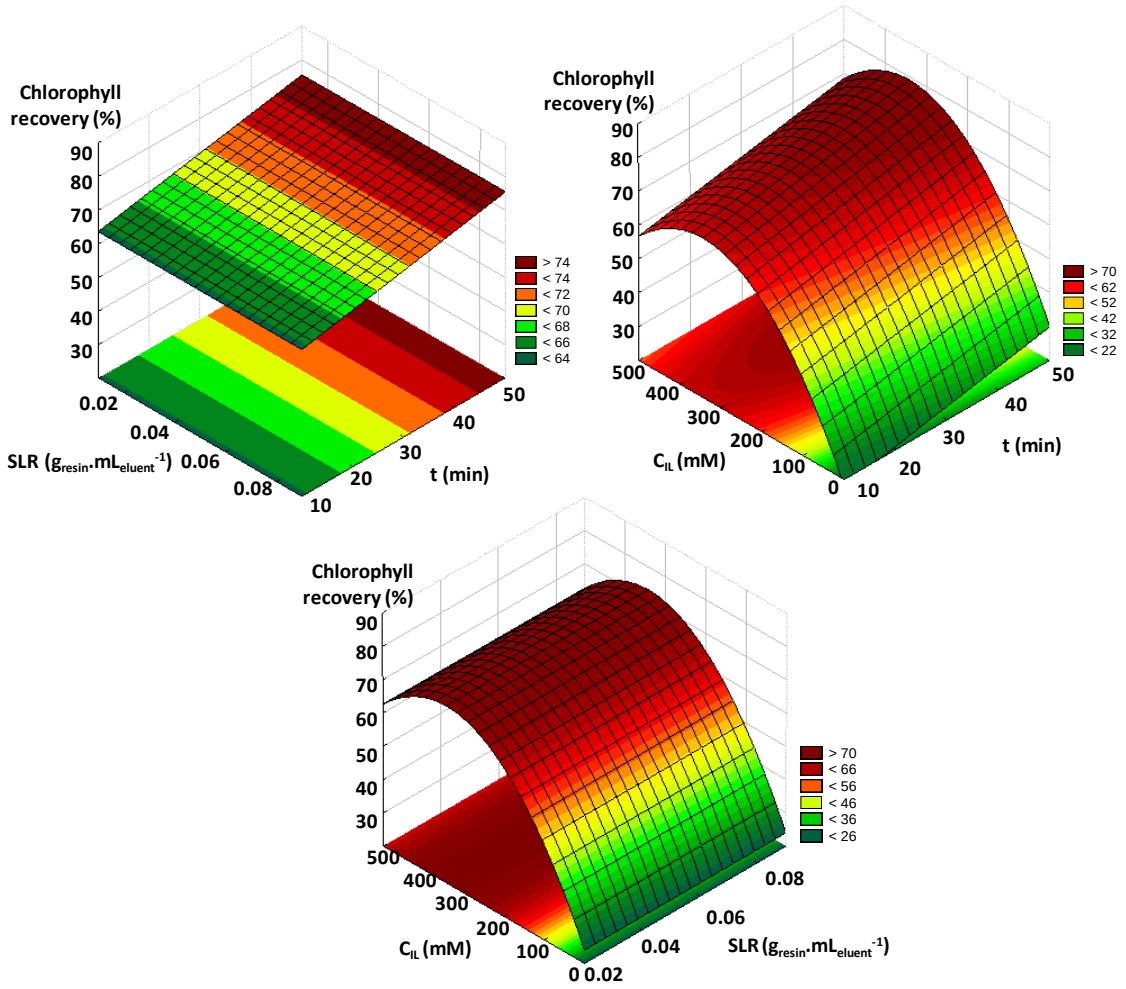

Figure 3.4.3. Response surface plots obtained for the CCRD (2^3) using an aqueous solution of $[P_{4,4,4,14}]Cl$ regarding: time of contact (t in min), IL concentration (C_{IL} in mM), and solid-liquid ratio (SLR in $g_{resin}.mL_{eluent}^{-1}$) in terms of percentage of the chlorophyll recovery from the resin.

Along with the aqueous solutions of $[P_{4,4,4,14}]Cl$, solutions of tensioactive ammonium-based ILs also showed to be very promising to recover chlorophylls from the AmberLite™ HPR900 OH resin (69 % of chlorophyll recovery). Therefore, the process was also optimized using $[N_{1,1,1,12}]Br$ in a similar approach to the previously done for $[P_{4,4,4,14}]Cl$. The maximum chlorophyll recovery was obtained in the run 8 (93.3 %), using a SLR fixed at 0.070 $g_{resin}.mL_{eluent}^{-1}$, and a C_{IL} of 370 mM, during 41 min of contact. The data obtained in the Box-Behnken (Table E.5 in Appendix E) experiment was converted into a second-

order polynomial equation with three independent variables (X1, X2, and X3), as described by Eq. 3.

$$\text{Chlorophyll recovery } (\%) = -15.30 + 322.06(\text{X1}) - 5751.61(\text{X1})^2 + 1.17(\text{X2}) - 0.02(\text{X2})^2 + 0.30(\text{X3}) + 0.62(\text{X1} \times \text{X3}) \quad \textbf{Eq. 3}$$

The Pareto Chart shows that the most significant independent variables influencing the chlorophyll recovery are the C_{IL}, both linear and quadratic, but the other variables also present an important role in the optimization process (Figure E.6 in Appendix E). The predicted and observed values were close to each other (Figure E.7 in Appendix E), making the model adequate. By applying ANOVA, the regression model was considered significant ($p < 0.05$, Table E.5 in Appendix E), and thus useful in predicting the effects of the three different level factors in the recovery of chlorophylls. Interpreting together the response surfaces displayed in Figure 3.4.4, it is possible to highlight an ideal recovery condition as SLR at 0.070 $g_{resin}.mL_{eluent}^{-1}$, C_{IL} of 370 mM, and 48.5 min of time of contact. This condition reflects in the prediction of almost 100 % of recovery from the chlorophylls fixed in the resin (predicted value). Although the observed recovery value under the optimum condition was 97.0 ± 0.9 %, this model is considered valid and accurate, since a small relative deviation was observed (3.0 %, Table E.6 in Appendix E).

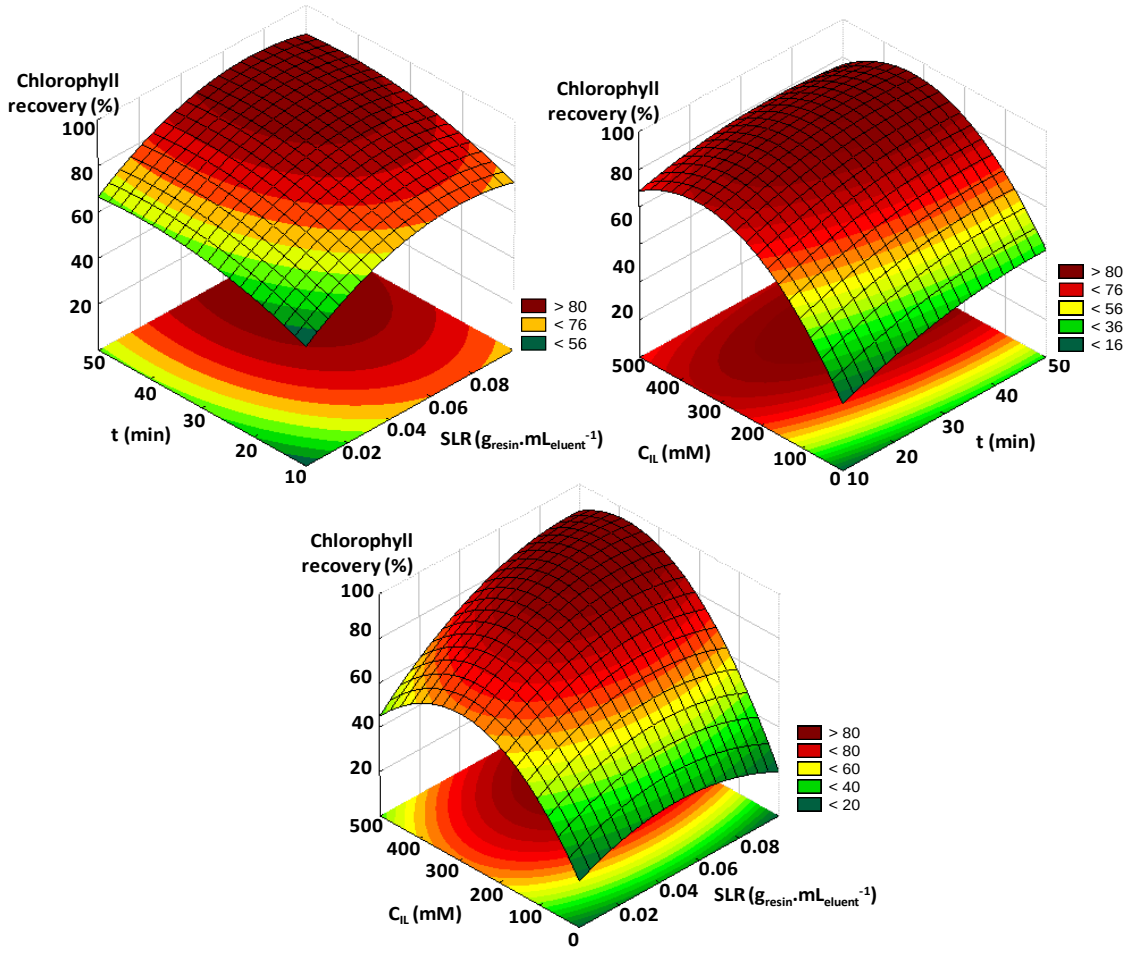

Figure 3.4.4. Response surface plots obtained for the CCRD (2^3) using an aqueous solution of $[N_{1,1,1,12}]Br$ regarding the time of contact (t in min), IL concentration (C_{IL} in mM), and solid-liquid ratio (SLR in $g_{resin}.mL_{eluent}^{-1}$) in terms of chlorophyll recovery (in percentage) from the resin.

As shown in Table 3.4.3, apart from the SLR, which has no significant value in the case of $[P_{4,4,4,14}]Cl$, the best results in terms of chlorophyll recovery were found for the same concentration of IL and time of contact for both approaches. Major differences come in what respects to the outputs. An increase in the chlorophyll recovery from 80 ± 2 to 97.0 ± 0.9 % was seen when an aqueous solution of $[N_{1,1,1,12}]Br$ was used as eluent instead of an aqueous solution of $[P_{4,4,4,14}]Cl$.

Table 3.4.3. Summary of the best conditions and results found in CCRD for both aqueous systems with $[P_{4,4,4,14}]Cl$ and $[N_{1,1,1,12}]Br$ as chlorophyll eluents.

	$[P_{4,4,4,14}]Cl$	**$[N_{1,1,1,12}]Br$**
C_{IL} (mM)	370	370
SLR ($g_{resin}.mL_{eluent}^{-1}$)	0.050	0.070
t (min)	48.5	48.5
Chlorophyll recovery (%)	80 ± 2	97.0 ± 0.9

Although the results seem to suggest $[N_{1,1,1,12}]Br$ to be the best eluent, the aqueous solutions of both eluents were tested again, in the elution of chlorophyll, at their optimum conditions (Table 3.4.3) considering the reuse of the commercial resin up to five times (Figure 3.4.5), including three stages: (i) the loading of a new ethanolic extract, (ii) the elution with aqueous solutions of IL and (iii) the regeneration with NaOH. In each cycle, three fractions were collected: the carotenoid rich extract (in ethanol), the eluted solution (aqueous solution of IL) rich in chlorophylls, and the aqueous solution of NaOH used to regenerate the resin. The xanthophyll content was analysed in terms of fucoxanthin content (Figure 3.4.5 A). The concentration of fucoxanthin was kept constant from cycle 1 to cycle 5, regardless the aqueous solution of IL used in the elution step, showing that the carotenoid fraction is not affected even considering five cycles of reusing the resin. As depicted in Figure 3.4.5 B, the three collected fractions were also analysed in terms of chlorophyll when compared to the initial extract (before the solid-phase extraction).

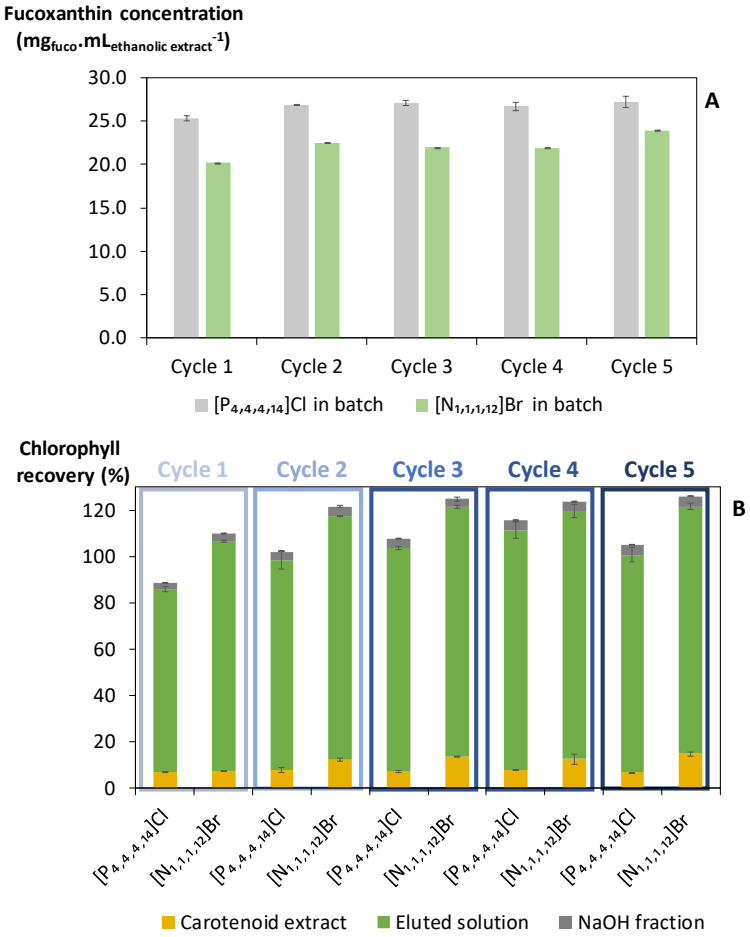

Figure 3.4.5. Comparison of the process using aqueous solution of [$N_{1,1,1,12}$]Br and [$P_{4,4,4,14}$]Cl as eluents at the optimized conditions previously selected along five cycles of reuse of the resin, considering (A) fucoxanthin concentration in the carotenoid ethanolic extract (first fraction collected from the resin); and (B) chlorophyll recovery in the different fractions collected from the resin.

Note: Different initial values in (A) are due to the need of preparing new initial extracts at the beginning of each experiment.

It is important to note that values of chlorophyll recovery greater than 100 % in Figure 3.4.5 B can be a result of the interference of slight amounts of different solvents in the fraction being analysed, changing the behaviour of the calibration curve, but it only means that the recovery is complete. Regarding the carotenoid rich extract, from the second cycle onwards, if the [$N_{1,1,1,12}$]Br aqueous solution is used as eluent, there is a slightly increase in the content of chlorophylls in the extract of carotenoids. Additionally, for each IL individually, the behaviour in each cycle (and each fraction) seems to be similar after the second cycle, leading to the idea that more cycles could be done using

the same resin and procedure. The chlorophyll content in the NaOH fractions has no significant differences regardless the eluent used in the elution step, being very low in all cycles. Moreover, it can be observed that, in all cases, most of the chlorophyll is in the eluted solution, *i.e.* in aqueous solution of IL, for both ILs. Photographs of the resin along with the adsorption and elution of chlorophyll, and regeneration of the resin can be seen in Figure E.2 in Appendix E. In addition to the best elution performance of chlorophyll from the AmberLite™ HPR900 OH, tensioactive ammonium-based ILs are commonly used in the industry due to its lower price,[103] having at the same time lower associated environmental impacts when compared with other families of ILs.[224]

In the end and, after a deep analysis of all parameters optimized and results obtained, it was concluded that the ammonium-based IL has provided the best results in terms of elution performance in comparison with the phosphonium-based IL. After the selection of the best eluent, the composition of the extracts at the different stages of the process was checked by UHLPC-MS. In this sense, the initial extract (obtained after the solid-liquid extraction with ethanol from *Isochrysis galbana*), the carotenoid extract (obtained after passing the initial extract through the resin), and the chlorophyll extract (obtained after the use of $[N_{1,1,1,12}]$Br aqueous solution as eluent and polishing of the IL) were analysed, being the main results obtained depicted in Table 3.4.4 (see also Figure E.3 in Appendix E). From the data collected using UHPLC-MS it was possible to confirm, in the initial extracts, the presence of the chlorophyll derivatives (pheophorbide *a* and pheophytin *a*) and also the presence the xanthophylls (fucoxanthin and diatoxanthin). Moreover, it was confirmed the affinity of the resin to preferably adsorb the chlorophyll derivatives allowing their separation from the carotenoid extract and the efficiency of the ammonium-based aqueous solution to recover afterwards the adsorbed chlorophyll derivatives. Furthermore, the UHPLC-MS analysis confirmed small structural alterations in fucoxanthin (hydrolysis of the ester group and dehydration) affording a fucoxanthin derivative with the protonated molecular ion at *m/z* = 599. Concerning the chlorophyll extract, the results suggest the hydrolysis of the methyl ester in pheophorbide *a* and addition of water affording a pheophorbide a derivative with *m/z* at 597. No structural alterations were detected in diatoxanthin and in pheophytin *a*. Based on this, we believe that the selective removal of chlorophylls during the strong basic resin treatment can be attributed to a compromise between interionic (an anion exchange mechanism) and

intermolecular forces such as hydrogen bonding and dipole–dipole interactions between the polar moieties of the resin and the chlorophyll derivatives. Similar interactions are responsible for the removal of the chlorophyll derivatives from the resin with the cationic IL (see Figure E.3 in Appendix E).

Table 3.4.4. Composition of the initial extract (the one obtained after the solid-liquid extraction with ethanol from *Isochrysis galbana*), the carotenoid extract (obtained after passing the initial extract through the resin), and the chlorophyll extract (obtained after the use of $[N_{1,1,1,12}]Br$ aqueous solution as eluent and polishing of the IL) performed by UHPLC-MS.

	Compound	**Retention time (min)**	**$[M+H]^+$ (m/z)**	**UV-Vis (nm)**	**Compound abundance (%)**
Initial Extract	Fucoxanthin	2.69	659	450	26.7 (450 nm)
	Pheophorbide *a*	4.01	593	450, 470, 655	11.7 (450 nm) 5.5 (470 nm) 45.3 (655 nm)
	Diatoxanthin	5.55	567	450, 470	51.7 (450 nm) 75.7 (470 nm)
	Pheophytin *a*	23.24	871	450, 470, 655	9.0 (450 nm) 18.8 (470 nm) 54.7 (655 nm)
Carotenoid extract	Fucoxanthin derivative	1.58	599	450	26.6 (450 nm)
	Diatoxanthin	5.97	567	450, 470	73.4 (450 nm) > 90.0 (470 nm)
Chlorophyll extract	Pheophorbide *a* derivative	2.49	597	655	43.6 (655 nm)
	Pheophytin *a*	17.27	871	655	56.4 (655 nm)

Continuous process in column

To study the adsorption and elution of chlorophylls, and regeneration of the resin in a continuous mode, a solid-phase extraction cartridge filled with the resin was used. The extract, eluent and regeneration solution used to perform the continuous studies were the same as in batch adsorption, elution, and regeneration process, with the flow kept constant at 45 $\mu L.s^{-1}$. In each cycle, and as previously defined, the three fractions were collected, namely the carotenoid rich extract after passing through the column (in ethanol), the eluted solution (aqueous solution of IL) rich in chlorophylls, and the aqueous solution of NaOH used to regenerate the resin. All fractions were analysed, being the results depicted in Figure 3.4.6.

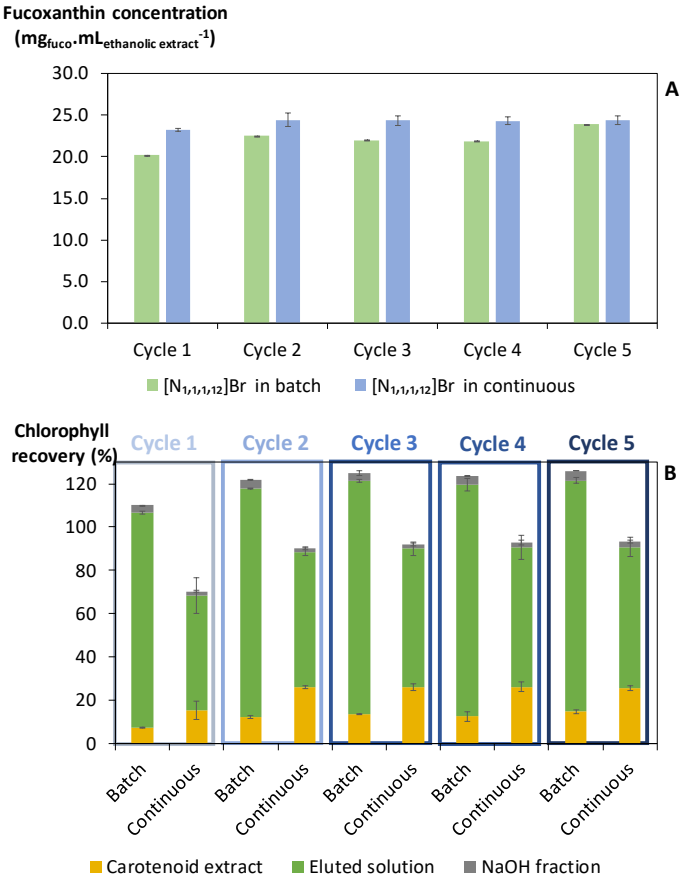

Figure 3.4.6. Comparison of the process using aqueous solution of $[N_{1,1,1,12}]Br$ as eluent both in batch and in continuous regime along five cycles of reuse of the resin, considering (A) fucoxanthin concentration in the carotenoid ethanolic extract (first fraction collected from the resin); and (B) chlorophyll recovery in the different fractions collected from the resin. Results regarding the batch process are once more displayed to facilitate the comparison.

Note that different initial values in (A) are due to the need of preparing new initial extracts in the beginning of each experiment.

As previously observed for the batch process, the concentration of fucoxanthin (Figure 3.4.6 A) was kept constant from cycle 1 to cycle 5, with no loss in efficiency in the collection of fucoxanthin. It can also be observed (Figure 3.4.6 B) that, from the second cycle onwards, the amount of chlorophyll in the different fractions analysed remains constant for the following adsorption, elution and regeneration cycles. Nevertheless, the chlorophyll content on the ethanol extract rich in xanthophylls after contact with the resin is higher than that observed at the batch process (26.0 % and 12.2 %, respectively, in the 2nd cycle). This decrease in the amount of chlorophyll adsorbed by the resin may be due to a low residence time of the adsorbate (chlorophyll) in the adsorbent (resin). Due to experimental constraints the flow rate of 45 µL.s^{-1} was used since this was the minimum that could be achieved in the experimental setup. This flow seems to be somewhat high for this adsorption process, not allowing the time for the complete adsorption of the chlorophyll to the adsorbent. Since the residence time is a relevant parameter in the adsorption of chlorophylls by this resin (as well as in the elution of chlorophyll, as seen in the response surface methodology), the flow rate should be lower to achieve better results.

Chlorophylls polishing and proposal of an integrated process

At the end of the elution, an aqueous solution of [$N_{1,1,1,12}$]Br with chlorophyll was collected. A process to achieve the polishing of chlorophyll was developed in order to have the chlorophyll free of IL to be used in any application and/or to allow the reuse of the IL in new cycles of elution. As explained in the methodology section, the water content was completely removed by freeze-drying. The resulting powder (a mixture of IL and chlorophylls) was dissolved in pure ethanol in the proportion of 10:3 ($V_{ethanol}$:$V_{initial\ aqueous\ solution}$). The liquid solution was stocked at -80 °C during three days. As a result, a pellet at the bottom and a green liquid on the top of the flask were obtained corresponding to the IL and ethanolic fractions rich in chlorophylls, respectively. The ^{1}H NMR spectrometry analysis, performed in pure IL and in the ethanolic fraction rich in chlorophylls using D_2O as solvent, revealed the absence of IL (here seen as

contamination) in the ethanolic fraction (see Figure E.8 in Appendix E), allowing the reuse of IL. An integrated process was thus designed considering not only the methodology developed in this work but also a proposal of operations for the entire process, as sketched in Figure 3.4.7. In short, a solid-liquid extraction of chlorophylls and carotenoids from *Isochrysis galbana* is done using pure ethanol as solvent. The obtained initial extract is passed throught the commercial resin AmberLite^{TM} HPR900 OH in a continuous process allowing a carotenoid rich-extract to be collected by the adsorption of the chlorophylls to the resin. The chlorophylls are then eluted using an aqueous solution of $[N_{1,1,1,12}]Br$ at 370 mM, being that same solution passed throught the column until it reaches saturation. Lastly, the resin is regenerated by the replacement of the OH^- groups using a fresh solution of NaOH [4 % (w:v) in water]. The resin can be reused, with no loss in efficiency, at least up to five cycles (number of completed cycles tested). The chlorophylls in the aqueous solution of IL are recovered using ethanol, using the procedure previously described. The chlorophylls and carotenoids present in ethanolic extracts can then be recovered in dry form, if required by the final application, allowing the reuse of the ethanol, using a vaccum drying tecnique carried at low pressures and temperatures to avoid the degradation of the pigments.

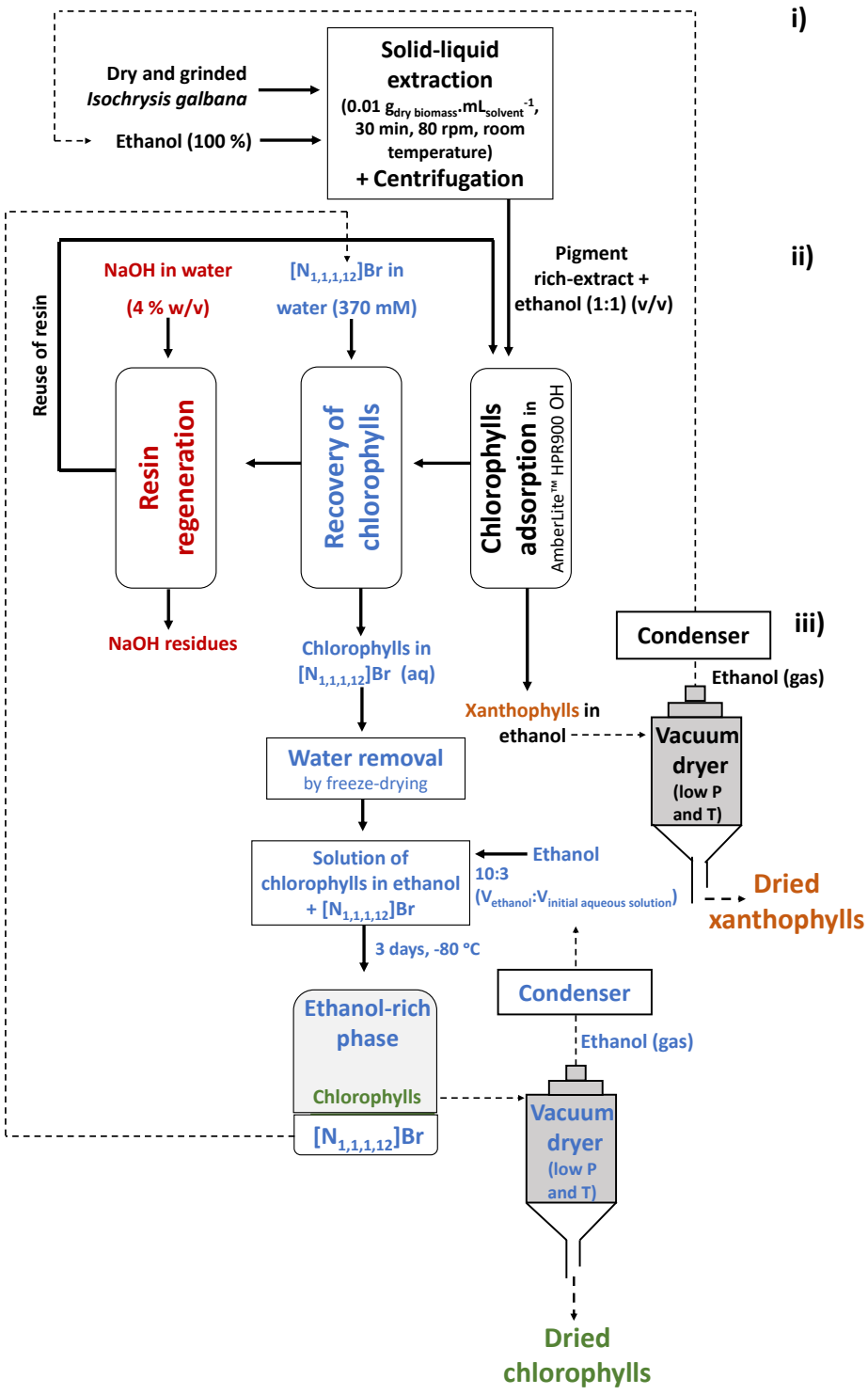

Figure 3.4.7. Schematic representation of the final process proposed in this work, where i) represents the solid-liquid extraction of pigments from the biomass; ii) the recovery of xanthophyll and chlorophyll through continuous process in column; and iii) the polishing of pigments and recovery of the solvents. Dashed lines were not experimentally tested, being just a proposal of what can be done.

Conclusions

The production of multiple products from a single resource is very important to increase the economic viability of biomass processing and, at the same time, to reduce the wastes associated with the process. While the extraction from biomass of a large number of natural bioactive pigments using IL as solvents has been proposed, in this work, aqueous solutions of ILs were instead used to elute adsorbed chlorophylls from a commercial resin. Unlike organic solvents and other solutions, aqueous solutions of tensioactive $[N_{1,1,1,12}]Br$ and $[P_{4,4,4,14}]Cl$ were successively applied as chlorophyll eluents. The elution conditions were optimized for batch processes and the elution of 97.0 ± 0.9 % of the initial chlorophyll loaded in the resin was eluted with aqueous solutions of $[N_{1,1,1,12}]Br$ (370 mM). At these conditions, the resin was reutilized 5 times without compromising the purity of the xanthophyll fraction or the resin's efficiency. A continuous process in a column was also performed and, based on the results reported here, an integrated process can be envisioned to obtain purified fractions of different pigments by solid-phase extraction with the reuse of the resin, the polishing of pigments and the recycling of the solvents.

CHAPTER 4. Alternative approach for purification of phycobiliproteins

4.1 Sustainable strategy based on induced precipitation for the purification of phycobiliproteins

This chapter is based on the manuscript

Margarida Martins, Bruna P. Soares, João H.P.M. Santos, Pankaj Bharmoria, Mario A. Torres-Acosta, Ana C.R.V. Dias, João A.P. Coutinho, Sónia P.M. Ventura,* "Sustainable strategy based on induced precipitation for the purification of phycobiliproteins", ACS Sustainable Chemistry & Engineering, 2021, DOI: 10.1021/acssuschemeng.0c09218.

*Contributions: M.M. and B.P.S., acquired the experimental data. M.M. performed the data analysis. P.B., M.A.T.-A., A.C.R.V.D. assessed the circular dichroism assays, economic impact, and environmental impact, respectively. M.M. wrote the manuscript with substantial contributions from the remaining authors.

Abstract

Phycobiliproteins are fluorescent proteins mainly produced by red macroalgae and cyanobacteria. These proteins, essential to the survival of these organisms, find application in many fields of interest, from medical, pharmaceutical, and cosmetic to food and textile industries. The biggest obstacle to its use is the lack of simple environmental and economical sustainable methodologies to obtain these proteins with a high purity.

In this work, a new purification process is proposed based on the induced precipitation of the target proteins followed by an ultrafiltration. Purities of 89.5 % of both phycobiliproteins and 87.3 % of R-phycoerythrin were achieved using ammonium sulfate and poly(acrylic acid) sodium salts as precipitation agents (followed by an ultrafiltration step), while maintaining high recovery yields and the protein structure stability. Environmental analysis performed to evaluate the proposed process show that the carbon footprint for the proposed process is much lower than those reported for alternative methodology, and the economic analysis reveals the cost-effective character associated to its high performance. This work is a step towards more sustainable and effective methodologies/processes with high industrial potential.

Keywords: *Gracilaria gracilis*, induced precipitation, purification, phycobiliproteins, R-phycoerythrin.

Introduction

The production of chemicals, materials and fuels from biomass is a growing trend in which academia and industry have invested significant efforts during the last decade.[8] The goal is to reduce the world dependence on a petroleum-based economy, gradually replacing it by a bioeconomy where the so-called biorefinery plays a major role.[2] The development of biorefinery processes is still much focused on the biofuels, power and heat production.[7] However, to achieve a full exploitation of the biomass, a complete cascade of different products should be obtained,[1] following an order that should be dependent on the market value of what is obtained and the sensitivity of the compounds to the conditions of extraction. By guaranteeing the stability of the bioactive compounds, the process value-chain should start by the recovery of low-volume high-value products.[8,64]

Macroalgae are an example of a biomass that could allow the development of a biorefinery focusing on a blue economy. Many high-value products, such as pigments,[156] phenols,[225] lipids,[226] and proteins,[227] are already being explored in what should be the beginning of the biorefinery cascade.[19]

Phycobiliproteins are a family of fluorescent and hydrophilic proteins involved in the light-harvesting processes in red macroalgae. This family of proteins, in red macroalgae, is mainly composed of R-phycoerythrin (R-PE) and R-phycocyanin (R-PC).[40] R-PE has a soft pink colour and orange fluorescence, composed of $(\alpha\beta)_6\gamma$ complexes and with 240 kDa, while R-PC has a blue colour and red fluorescence, composed of $(\alpha\beta)_3$ complexes.[40,228] Due to their spectroscopic and fluorescent properties, those proteins can be applied in different fields from biotechnology, biomedicine, pharmaceuticals, cosmetics, and food products.[229] More recently, extracts rich in phycobiliproteins were also studied as natural dyes to use as optical active centers for sustainable luminescent solar concentrators and proving their potential towards cheap and sustainable photovoltaic energy conversion.[51]

Despite the efforts from several researchers on the development of new processes to obtain pure phycobiliproteins, these are still far from industrialization. The purity level required is defined by the application/product demands, and the process to be implemented should take these requirements into account. Conventionally, the purification of phycobiliproteins can be achieved by a set of unit operations that may

include (i) a pre-purification step commonly applying ammonium sulfate precipitation, (ii) one or more purification steps applying membrane separation processes (*i.e.* ultrafiltration (UF) and cross-flow ultrafiltration) and/or chromatographic processes which are usually column chromatography (*i.e.* size exclusion-, ion exchange-, hydrophobic interaction-, and affinity-chromatography), and iii) a last step of dialysis to completely remove, replace, or decrease the concentration of salts or solvents from the purified extracts.[43,70,74–77,230] Recently, alternative methodologies of protein purification have been proposed, such as membrane chromatography,[231] centrifugal precipitation chromatography,[232] electrophoretic elution,[233] vortex flow reactor in an adsorption experiment,[234] and aqueous micellar two-phase systems.[235] However, most of them have disadvantages related to complexity, difficulty to scale-up and high associated costs, limiting the applicability of these processes at an industrial scale. This is also true for the process we have previously proposed based on the use of aqueous micellar two-phase systems.[235] Despite the good results achieved for the purification of phycobiliproteins, and R-PE in particular, the process included five main steps, comprising a first solid-liquid extraction, two units of purification applying aqueous micellar two-phase systems followed by two units of operation to separate the target proteins from the main solvents used. In this context, the present work will attempt the development of a simpler process to purify phycobiliproteins, and also R-PE. The first approach to be used was the elimination of the fourth and fifth steps of our previous process involving the separation of the target proteins after purification from the extraction solvents. For that, the use of induced precipitation seems to be a good strategy. The recovery and purification of proteins by precipitation is one of the most important operations in protein purification, recurrently used in laboratories and also industry.[236] This is achieved by the destabilization of a protein solution that is then separated from the liquid/supernatant by gravity settling, centrifugation, or filtration. The precipitation can be driven by the ionic strength of the medium, but also by size exclusion, pH and temperature variations.[237,238] Much work has been done regarding the use of ammonium sulfate, which is a classic salting-out agent and usually the first choice in protein precipitation.[238] However, and despite its high efficiency promoting precipitation, it is not selective, which means that it will precipitate most of the proteins in the solution. It is also known that many other compounds can act as precipitation

agents, such as polymers, copolymers, and polyelectrolytes by different phenomena such as crowding or by direct interaction between the protein and the precipitation agent that can tune the solubility decrease of the target protein from a crude extract, thus leading to a selective precipitation.[237–241]

Precipitation is normally used as a pre-treatment,[72,76] meaning that it is complemented by a set of other purification steps, including chromatographic[43] or non-chromatography[242] downstream processing steps. However, in this work, the main objective was to decrease the number of steps required to obtain pure phycobiliproteins from *Gracilaria gracilis*, in particular, R-PE, thus avoiding the application of other purification steps. The screening of various potential precipitating agents was studied from a large set of polymers, copolymers, and polyelectrolytes. After the design of a simple and efficient process to obtain the phycobiliproteins (and particularly, R-PE), a life cycle analysis was done to compare this process with the one previously proposed by us using aqueous micellar two-phase systems,[235] followed by an economic analysis, based on which the viability and sustainability of this process is discussed.

Experimental

Biomass

The biomass used in this work, fresh *Gracilaria gracilis*, was kindly provided by ALGAplus (Ílhavo, Portugal). ALGAplus farms the macroalgae at Ria de Aveiro lagoon (40°36'44.7" N, 8°40'27.0" W) in coastal Portugal under the European union organic aquaculture standards (EC710/2009). This aquaculture is performed in a land-based integrated multi-trophic aquaculture system (meaning that the nitrogen input is higher than in the outside natural lagoon due to the use of effluent water from fish production). Macroalgae samples were collected between April and December of 2019, washed with tap and distilled water, being frozen until needed, but never for longer than one month.

Chemicals

Ammonium sulfate ($(NH_4)_2SO_4$, 99.5 %, CAS 7783-20-2) was acquired from Merck. Poly(acrylic acid) sodium salts (CAS 9003-04-7) with average molecular weight of 1200 $g.mol^{-1}$ (NaPA 1200, 45 wt % in water solution) and 8000 $g.mol^{-1}$ (NaPA 8000, 45 wt% in water solution), polyethylene glycol (PEG) with average molecular weight of

8000 g.mol^{-1} (PEG 8000, pure, CAS 25322-68-3) and polypropylene glycol (PPG) polymer with average molecular weight of 400 g.mol^{-1} (PPG 400, pure, CAS 25322-68-3) were purchased from Sigma-Aldrich. PEG with average molecular weight of 10000 g.mol^{-1} (PEG 10000, pure, CAS 25322-68-3) was supplied from Fluka.

Nonionic copolymers composed of PEG and PPG blocks were also used (Figure 4.1.1, CAS 9003-11-6). Their commercial names were adopted throughout this work. Pluronic PE 6800 (PPG-PEG-*blocks* with approx. 8000 g.mol^{-1}, composed of 80 wt % PEG), Pluronic PE 6400 (PPG-PEG-*blocks* with approx. 2900 g.mol^{-1}, composed of 40 wt % PEG) and Pluronic PE 6200 (PPG-PEG-*blocks* with approx. 2450 g.mol^{-1}, composed of 20 wt % PEG) were purchased from BASF. Pluronic P 17R4 (PPG-PEG-PPG-*blocks* with approx. 2700 g.mol^{-1}, composed of 40 wt % PEG), Pluronic L81 (PEG-PPG-PEG-*blocks* with approx. 2800 g.mol^{-1}, composed of 10 wt % PEG), and Pluronic P123 (PEG-PPG-PEG-*blocks* with approx. 5800 g.mol^{-1}, composed of 30 wt % PEG) were acquired from Sigma-Aldrich.

As standard, commercial R-PE (CAS 11016-17-4) supplied by Sigma-Aldrich was used.

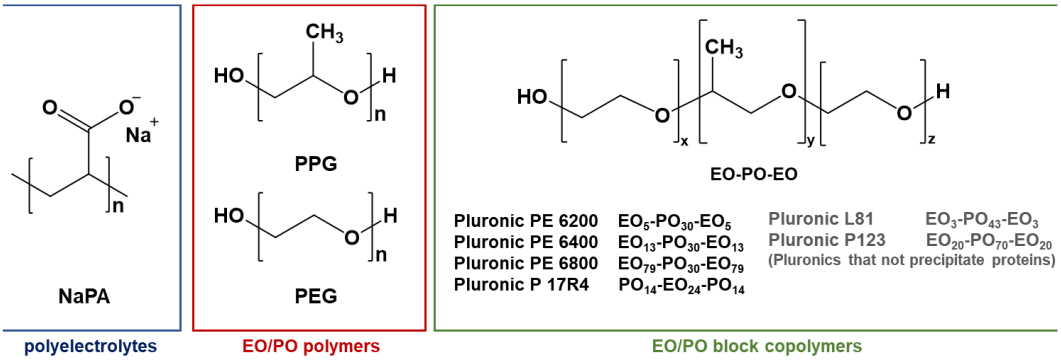

Figure 4.1.1. Structures of the precipitation agents studied in this work.

Solid-liquid extraction

The solid-liquid extraction procedure was adopted from Martins et al.[100] but with some modifications. Briefly, fresh *Gracilaria gracilis* was ground in a coffee mill after being frozen with liquid nitrogen for a more efficient extraction. The extraction was performed using distilled water as solvent in a solid-liquid ratio of 0.5 $g_{fresh\ biomass}.mL_{solvent}^{-1}$ during 20 min at room temperature (20–25 °C) in an orbital shaker (IKA KS 4000 ic control) at 250 rpm and protected from light. The crude extract was obtained after centrifugation at 14000 *g*, 20 min, at room temperature (20–25 °C) in a VWR microstar 17 centrifuge.

Induced precipitation

Several precipitation agents were tested at three different concentrations (100, 200 and 300 $g.L^{-1}$). Each precipitation agent was dissolved in the crude extract and left overnight at 4 °C. Pellet and supernatant phases were induced by centrifugation at 900 *g*, for 15 min at room temperature (20–25 °C) in the VWR microstar 17 centrifuge using the same conditions described in the section *Solid-liquid extraction*. After centrifugation, the pellet was resuspended in the same initial volume using distilled water. When particles not soluble in water are observed in the resuspended pellets (that happened in PEG 10000 and Pluronic PE 6200), a vigorous centrifugation at 9600 *g* for 5 min was applied to remove these solids before further analysis.

Ultrafiltration (UF)

500 μL of sample was added in each Amicon Ultra-0.5 mL Centrifugal Filter Unit 100 K. The sample was centrifuged at 14000 *g* during 15 min. The permeate was discarded and 400 μL of ultrapure water was added to the concentrate and centrifuged in the same conditions, being this last step repeated twice. Lastly, 500 μL of ultrapure water was added to recover the concentrated sample after a centrifugation of 2 min, at 1000 *g*.

Spectroscopic methods

The absorption spectra of different fractions were measured between 200 and 700 nm using a UV-Vis microplate reader (Synergy HT microplate reader – BioTek). This technique was used in the initial screening of precipitation agents, in which the phycobiliproteins were quantified directly at 565 nm, and the total amount of proteins was quantified by the Bicinchoninic Acid (BCA) method at 562 nm, considering two calibration curves previously prepared (R^2 = 0.999 and R^2 = 0.998, for phycobiliproteins and total proteins, respectively). The total protein concentration was determined with the Pierce™ BCA Protein Assay and Micro BCA Protein Assay (Thermo Scientific, Schwerte, Germany) according to the supplier recommendations. Bovine serum albumin (from Fisher Scientific) was used as the standard protein. The purity parameter was obtained as the ratio between the phycobiliproteins concentration and the total proteins concentration in the resuspended pellet, these values being presented as a percentage. The yield was calculated as the ratio between the phycobiliproteins

concentration in the resuspended pellet and the phycobiliproteins concentration in the initial extract.

Parameters as selectivity and *R-PC index* were calculated according to Vicente et al.[235] In order to determine the selectivity, the partition coefficient of R-PE ($K_{R\text{-}PE}$) and total proteins ($K_{Total\ proteins}$) were firstly calculated (Eqs. 1 and 2, respectively). This parameter is the ratio between the concentration of R-PE (or total proteins) in the purified fraction and the discarded phases along the purification steps. Knowing the partition coefficient of both R-PE and total proteins, the selectivity of the proposed method was determined according to Eq. 3.

$$K_{R-PE} = \frac{[\text{R–PE}]_{\text{purified fraction}}}{[\text{R–PE}]_{\text{discarded fraction}}} \quad \textbf{Eq. 1}$$

$$K_{Total\ proteins} = \frac{[\text{Total proteins}]_{\text{purified fraction}}}{[\text{Total proteins}]_{\text{discarded fraction}}} \quad \textbf{Eq. 2}$$

$$Selectivity = \frac{K_{\text{R–PE}}}{K_{\text{Total proteins}}} \quad \textbf{Eq. 3}$$

The *R-PC index* relates the amount of R-PC and R-PE in a sample and it was calculated by the ratio between the maximum absorbance of R-PC and R-PE, *i.e.* the absorbance at 617 nm and 565 nm, respectively (Eq. 4).

$$R\text{–}PC\ index = \frac{\text{Abs}_{617\ \text{nm}}}{\text{Abs}_{565\ \text{nm}}} \quad \textbf{Eq. 4}$$

High-performance liquid chromatography (HPLC) using the equipment Chromaster HPLC system (VWR Hitachi) equipped with a binary pump, column oven, temperature-controlled auto-sampler, DAD detector (HPLC-DAD) and an analytical column Shodex Protein KW-802.5 (8 mm×300 mm) was applied. A 100 mM phosphate buffer pH 7.0 was run isocratically with a flow rate of 0.5 mL.min^{-1} and the injection volume was 10 µL. All samples were previously filtered with the 25 mm GHP Acrodisc syringe filters with a pore size of 0.45 µm. The wavelength was set at 280, 565 and 617 nm. All spectra were treated using OriginPro 2018 program. The peaks were deconvoluted and the obtained areas were used, namely the total area and the area of the R-PE and R-PC specific peaks. The purity was obtained by the ratio of the areas of R-PE or R-PC specific peaks and the total area of the spectrum, in percentage. The yield was calculated by the ratio of the

areas of R-PE or R-PC specific peaks in the purified extract and the areas of R-PE or R-PC specific peaks in the initial extract, in percentage.

Circular dichroism spectra were recorded using a Jasco J-815 circular dichroism spectrometer at 25 °C in the far UV region (λ = 180−260 nm). Spectra were collected in a 0.1 cm path length quartz cuvette at a scan rate of 100 $nm.min^{-1}$ and sensitivity of 100 mdeg. The response time and the bandwidth were 2 s and 0.5 nm, respectively. The samples were solubilized in distilled water up to a dilution where the influence of the sample interferences was negligible, being in those conditions the circular dichroism spectra obtained with high tension voltage below 600 (Figure F.1 in Appendix F).

SDS-PAGE

The phycobiliprotein crude extract was analysed through electrophoresis that was prepared on polyacrylamide gel (stacking: 4 % and resolving: 20 %) with a running buffer consisting of 250 $mmol.L^{-1}$ of Tris-HCl, 1.92 $mol.L^{-1}$ of glycine, and 1 % of SDS. The proteins were stained with the usual staining procedure [Coomassie Brilliant Blue G-250 0.1 % (w:v), methanol 50 % (v:v), acetic 7 % (v:v), and water 42.9 % (v:v)] in an orbital shaker, at moderate speed, for 2–3 hours at room temperature (20–25 °C). The gels were detained in a solution containing acetic acid 7 % (v:v), methanol 20 % (v:v), and water 73 % (v:v) in an orbital shaker at ± 60 rpm for 3–4 hours at room temperature (20–25 °C). The molecular weight marker used was the NZYColour Protein Marker II from NZYTech.

Environmental analysis by life cycle assessment

The environmental profile of two scenarios to purify phycobiliproteins was evaluated by life cycle assessment, according to ISO 14040 standard,[192] and covering the impacts from the production of the chemicals used in the processes, water and also the electricity consumption. Table F.1 of Appendix F shows the amounts of chemicals and water consumed during the experimental procedure, as well as the amount of electricity spent. The latter parameter was calculated for each equipment based on the time of operation, nominal power and fraction of occupancy over total capacity. These amounts are expressed *per* mg of R-PE obtained to allow comparison between the two scenarios proposed in this work. The impact factors associated with the production of chemicals

and electricity (Portuguese mix) were taken from the Ecoinvent 3.6 database.[194] The impact factors for distilled and ultrapure water result from tap water production[243] and electricity consumed during the distillation and ultrafiltration.[194] The impact assessment method was the ReCiPe 2008 Midpoint at the Hierarchist perspective,[205] considering the following impact categories: climate change (equivalent to the carbon footprint), photochemical oxidant formation, terrestrial acidification and fossil depletion. The results were compared with the ones obtained by Vicente and collaborators when applying aqueous micellar two-phase systems to purify phycobiliproteins.[235]

Economic analysis

To further expand this study and understand some of the potential economic constrains of implementing the process optimized in this work into an industrial scenario, an economic analysis was performed considering the traditional approach [using $(NH_4)_2SO_4$] and the alternative precipitation method proposed in this work. In this analysis, the production cost was calculated *per* mg of R-PE (CoG.mg^{-1}).[156,244] Briefly, three areas need to be fulfilled to have a complete process: to set up a target output or production scenarios, then to determine the sequence of unit operations and their process parameters, and finally to collect the economic datasets to populate the model. For the process developed in this work, the production scale to be used at the industrial stage has not been decided and for this reason, five different scales were analysed, namely 0.01 kg, 0.1 kg, 1 kg, 10 kg, and 100 kg. This will give a wide range of operations from the laboratory, to pilot and, finally, industrial scales. The sequence of unit operations is something that will be discussed in later sections as a result of all the analyses performed in this work, but briefly, it consists of a water extraction of R-PE from the biomass, then a centrifugation to remove the spent biomass. For the precipitation stage, it starts with the mixing of the extract with the precipitant in a tank, followed by the induced precipitation using a centrifugal step, and a re-suspension of the pellet. The process ended with an ultrafiltration/diafiltration step to remove the non-suspended proteins, allowing also the final polishing.

The economic datasets are composed of different areas. For the capital investment (mainly equipment acquisition costs), cost of equipment was obtained from the database on the software Biosolve Process (Biopharm Services Ltd., Buckinghamshire,

UK), then different regressions were determined to interpolate the results considering the different scales needed. The same strategy was employed for consumables (vessel filters and ultrafiltration/diafiltration membranes). For materials costs (chemicals), as this analysis comprised small and large scales, their costs were obtained from Sigma-Aldrich and Alibaba, respectively. Labor has been reported to be approximately 15 % of the total production costs,[245] so this approach was taken here. Lastly, an additional economic aspect was denoted as "others", in which utilities and maintenance costs were included. This was calculated following Biosolve Process approach, which estimates these costs as 4 % of the capital investment. Full data for process and economic parameters employed here are included in Table F.2 in Appendix F.

After the completion of the model construction, different analyses were performed to understand how the CoG.mg^{-1} of the R-PE behaves. First, different production scales were evaluated, for the whole range mentioned before (0.01 kg to 100 kg), following incremental steps of 0.1 kg. Then, using only the discrete range of production scales (0.01 kg, 0.1 kg, 1 kg, 10 kg, and 100 kg), a sensitivity analysis was performed by systematically varying the values of the amount of R-PE content in the biomass (mg of R-PE *per* kg of fresh biomass), the materials cost variation and the duration of the process, all of them in a range from 10-fold above and below (± 10X). Additionally, the impact of the overall recovery yield was included, but due to the results obtained, the range was constrained, the worst-case scenario was 30 % less of what is reported in the following sections and the best scenario can only increase up to 100 %. This analysis can provide an insight on how each individual parameter affects the production costs and help potentially to devise strategies to control their variations. As a complement to the sensitivity analysis, a series of Monte Carlo simulations was performed varying the same parameters, with the same ranges, but under a triangular distribution and calculating their respective production costs (CoG.mg^{-1}) for each scenario. Afterwards, a multiple linear regression was calculated to obtain the coefficients and *p*-value for each parameter.

An additional approach was determined in this work, which results in the calculation of the potential income, or *Return*, that the product could provide and to understand how the different process parameters could affect it. Based on other reports,[103] Eq. 5 was defined to calculate the *Return* based on the results obtained from this work:

$$Return = \left[C_{prod} \times \$_{prod}\right] - \$_{biom} - \left[(\alpha) \times (\text{Production cost per kg of biomass})\right] \quad \textbf{Eq. 5}$$

In Eq. 5, *Return* stands for the *Return per* kg of processed fresh biomass, C_{prod} is the amount of product *per* kg of biomass, $\$_{prod}$ is the commercial price of R-PE on the market and $\$_{biom}$ is the cost associated with the acquisition of the biomass. While, in the second term, the production cost *per* kg of biomass is a conversion of the CoG.mg^{-1} of R-PE into a CoG.kg^{-1} of processed biomass. To obtain this, it is needed to obtain the production cost *per* batch (CoG/batch) and to divide it by the amount of biomass processed in that particular batch. The α is an additional term employed as a multiplier of the CoG.kg^{-1} in order to increase or decrease its impact consequently allowing us to analyse their effect in case the real production costs are higher or lower. As part of the *Return* analysis, a sensitivity analysis was performed by varying the C_{prod} by 0.5X, 1X, or 2X (half or double of the base concentration) and the α term was varied between 1X, 2X, or 5X. Additionally, R-PE has a wide range of prices depending on the application, purity and amount being acquired, and for this reason, the range of EU 5 to EU 5,000 *per* kg was analysed.

Results

Induced precipitation of proteins

Various phenomena can promote protein precipitation however, substances (generally in high concentration) changing the environment of the protein (e.g. some organic solvents, salts, and neutral polymers); or substances (generally at low concentration) interacting directly with the protein (e.g. acids, bases, polyelectrolytes and some metal ions), have been reported as the most relevant.[236] In this work, a screening of polymers, copolymers, and polyelectrolytes at different concentrations was performed, being their ability to induce protein precipitation reported in Table 4.1.1 and their performance compared with the results obtained for $(NH_4)_2SO_4$ (the conventional precipitation agent here used as control).

Table 4.1.1. List of precipitation agents screened according to their ability to precipitate phycobiliproteins from the raw extract at different concentrations. The symbols ✓ and X represent, respectively, the systems with and without protein precipitation occurring.

Precipitation agent	Concentration ($g.L^{-1}$)		
	100	200	300
NaPA 1200	✓	✓	✓
NaPA 8000	✓	✓	✓
PEG 8000	X	X	X
PEG 10000	✓	✓	✓
PPG 400	✓	✓	✓
Pluronic PE 6800	✓	✓	✓
Pluronic PE 6400	✓	✓	✓
Pluronic PE 6200	✓	✓	✓
Pluronic P 17R4	✓	✓	✓
Pluronic L81	X	X	X
Pluronic P123	X	X	X
$(NH_4)_2SO_4$	✓	✓	✓

NaPA 1200 and NaPA 8000 are included in the group of precipitation agents interacting directly with the proteins, while the rest of the substances screened, *i.e.* polymers and copolymers, act by promoting changes in the environment of the initial solvent. Although according to literature,[236] low concentrations of precipitation agents are required when their mechanism of action involves the direct interaction with proteins, both NaPA 1200 and NaPA 8000 were found to be able to induce the precipitation of phycobiliproteins at all concentrations tested. The worst results, without any precipitation of phycobiliproteins, were obtained for PEG 8000, Pluronic L81 and Pluronic P123, independently of the concentration applied.

As previously discussed in the literature,[246] the main phenomena behind the protein precipitation with polymers and copolymers is, in general, a result of the crowding effect, which happens when high concentrations of these molecules are introduced in the system, drastically reducing the volume of water molecules available for protein solvation. In this context, it is well established that PEGs with high molecular weights

more easily precipitate proteins, which can explain the difference in the behaviours of PEG 8000 and PEG 10000.

As the polymers, the copolymers can also decrease the solubility of proteins in solution due to their interaction with the water molecules and the volume they occupy in solution. According to the hydrophilic-lipophilic balance which is a parameter that helps to describe the higher or lower capacity of substances to interact with water molecules (data provided by their suppliers and displayed in Table F.3 of Appendix F), the screened Pluronic substances can be ordered as follows: PE 6800 > PE 6400 > P 17R4 ~ PE 6200 ~ P123 > L81. Considering the results of the hydrophilic-lipophilic balance, it is clear that the decrease in the hydrophilicity of the Pluronics screened makes them unable to precipitate the phycobiliproteins, as a result of their reduced capacity to interact with the water molecules present in the crude extract.

After selecting from Table 4.1.1 all the compounds able to precipitate the phycobiliproteins, and considering the viscosity of the solutions, and the colour intensity in the supernatants (which is a proxy for the residual amounts of phycobiliproteins in solution) only Pluronics, PPG 400 and NaPA 1200 and NaPA 8000 were retained to further evaluate the purity and yield parameters (Figure 4.1.2).

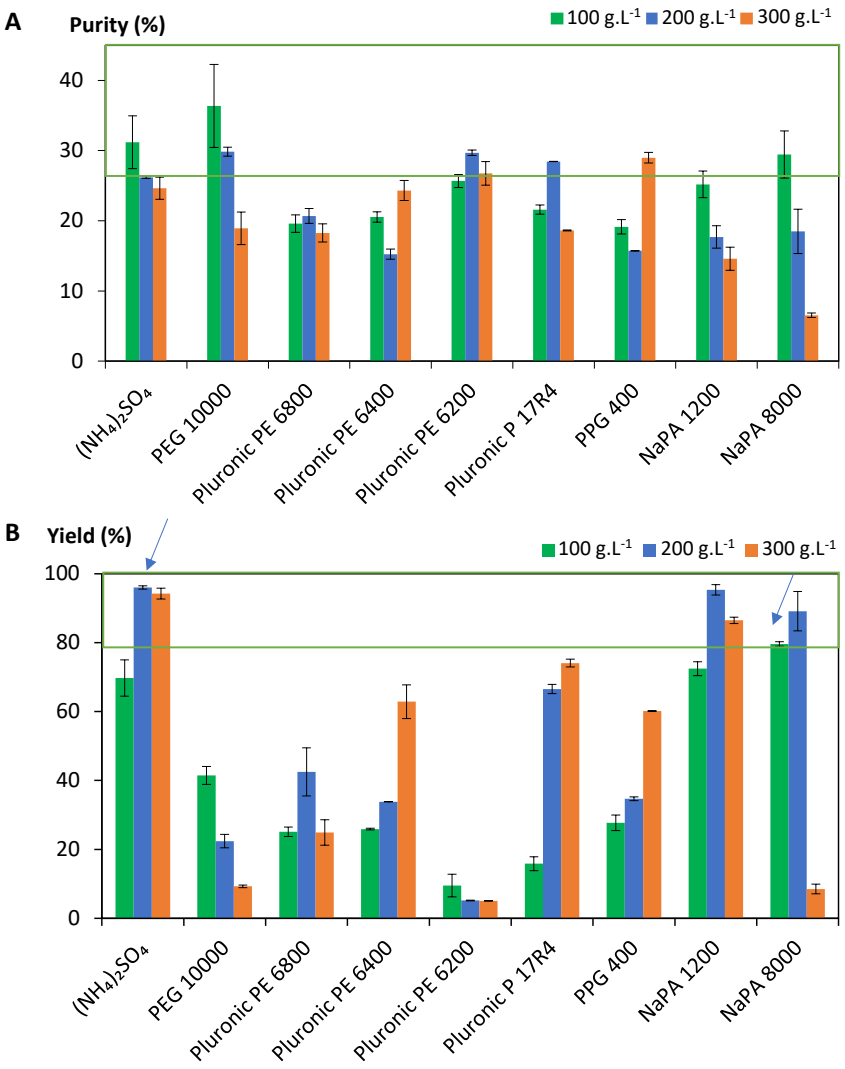

Figure 4.1.2. Results obtained for the (A) purity and (B) yield (%) obtained in the resuspended pellets after the precipitation step using different precipitation agents at three distinct concentrations (100, 200 and 300 $g.L^{-1}$). These analyses were assessed by UV-Vis absorption spectroscopy.

In the view to find the best precipitation agent, a compromise between purity and yield of precipitation was required. The objective was to select the system providing the highest purity levels of phycobiliproteins without reduce the yields of precipitation. After the interpretation of the data presented in Figure 4.1.2 and aiming to proceed with the analysis, the criteria selected was the following: to identify the precipitation agents able to simultaneously provide purities and yields higher than 25 % and 80 %, respectively. The systems fulfilling this criteria were the traditional $(NH_4)_2SO_4$ at 200 $g.L^{-1}$ (purity = 26.2 ± 0.1 % and yield = 96.0 ± 0.5 %) and the polyelectrolyte NaPA 8000

(purity = 29 ± 3 % and yield = 79.6 ± 0.7 %). After choosing the best systems and respective concentrations to induce the precipitation of phycobiliproteins, the extracts obtained were further analysed by HPLC-DAD (Table 4.1.2). This analysis identifies which phycobiliprotein (R-PE or R-PC, the two most relevant phycobiliproteins present in the initial extract)[100,235] and in what extent, was precipitated. Moreover, it also enabled us to infer on the selectivity (capacity to separate R-PE from R-PC) of each system (*i.e.* precipitation agent and its concentration).

Table 4.1.2. Purity and yield (%) obtained in different fractions separately for R-PE and R-PC based on HPLC-DAD analysis.

	Purity (%)			**Yield (%)**	
	R-PE	**R-PC**	**Phycobiliproteins (R-PE + R-PC)**	**R-PE**	**R-PC**
Initial extract	4.4 ± 1.0	3.2 ± 1.5	7.4	-	-
$(NH_4)_2SO_4$ at 200 $g.L^{-1}$	35.0 ± 2.4	18.5 ± 1.2	53.4	100.0 ± 2.6	81.1 ± 1.3
NaPA 8000 at 100 $g.L^{-1}$	50.5 ± 7.4	-	50.5	79.5 ± 3.6	-

The results reported in Table 4.1.2 show that the $(NH_4)_2SO_4$ at 200 $g.L^{-1}$ can precipitate both R-PE and R-PC, while NaPA 8000 at 100 $g.L^{-1}$ is selective for R-PE, *i.e.* it only causes the precipitation of R-PE, while the other phycobiliproteins remain solubilized in the crude extract. The results of $(NH_4)_2SO_4$ are not surprising, since it is well known that, despite its high capacity to induce the precipitation of proteins, it is not selective. It is efficient in precipitating the R-PE because of its very high molecular weight (240 kDa)[76]. Although R-PC (~ 112 kDa)[247] has a lower molecular weight than R-PE, due to the difference between their complexes ($(\alpha\beta)_3$ for R-PC and $(\alpha\beta)_6\gamma$ for R-PE), the R-PC precipitation might be induced due to the proximity between the pH of the aqueous solution of $(NH_4)_2SO_4$ (5.5) and the R-PC isoelectric point, 5.7.[247] In the other hand, NaPA 8000 at 100 $g.L^{-1}$ interacts directly with R-PE, establishing soluble complexes, but not with R-PC, promoting a selective precipitation. Since NaPA 8000 is a polyanion, and at the conditions of the solution, R-PE is negatively charged [pH (8.1) > R-PE isoelectric

point (4.2)[248]], site-specific local interactions might be happening, thus justifying the establishment of soluble complexes.[239]

Although the purity has increased after the precipitation step, the extracts are still not very pure (maximum purity up to this point around 50 %). For that reason, the resuspended pellets obtained after the precipitation with NaPA 8000 and $(NH_4)_2SO_4$ were subjected to an additional step of purification using ultrafiltration. As previously detailed in the Experimental section, filters with a cutoff of 100 kDa were applied to remove the small and medium-size contaminant proteins present in the macroalgae.[235] Yields and purity obtained before and after ultrafiltration are plotted in Figure 4.1.3 (with more details in Table F.4 of Appendix F).

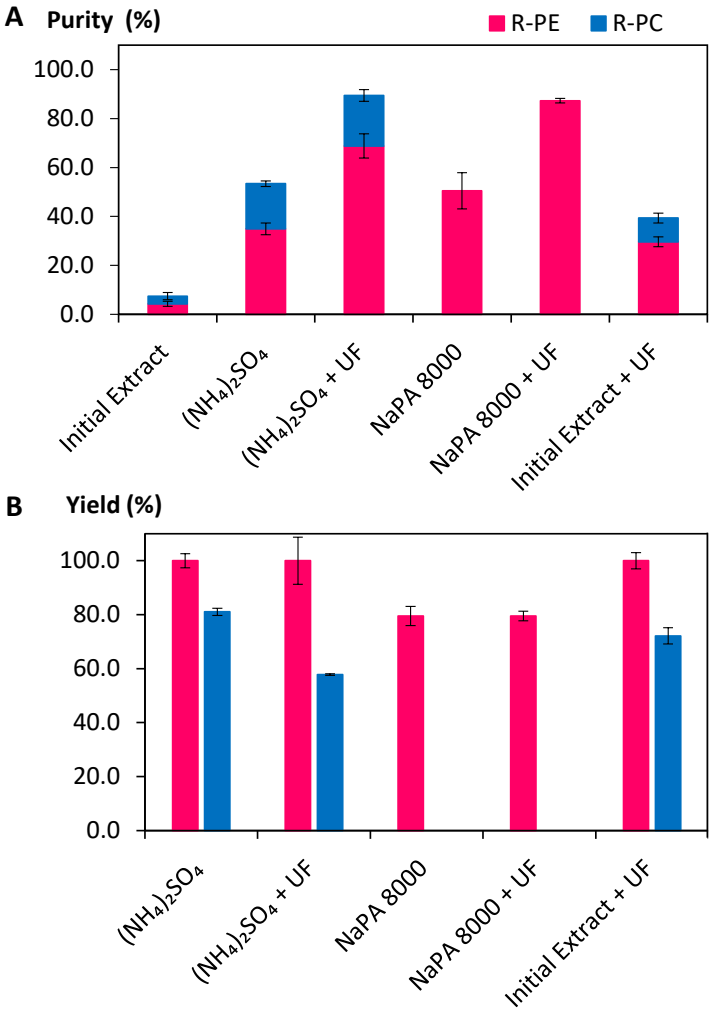

Figure 4.1.3. Summary of the results obtained by HPLC-DAD for the (A) purity and (B) yield obtained in different fractions, namely the initial extract, the resuspended pellets after precipitation using $(NH_4)_2SO_4$ at 200 $g.L^{-1}$, $(NH_4)_2SO_4$ at 200 $g.L^{-1}$ followed by an ultrafiltration step, and NaPA 8000 at 100 $g.L^{-1}$, NaPA 8000 at 100 $g.L^{-1}$ followed by an

ultrafiltration step, and lastly initial extract purified by an ultrafiltration step) separately for R-PE (pink bars) and R-PC (blue bars).

Summing up the results, the initial extract has a purity in phycobiliproteins around 7.4 % (this representing 100 % of both R-PE and R-PC extracted from the biomass). By submitting the extract to a precipitation step using $(NH_4)_2SO_4$ at 200 $g.L^{-1}$, the purity of both phycobiliproteins increased to 53.4 % without compromising the yield of precipitation. By adding an ultrafiltration step, the purity increased to 89.5 % in phycobiliproteins, without affecting the yield of precipitation of R-PE. In the other hand, and as previously analysed, after precipitation with NaPA 8000 at 100 $g.L^{-1}$ only R-PE precipitated with a purity of 50.4 % (R-PC remained in solution). Meanwhile, and after applying the ultrafiltration step, the purity of the extract increased from 50.5 % to 87.3 % in R-PE with a yield of 79.5 %.

The selectivity and *R-PC index* of the purified extract obtained from both purification methodologies proposed in this work were also calculated and compared with the results obtained for the process using aqueous micellar two-phase systems[235] for the purification of R-PE (Table 4.1.3). In terms of selectivity, it was found that both processes proposed in this work are superior to the systems previously reported by Vicente et al.[235] The *R-PC index* in the extracts purified by $(NH_4)_2SO_4$ (200 $g.L^{-1}$) precipitation with an additional ultrafiltration step is higher than the NaPA 8000 (100 $g.L^{-1}$) precipitation with an additional ultrafiltration, supporting the selectivity of the induced precipitation process based in NaPA 8000. Moreover, the induced precipitation with $(NH_4)_2SO_4$ has a higher *R-PC index* than those presented by Vicente et al., showing its ability in preserve the R-PC content. In the other hand, systems of purification with NaPA 8000 have the lowest *R-PC index* in comparison with all systems presented by Vicente and co-authors being in the purity range of the standard R-PE sold by Sigma-Aldrich[249] (which is < 0.03) showing its extremely low contamination with R-PC, as intended.

Table 4.1.3. Selectivity and *R-PC index* of both purification methodologies proposed.

	$(NH_4)_2SO_4$ (200 $g.L^{-1}$) + ultrafiltration (this work)	**NaPA 8000 (100 $g.L^{-1}$) + ultrafiltration (this work)**	***Aqueous micellar two-phase system**[235]	**Sigma-Aldrich**[249]
Selectivity	19.6 ± 0.1	15.3 ± 0.4	13.6 ± 0.0	---
R-PC index	0.23 ± 0.01	0.011 ± 0.001	0.047 ± 0.004	< 0.03

*best system proposed by Vicente and co-authors.[235]

Lastly, an ultrafiltration step was applied to the initial extract without any previous precipitation step in order to understand if the same results could be obtained by skipping the precipitation procedure. At this point, the purity obtained was only of 39.4 % in phycobiliproteins, which represents much lower values than those discussed previously with induced precipitation as a first step, thus showing the need of both steps in the proposed process.

To confirm the results represented in Figure 4.1.3 on the increase of purity of the extracts in the different scenarios tested, a SDS-PAGE electrophoresis was carried out being the results depicted in Figure 4.1.4.

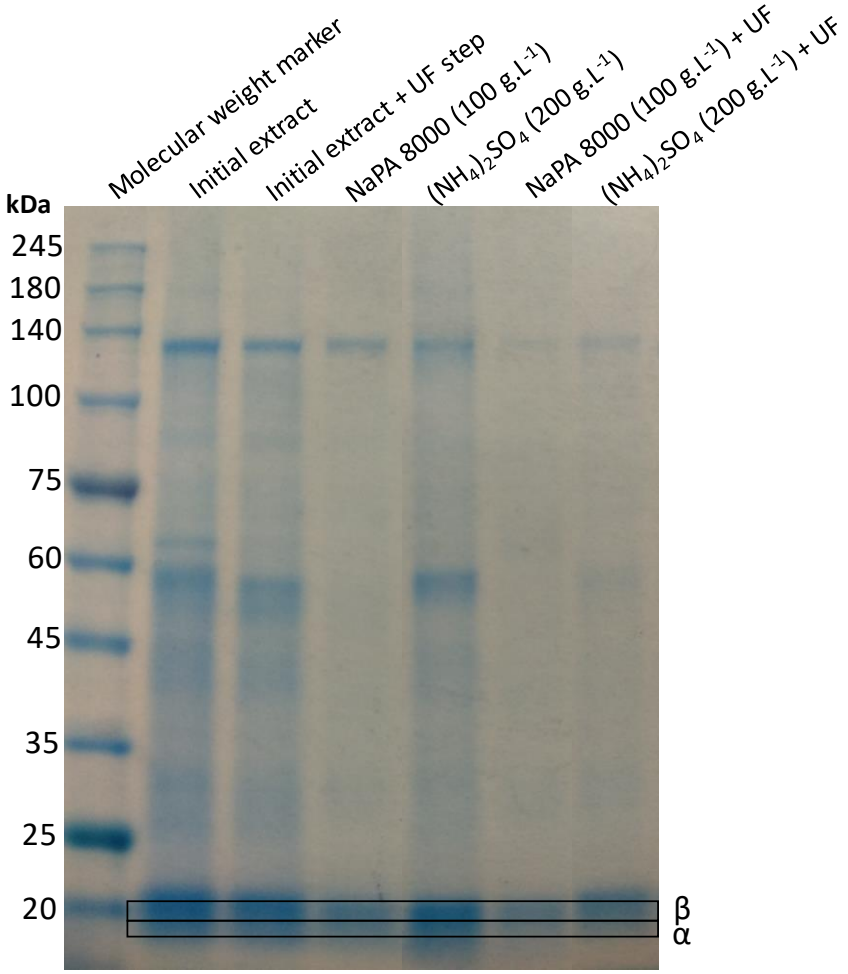

Figure 4.1.4. SDS-PAGE analysis of different fractions obtained after testing the different scenarios under study. UF stands for ultrafiltration.

As previously mentioned, R-PE and R-PC are composed of $(\alpha\beta)_6\gamma$ and $(\alpha\beta)_3$ complexes,[228] respectively. Although there are slight differences among the α and β subunits present in the phycobiliproteins, their weight is quite similar, being 18–20 kDa (for α) and 19.4–21 kDa (for β), and for R-PE an additional γ subunit of ~ 30 kDa is also present.[48,75,247] This said, the presence of α and β subunits is a constant in all samples represented in Figure 4.1.4. It is also evident the high contamination of the initial extract with other proteins. Despite the removal of some impurities when applied an ultrafiltration step to treat the initial extract, it is not enough to achieve a significant increment in purity. The step of precipitation of phycobiliproteins by itself (53.4 %) is more effective in the purification than the ultrafiltration alone (39.4 %), as proved by HPLC-DAD (data depicted in Table F.4 in Appendix F). With the application of ultrafiltration after precipitation with polyelectrolyte, an extract with high purity in R-PE was obtained, with

just a tenuous band of contaminating protein (~ 120 kDa) present, which is in agreement with the results depicted in Figure 4.1.3. It is then evident that the combination of both steps is able to remove most proteins and peptides apart from α and β subunits, characteristic of phycobiliproteins.

After assessing the purity of the samples by SDS-PAGE electrophoresis, the structural integrity of the phycobiliproteins was checked using circular dichroism. With this technique, the secondary structure of the proteins along the different stages of purification using NaPA 8000 were evaluated and compared with pure commercial R-PE. The results are depicted in Figure 4.1.5, with the high-tension voltage graph displayed in Figure F.1 in Appendix F.

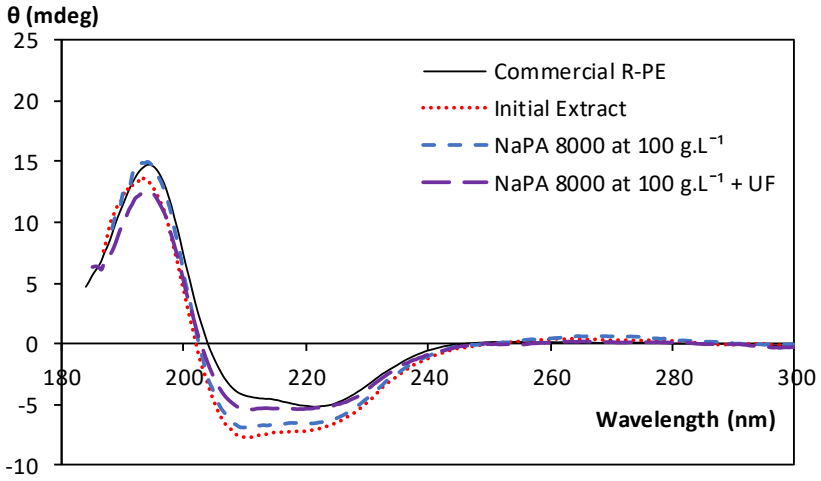

Figure 4.1.5. Circular dichroism spectra of the initial extract (dotted line), resuspended pellet after precipitation using NaPA 8000 at 100 g.L^{-1} (smaller dashed line), and resuspended pellet after precipitation using NaPA 8000 at 100 g.L^{-1} followed by an ultrafiltration step (larger dashed line), and commercial R-PE from Sigma-Aldrich (solid line).

The results show that, as the purity of the extracts increases, the better the spectrum fits the commercial R-PE spectra, being indicative of the preservation of the secondary structure of R-PE after purification. The removal of contaminant proteins with different conformations allows the extract to show a spectrum more similar to the commercial R-PE. Besides, and according to literature for R-PE from *Gracilaria chilensis*, the R-PE is mainly composed of α-helixes (71 %) and a minor content in β-sheets and random coils (12 and 17 %, respectively).[250] This also suggests the preservation of the structural integrity of R-PE after precipitation, since the circular dichroism spectra shows the

maxima of negative signals at ca. 222 and 210 nm, typical of proteins with a high α-helical content.

In conclusion, the proposed processes for purification of phycobiliproteins (A) and for the selective recovery of R-PE (B) from *Gracilaria gracilis* are represented in Figure 4.1.6.

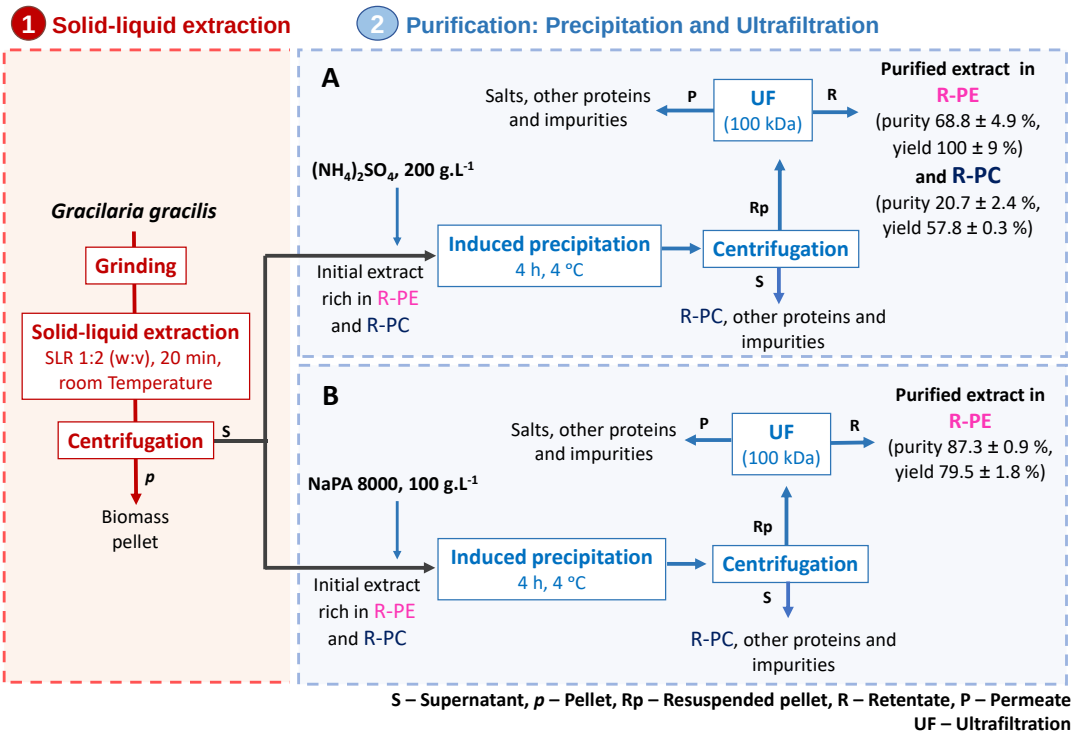

Figure 4.1.6. Process flow diagram proposed to obtain a purified extract in phycobiliproteins (A) and an extract with only R-PE (B).

Environmental analysis by life cycle assessment

Aiming to understand the potential environmental impact of the processes developed in this work, and how they do compare with the process already reported using aqueous micellar two-phase systems,[235] the assessment of their environmental impacts was performed. The results of the life cycle assessment, expressed *per* 1 mg of R-PE, show that the impacts of the scenario where $(NH_4)_2SO_4$ is used are 23–25 % smaller than the impacts of the scenario with NaPA 8000 (Table 4.1.4 and Figure F.2 in Appendix F). The main reason for this result is the higher yield when $(NH_4)_2SO_4$ is used, which leads to lower values of electricity consumption for obtaining the same amount of R-PE. Another reason is the smaller impacts associated with $(NH_4)_2SO_4$ in comparison with NaPA 8000. The purification step has the largest impact in both scenarios, mainly due to electricity

consumption during the cycles of ultrafiltration, which contributes to 70–73% of the total impacts (Figure 4.1.7).

Table 4.1.4. Life cycle assessment for 1 mg of R-PE obtained in both scenarios under study. Scenario 1 represents NaPA 8000 and scenario 2 represents the $(NH_4)_2SO_4$.

Life cycle assessment parameters	Scenario 1	Scenario 2
Climate change (g_{CO2eq})	11.6	8.84
Photochemical oxidant formation ($g_{NMVOCeq}$)	0.0453	0.0349
Terrestrial acidification (g_{SO2eq})	0.0803	0.0622
Fossil depletion (g_{oileq})	3.66	2.73

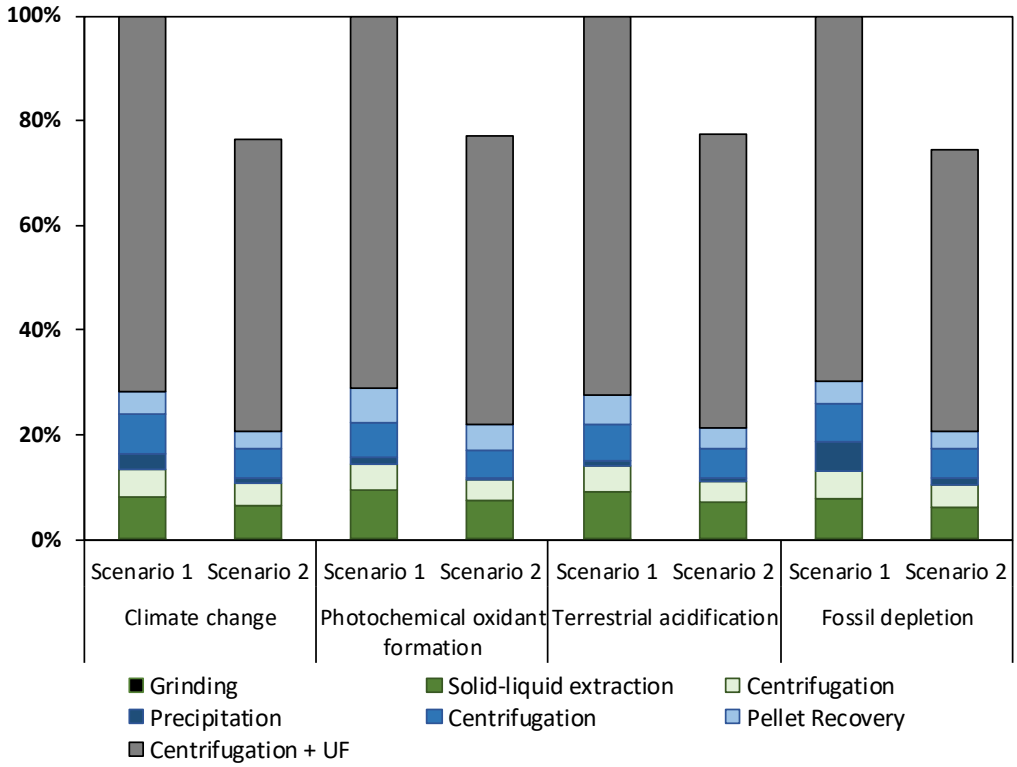

Figure 4.1.7. Relative contribution of the operations for the results of life cycle assessment, considering scenario 1 representing NaPA 8000 and scenario 2 representing $(NH_4)_2SO_4$. Greenish bars are related to the recovery of phycobiliproteins from the biomass, blueish bars are related to the precipitation step in the purification approach, and grey bar is related to ultrafiltration.

Despite the small difference between the two scenarios, 1 (NaPA 8000) and 2 ($(NH_4)_2SO_4$), the carbon footprint (corresponding to the climate change results) obtained

are much smaller than those reported by Vicente et al.[235] (68.14 and 81.30 kg CO_{2eq}.$mg_{R\text{-}PE}^{-1}$) as a result of a much lower electricity consumption in the current process. The process developed in this work proved, not only to be efficient regarding the purification of phycobiliproteins and R-PE in particular, but also to have a low environmental impact.

Economic analysis

Envisioning the potential industrialization of the process here developed, a detailed economic analysis was performed for both systems, scenario 1 using NaPA 8000 and scenario 2 using $(NH_4)_2SO_4$) as precipitating agents. The production cost *per* mg of R-PE is highly variable and deeply influenced by the process scale (Figure 4.1.8). It is important to mention that, as there is not a guide of when to incorporate materials prices for bulk acquisitions, this analysis was performed using the laboratory-scale prices (Table F.2 in Appendix F). Depending on the precipitating agent used, the CoG.mg^{-1} tends to stabilize on EU 0.93 *per* mg and EU 0.32 *per* mg for NaPA 8000 and $(NH_4)_2SO_4$, respectively.

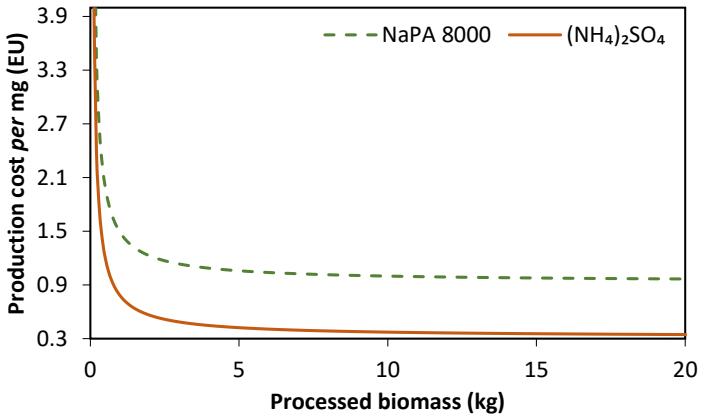

Figure 4.1.8. Analysis of production scale (amount of biomass processed).

In practice, different aspects of the bioprocess tend to vary, and thus, a model is very helpful as it is possible to create a wide range of values for different variables to understand how production costs can be affected. For this reason, a sensitivity analysis was performed on the amount of R-PE content in the biomass (mg of R-PE *per* kg of biomass), on the materials cost variation and on the duration of the process. For these three variables, the range of variation was 10-fold (either above or below the amount used for the model construction). Also, the recovery yield of the process was analysed by a decrease of up to 30 % (worst case scenario), while the best scenario could not be

done up to 30 % because of their current level (it will result in recoveries above 100 %), for this reason, the optimal results were fixed at 100 %. The data collected indicated the content of product in the processed biomass as the most important parameter, followed by the materials costs (Figure 4.1.9). In general, the impact of all parameters decreases as the production scale increases, which is related to the amount of product being generated, as it dilutes the cost variations. Furthermore, the impact of the amount of product being generated has been reported continuously to be one of the most important parameters governing the production costs.[251–253] Finally, it is critical to note that for NaPA 8000, the variation on materials costs is more noticeable than for $(NH_4)_2SO_4$. This is because NaPA is a much more expensive material at both laboratory and large scales (Table F.2 in Appendix F).

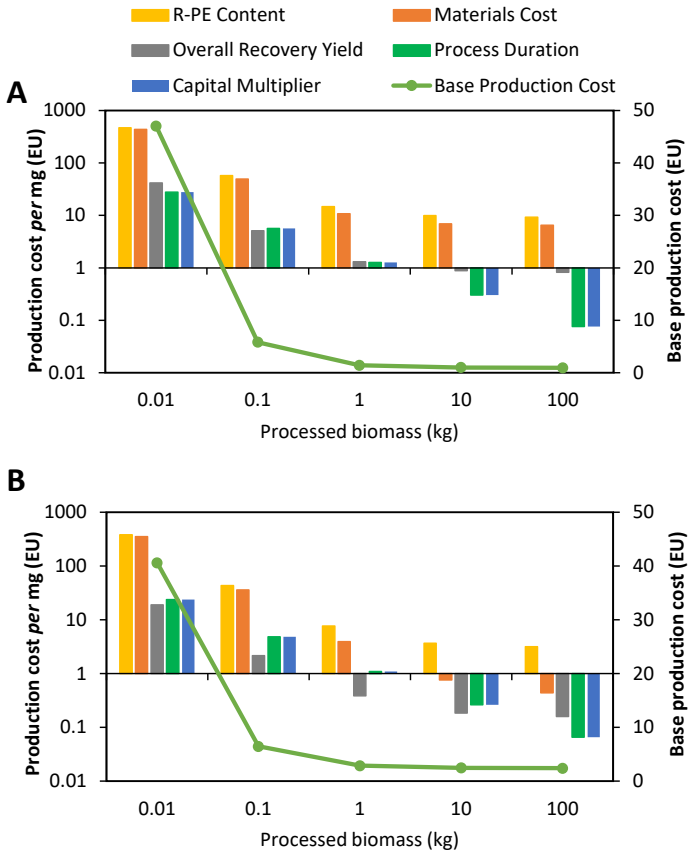

Figure 4.1.9. Result for the sensitivity analysis of the complete bioprocess of NaPA 8000 (A) and $(NH_4)_2SO_4$ (B).

Note: Results are expressed as the difference of the highest and lowest production costs calculated after varying between the worst and best scenarios. Additionally, for reference, the base production cost is shown as the green line (right Y-axis). The left Y-axis is presented in a logarithmic scale.

Using the same variables and ranges, a series of Monte Carlo simulations were run to understand how the simultaneous variation of the main parameters affects the production costs. This was done for scales of 0.01 kg and 100 kg (full data is presented in Table F.5 in Appendix F). This results in a collection of statistical data that can show the significance or not of a variable. The main results confirm the importance of the product content in the biomass and of the materials cost variation, but the effect of the second is almost ten times bigger for NaPA 8000 than for $(NH_4)_2SO_4$ at large-scale (Table 4.1.5). Interestingly, for all the analysed scales, the duration of the process is not statistically significant, which means that, if the process is shorter or longer, it will have a negligible effect on the production cost.

Table 4.1.5. Monte Carlo simulations and multiple linear regression.

Note: Input variables were in the corresponding multiplier or modifier from the sensitivity analysis. To calculate the CoG.mg^{-1} for R-PE content, materials costs and process duration can be any value that represents a multiplier (used for the modelling were from 0.1X to 10X), while for the recovery yield it is a modifier (± 30 %).

	NaPA 8000				**$(NH_4)_2SO_4$**			
	0.01 kg	*p*-value	100 kg	*p*-value	0.01 kg	*p*-value	100 kg	*p*-value
Intercept (βo)	110.06	$1.17x10^{-9}$	1.864961	$4.19x10^{-11}$	78.82741	$4.67x10^{-8}$	0.344542	$2.88x10^{-22}$
R-PE content (β1)	-28.5299	$1.43x10^{-24}$	-0.44852	$5.06x10^{-25}$	-23.5171	$4.22x10^{-30}$	-0.06802	$1.42x10^{-41}$
Overall recovery yield (β2)	-0.94794	0.042111	-0.01485	0.04056	-0.71106	0.202417	-0.00254	0.0505
Materials cost (β3)	22.79631	$3.47x10^{-18}$	0.340471	$4.68x10^{-17}$	20.58119	$1.39 x10^{-25}$	0.030776	$1.39x10^{-12}$
Process duration (β4)	-1.3313	0.586571	-0.0387	0.309369	0.020603	0.991292	0.000104	0.981153

Equations have the form (Eq. 6):

$$Production\ Cost\ [\text{EU } per \text{ mg}] = \beta o + \beta 1 \times \text{R} - \text{PE Content} + \beta 2 \times \text{Overall Recovery Yield} + \beta 3 \times \text{Materials Cost} + \beta 4 \times \text{Process Duration} \quad \textbf{Eq. 6}$$

Lastly, the *Return per* kg of processed biomass was performed at laboratorial-scale (0.01 kg) and large-scale (100 kg) using prices from Sigma-Aldrich (for 0.01 kg) and Alibaba (for 100 kg), the latest being considered as an example of a real-life value. Additionally, the amount of product in the biomass (C_{prod}) was varied by 0.5X, 1X or 2X and the CoG.kg^{-1} of processed biomass was varied by a factor of 1X, 2X and 5X. Moreover, the $\$_{prod}$ was varied from EU 5 *per* kg to EU 5,000 *per* kg. Results provide an in-depth look into different scenarios and how they can influence the potential economic return for this process (Figure 4.1.10 and Figure F.3 in Appendix F).

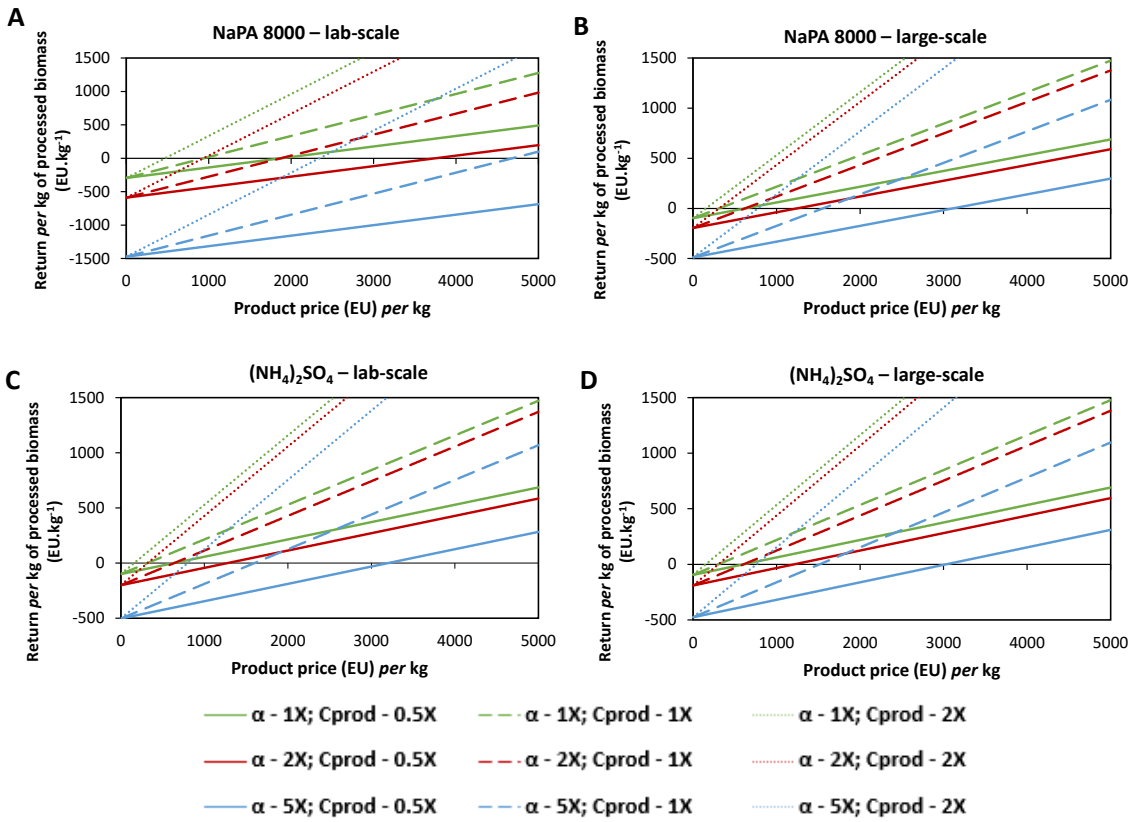

Figure 4.1.10. *Return* analysis for NaPA 8000 and $(NH_4)_2SO_4$. NaPA 8000 results are presented in (A) for laboratory-scale (0.01 kg) and (B) for large-scale (100 kg), while for $(NH_4)_2SO_4$ are (C) laboratory-scale (0.01 kg) and (D) for large-scale (100 kg). Green lines are for an alpha of 1X, red for alpha of 2X and blue for alpha of 5X; solid lines for a C_{prod} of 0.5X, dash lines for C_{prod} of 1X and dot lines for C_{prod} of 2X.

Results from this analysis can help to appreciate different issues considered relevant for the efficiency and sustainability of the process. The slope of each line is the influence of the C_{prod} on the *Return*: the higher the C_{prod}, the more vertical the line will be. Additionally, the position where the lines intercept with the y-axis (the point where $\$_{prod}$ is 0), is dictated by the CoG.kg^{-1} of biomass. The most evident result is the abrupt difference on the y-axis intercept for Figure 4.1.10 A and B, indicating the impact that the change in the price of the materials has on the CoG.kg^{-1} of biomass. From the data on Table F.2 in Appendix F, the price reduction of NaPA 8000 from laboratory to large-scale is much larger compared to the decrease of $(NH_4)_2SO_4$ price, which can be related to the extensive use of $(NH_4)_2SO_4$. Moreover, this dramatic change becomes the critical aspect for determining, for specific conditions, if there is any *Return* at all.

Given the results obtained here, even after increasing the potential CoG.kg^{-1} of biomass by 5-fold, reducing the C_{prod} by half, it is possible to have a positive *Return* and possible above the EU 1,000 *per* kg of product. This can be ensured and enhanced if the bioprocess developed here can increase the purity of the product, then its market price can be increased. As a reference, commercial price of R-PE from Sigma-Aldrich (Product 52412) sells at EU 155 *per* mg (EU 155,000,000 *per* kg).

Conclusions

In this work, a new approach, easy to implement, using induced precipitation, is proposed for the purification of phycobiliproteins, in particular R-PE. A set of polymers, copolymers, and polyelectrolytes was screened correlating their ability to selective precipitate proteins from a raw extract of phycobiliproteins regarding the purification of fluorescent proteins. It was found that the most common used precipitation agent in proteins – $(NH_4)_2SO_4$ – at 200 g.L^{-1} is able to precipitate both R-PE and R-PC but it is not selective, while the polyelectrolyte NaPA 8000, even at low concentrations (100 g.L^{-1}), can selectively induce the precipitation of R-PE among the set of phycobiliproteins present in the extract. By further using an ultrafiltration step, purities of 89.5 % and 87.3 % were achieved, respectively for the two phycobiliproteins using the strategy of $(NH_4)_2SO_4$ followed by ultrafiltration and for only R-PE using NaPA 8000 followed by ultrafiltration, having this last one its structural integrity preserved. Summing up, and

despite the regular use of $(NH_4)_2SO_4$, its use did not allow the development of a selective induced precipitation, which is surpassed by the use of NaPA 8000.

Taking into account the results of selectivity for the system using NaPA 8000, the environmental impact was determined and compared with one of the most recent reports of processes optimized for the purification of R-PE using aqueous micellar two-phase systems. The low carbon footprint of the process optimized by using induced precipitation with NaPA 8000, shows that the process here proposed has a lower environmental impact. Using the current process results combined with the economic analysis, it was concluded that a potential real-life application can provide return dependent on the market price of the R-PE product. Some of the major factors to determine the required price are the amount of R-PE content in the biomass (or the amount extracted from it) and the price of the materials during a large-scale operation. The use of NaPA 8000 or $(NH_4)_2SO_4$ provides cost-effective results and, ultimately, the decision on their selection can be based on process-oriented results, such as the purity required of the product for the desired application, along with the possible commercial price of the product.

CHAPTER 5. Final remarks and future perspectives

Industry and academia must work together to supply consumers needs without compromise the environment and health of today's and future generations. To achieve this goal, instead of a linear and fossil-based economy, a bioeconomy based on natural and renewable resources is desirable. All routes associated to a certain process, from inputs to outputs, should be analysed in terms of their environmental and economic viability. The blue biorefinery must be seen as part of a circular economy, allowing the maximum profit out of a certain natural resource with minimal waste.

In this work, various methodologies to extract and purify different pigments from algae were studied and proposed. The main objective was to overcome issues related to conventional techniques, such as the excessive use of volatile organic solvents, high energy consumption, high demand in specific and complex equipment, which leads to high associated costs and environmental impacts. Alternative methodologies were designed in which the solvents choice was always of utmost importance, giving preference to water, the cheapest and greenest among all solvents. Indeed, water is the basis of all methodologies proposed in this thesis. Besides its known biocompatibility and environmental safety, the use of water also decreases the viscosity of the systems – enhancing mass transfer on the extraction processes – while having a very low volatility and no health or environmental impact in comparison with organic solvents. Besides the attention given to the choice of solvents, in all works operational conditions were always optimized in order to minimize energy and solvents consumption, without compromising yields of extraction, purities, and selectivity.

This thesis is divided in three main chapters: (i) solid-liquid extraction of hydrophobic pigments (2nd chapter); (ii) fractionation of hydrophobic pigments (3rd chapter); and (iii) purification of hydrophilic pigmented proteins (4th chapter). Regarding the recovery of more hydrophobic pigments, such as chlorophylls and carotenoids, aqueous solutions of tensioactive ILs, namely those based on ammonium and phosphonium cations, have shown an amazing performance due to their ability to interact with the phospholipid bilayer of the cell membranes and also due to their ability to form micelles, creating the perfect environment to extract hydrophobic compounds with water/aqueous solutions. The good performance of these ILs was seen not only in procedures of solid-liquid extraction (chapter 2) but also in procedures of fractionation/separation of chlorophylls and carotenoids (chapter 3.2 and 3.3) and in the recovery of adsorbed chlorophyll from

a resin allowing, consequently, the recycle of the resin in several cycles (chapter 3.4). It is also important to note that the scope of this thesis was never to propose the complete replacement of organic solvents in industrial processes. In fact, these solvents were used several times in the proposed methodologies. However, in these cases preference was given to natural solvents, such as ethanol and vegetable oils, yet at times petroleum-based solvents such as ethyl acetate, toluene, and hexane were also used, making their recovery and recycling even more important.

In chapter 3.1 very good results were obtained for the recovery of chlorophyll and xanthophylls, mainly by using organic solvents. However, it is interesting to see how their potential can grow together with other types of solvents such as aqueous solutions of ILs. A good example in this thesis was the application of organic solvents to polish the target compound by back-extraction (chapter 3.2 and 3.3) but also the other way around. In chapter 3.4, ethanol has worked as an extractive solvent and aqueous solutions of ILs as washing solutions to recover the pigments adsorbed in a resin. It is possible to conclude from all proposed methodologies in chapters 2 and 3 that the selection of the most appropriate methodology has no easy answer. Many parameters should be taken into consideration and all comes down to: (i) the desired pigment(s) or derivatives, (ii) the desired purity, (iii) the final application of the pigments and any limitation in terms of solvent (even residual), (iv) technical limitations associated for example to the use of highly viscous solutions (such as vegetable oil), and of course, (v) associated costs, and (vi) environmental impact.

In chapter 4, pigmented proteins were studied. Due to their hydrophilicity and protein nature neither tensioactive ILs or organic solvents were considered in the extraction and purification processes. Instead, the use of induced precipitation with polymers and polyelectrolytes coupled to an ultrafiltration step lead to high yields and purity when compared with previous works in literature. Besides, an analysis on environmental and economic impacts was done, showing high potential under both perspectives.

In the presented works, higher yields of extraction, purities and selectivity were, in general, obtained when compared to conventional approaches. Simple and easy to implement processes were proposed, with no use of temperature and pressure – allowing savings in energy – and with no use of special devices or equipment, leading to cost effective processes with low environmental impact. For the works for which

environmental and economic analysis were performed, very encouraging results were achieved showing their potential as alternatives methodologies.

In the other hand, although a view of integrated processes was given in most of the processes developed in this thesis, more and deeper studies need to be carried out. This means to completely recover the target compound from the solvent to allow a small loss of solvent that should reenter the process (recycle) and to allow the production of biomolecules free of solvents, such as ILs, to be applied in any field. Additionally, these studies should be seen under the light of continuous processes instead of batch processes. The scale-up to pilot and industrial scales is crucial, being at the same time fundamental to analyse all impacts associated. Further studies on pigments stabilization and products shelf life should be addressed, namely by the use of extraction solvent itself as stabilizer or part of the stabilizing solution. Finally, innovative applications increase the value of the products, namely in the cosmetic and biomedical fields due to the plethora of recognized biological activities these natural pigments have.

Due to their high market value and small market size, pigments should be one of the first compounds/classes of bioactive compounds to be recovered in a biorefinery chain. These processes here proposed can serve as an initial basis for that purpose. Although these works were focused on pigments, they should be seen as an example of what can be done for other bioactive compounds, in a larger perspective, from algae or other natural sources. To understand how solvents work within the cells in each type of biomass and, the interactions between them and the target compound is crucial, and we believe that these systems can be also applied in other situations, and to other products. The ultimate scope is undoubtedly to incentive industries to change their products going towards the natural products without compromise efficiencies, economics and environment.

CHAPTER 6. Scientific contribution

All the presented items only refer to the PhD grant period (from March 2017 to April 2021).

Patents

1. S.P.M. Ventura, **M. Martins**, J.A.P. Coutinho, M. Bisht; Método para a extração de colagénio purificado de matrizes biológicas; Depositor: Universidade de Aveiro, Deposit Date: 2020/12/03; Request number: 116915; provisional application in the portuguese deposit.
2. S.P.M. Ventura, **M. Martins**, J.A.P. Coutinho, M.G.P.M.S. Neves, D.C.G.A. Pinto, M.A.F. Faustino; Method to extract and purify chlorophylls and xanthophylls from biomass; Depositor: Universidade de Aveiro, Deposit Date: 2019/12/13; Request number: 115969; definitive application in the portuguese deposit; PAT 2019/58404; PN 115969.
3. S.P.M. Ventura, **M. Martins**, J.A.P. Coutinho, M.G.P.M.S. Neves, D.C.G.A. Pinto, Método de extração e purificação de clorofilas e xantofilas de biomassa; Depositor: Universidade de Aveiro, Deposit Date: 2019/12/13; Request number: 115968; definitive application in the portuguese deposit; PAT 2019/30886; PN 115968.

Published papers

1. **M. Martins**, L.M. de Souza Mesquita, B.M.C. Vaz, A.C.R.V. Dias, B. Quéguineur, J.A.P. Coutinho, S.P.M. Ventura, Extraction and fractionation of pigments from *Saccharina latissima* (Linnaeus, 2006) using an ionic liquid+oil+water system, **ACS Sustainable Chemistry & Engineering**, 2021, DOI: 10.1021/acssuschemeng.0c0911.
2. P.J. Bharmoria, M. Bisht, M.C. Gomes, **M. Martins**, M.C. Neves, J.F. Mano, Igor Bdikin, J.A.P. Coutinho, S.P.M. Ventura, Protein-Olive Oil-in-Water Nanoemulsions as Encapsulation Materials for Curcumin Acting as Anticancer Agent towards MDA-MB-231 Cells, **Scientific Reports**, 2021 – accepted.
3. **M. Martins**, B.P. Soares, J.H.P.M. Santos, P. Bharmoria, M.A. Torres-Acosta, A.C.R.V. Dias, J.A.P. Coutinho, S.P.M. Ventura, Sustainable strategy based on induced precipitation for the purification of phycobiliproteins, **ACS Sustainable Chemistry & Engineering**, 2021, 9(10), 3942-3954, DOI: 10.1021/acssuschemeng.0c09218.
4. **M. Martins**, C.M. Albuquerque, C.F. Pereira, J.A.P. Coutinho, M.G.P.M.S. Neves, D.C.G.A. Pinto, M.A.F. Faustino, S.P.M. Ventura, Recovery of chlorophyll *a* derivative

from *Spirulina maxima*, its purification and photosensitizing potential, **ACS Sustainable Chemistry & Engineering**, 2021, 9(4), 1772-1780, DOI: 10.1021/acssuschemeng.0c07880.

5. L.M. de Souza Mesquita, **M. Martins**, L.P. Pisani, S.P.M. Ventura, V.V. Rosso, Insights on the use of alternative solvents and technologies to recover bio-based food pigments, **Comprehensive Reviews in Food Science and Food Safety – IFT**, 2021, 20(1), 787-818, DOI: 10.1111/1541-4337.12685.

6. **M. Martins**, R. Oliveira, J.A.P. Coutinho, M.A.F. Faustino, M.G.P.M.S. Neves, D.C.G.A. Pinto, S.P.M. Ventura, Recovery of pigments from *Ulva rigida*, **Separation and Purification Technology**, 2021, 255, 117723, DOI: 10.1016/j.seppur.2020.117723.

7. B.P. Soares, J.H.P.M. Santos, **M. Martins**, M.R. Almeida, N.V. Santos, M.G. Freire, V.C. Santos-Ebinuma, J.A.P. Coutinho, J.F.B. Pereira, S.P.M. Ventura, Purification of green fluorescent protein using centrifugal partition chromatography, **Separation and Purification Technology**, 2020, 117648, DOI: 10.1016/j.seppur.2020.117648.

8. **M. Martins**, A.P.M. Fernandes, M.A. Torres-Acosta, P.N. Collén, M.H. Abreu, S.P.M. Ventura, Extraction of chlorophyll from wild and farmed *Ulva* spp. using aqueous solutions of ionic liquids, **Separation and Purification Technology**, 2021, 254, 117589, DOI: 10.1016/j.seppur.2020.117589.

9. B. Colombo, J. Pereira, **M. Martins**, M.A. Torres-Acosta, A.C.R.V. Dias, P.C. Lemos, S.P.M. Ventura, G. Eisele, Anna Alekseeva, F. Adani, L.S. Serafim, Recovering PHAs from mixed microbial cultures: using non-ionic surfactants as a pre-treatment step, **Separation and Purification Technology**, 2020, 253, 117521, DOI: 10.1016/j.seppur.2020.117521.

10. T.E. Sintra, S.S. Bagagem, F.G. Ahsaie, A.P.M. Fernandes, **M. Martins**, I.P.E. Macário, J.L. Pereira, F.J.M. Gonçalves, G. Pazuki, J.A.P. Coutinho, S.P.M. Ventura, Sequential recovery of C-phycocyanin and chlorophylls from *Anabaena cylindrica,* **Separation and Purification Technology**, 2021, 255, 117538, DOI: 10.1016/j.seppur.2020.117538.

11. P. Bharmoria, S.F.H. Correia, **M. Martins**, M.A. Hernández-Rodríguez, S.P.M. Ventura, R.A.S. Ferreira, L.D. Carlos, J.A.P. Coutinho, Protein Co-habitation: Improving the Photo-Chemical Stability of R-Phycoerythrin in Solid State, **The Journal of Physical Chemistry Letters**, 2020, 11(15), 6249-6255, DOI: 10.1021/acs.jpclett.0c01491.

12. C.P.A. Carlos, S.F.H. Correia, **M. Martins**, O.A. Savchuk, J.A.P. Coutinho, P.S. André, J.B. Nieder, S.P.M. Ventura, R.A.S. Ferreira, Environmentally-friendly luminescent solar concentrators based on optically efficient and stable green fluorescent protein, **Green Chemistry**, 2020, 22(15), 4943-4951, DOI: 10.1039/D0GC01742F.

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15. L.M. de Souza Mesquita, **M. Martins**, É. Maricato, C. Nunes, P.S.G.N. Quinteiro, A.C.R.V. Dias, J.A.P. Coutinho, L.P. Pisani, V.V. Rosso, S.P.M. Ventura, Ionic liquid-mediated recovery of carotenoids from the *Bactris gasipaes* fruit waste and their application in food-packaging chitosan films, **ACS Sustainable Chemistry & Engineering**, 2020, 8(10), 4085-4095, DOI: 10.1021/acssuschemeng.9b06424 – **front cover of the journal** (Volume 8, Number 10, 16 March 2020).

16. M. Kholany, P. Trebulle, **M. Martins**, S.P.M. Ventura, J.-M. Nicaud, J.A.P. Coutinho, Extraction and purification of violacein from *Yarrowia lipolytica* cells using aqueous solutions of surfactants, **Journal of Chemical Technology & Biotechnology**, 2020, 95(4), 1126-1134, DOI: 10.1002/jctb.6297.

17. F.A. Vicente, I.S. Cardoso, **M. Martins**, C.V.M. Gonçalves, A.C.R.V. Dias, P. Domingues, J.A.P. Coutinho, S.P.M. Ventura, R-phycoerythrin extraction and purification from fresh *Gracilaria* sp. using thermo-responsive systems, **Green Chemistry**, 2019, 21, 3816-3826, DOI: 10.1039/C9GC00104B – **front cover of the journal** (Volume 21, Number 14, 21 July 2019).

18. T.E. Sintra, M. Vilas, **M. Martins**, S.P.M. Ventura, A.I.M.C.L. Ferreira, L.M.N.B.F. Santos, F.J.M. Gonçalves, E. Tojo, J.A.P. Coutinho, Synthesis and characterization of surface-active ionic liquids used in the disruption of *Escherichia coli* cells, **ChemPhysChem**, 2019, 20(5), 727-735, DOI: 10.1002/cphc.20180112.

19. A.R. Frias, S.F.H. Correia, **M. Martins**, S.P.M. Ventura, E. Pecoraro, S.J.L. Ribeiro, P.S. André, R.A.S. Ferreira, J.A.P. Coutinho, L.D. Carlos, Sustainable Liquid Luminescent Solar Concentrators, **Advanced Sustainable Systems**, 2019, 3(3), 1800134, DOI: 10.1002/adsu.201800134.

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21. N.V. dos Santos, **M. Martins**, V.C. Santos-Ebinuma, S.P.M. Ventura, J.A.P. Coutinho, S.R. Valentini, J.F.B. Pereira, Aqueous Biphasic Systems Composed of Cholinium Chloride and Polymers as Effective Platforms for the Purification of Recombinant Green Fluorescent Protein, **ACS Sustainable Chemistry and Engineering**, 2018, 6, 9383-9393, DOI: 10.1021/acssuschemeng.8b01730.

Published book chapters

1. **M. Martins**, S.P.M. Ventura, Emerging Seaweed Extraction Techniques using ionic Liquids, Sustainable Seaweed Technologies – **Advances in Green and Sustainable Chemistry**, Elsevier, 2020, 287-311, DOI: 10.1016/B978-0-12-817943-7.00011-1.

Submitted papers

1. **M. Martins**, B.M.C. Vaz, L.M. de Souza Mesquita, M.C. Neves, A.P.M. Fernandes, M.G.P.M.S. Neves, J.A.P. Coutinho, S.P.M. Ventura, Ionic liquids as eluents in solid-phase extraction to purify pigments recovered from *Isochrysis galbana*, **Chemical Engineering Journal**.

Internship in industry

1. Research and development team, *Extraction of biological active ingredients from seaweed using eutectic solvents*, Olmix Group Internship, additional supervision of Clément Le Verge and Pi Nyvall Collén, September to December 2019, Bréhan, France.

Entrepreneurship

1. Conceptual development of the brand *Seauth – Natural Solutions*, BlueBioValue Ideation 2020 program promoted by Oceano Azul Foundation and Calouste Gulbenkian Foundation, organized by Fábrica de Startups, in collaboration with BlueBio Alliance and Faber Ventures, February to March 2020**.**

Awards and distinctions

1. **Scientific highlight in a newsletter article** regarding the publication "Purification of green fluorescent protein using fast centrifugal partition chromatography", by UAveiro Research Newsletter #234, available in https://mailchi.mp/7524680bc85a/uaveiro-research-newsletter-234?e=1ea214807b , 26 February 2021, **2021**.
2. **Front cover of the journal** ACS Sustainable Chemistry & Engineering: Volume 8, Number 10, 16 March 2020, **2020.**
3. **4th place in the BlueBioValue Ideation 2020 program** promoted by Oceano Azul Foundation and Calouste Gulbenkian Foundation, organized by Fábrica de Startups, in collaboration with BlueBio Alliance and Faber Ventures, **2020.**
4. **Best pitch** award entitled of "From salty water To salty water: problems with colorful solutions" – Research Summit 2019 at University of Aveiro – awarded by Vice-Rector for Research, Innovation and 3rd Cycle of University of Aveiro, **2019.**
5. **Front cover of the journal** Green Chemistry: Volume 21, Number 14, 21 July 2019, **2019.**
6. **Best pitch** award entitled of "SEA: Sustainability Extracted from Algae" – Research Summit 2018 at University of Aveiro – awarded by Vice-Rector for Research, Innovation and 3rd Cycle of University of Aveiro, **2018.**
7. **Best Rising Science Communicator** – Jornadas do CICECO 2018, Living Science Together – Honorable Mention – awarded by the organizing committee, **2018.**
8. **1st place in the in the doctoral contest** in the Bioengineering and Biotechnology panel, Fundação para a Ciência e a Tecnologia (Portuguese national funding agency for science, research and technology), **2017**.

Other scientific contributions

Oral communications in national or international events: 19 (+5 accepted, +1 abstract submitted)

Panel communications in conferences and seminars: 27 (+1 accepted)

Participation in conferences, seminars, and workshops: 22

Participation in events as organizer or exhibitor: 4

Experimental supervisions of younger students: 15

CHAPTER 7. Bibliography

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(263) Merck. Poly(ethylene glycol)-block-poly(propylene glycol)-block-poly(ethylene glycol) average Mn ~2,800 https://www.sigmaaldrich.com/catalog/product/aldrich/435430?lang=pt&region=PT.

CHAPTER 8. Appendix

Appendix A

Extraction of chlorophyll from wild and farmed *Ulva* spp. using aqueous solutions of ionic liquids

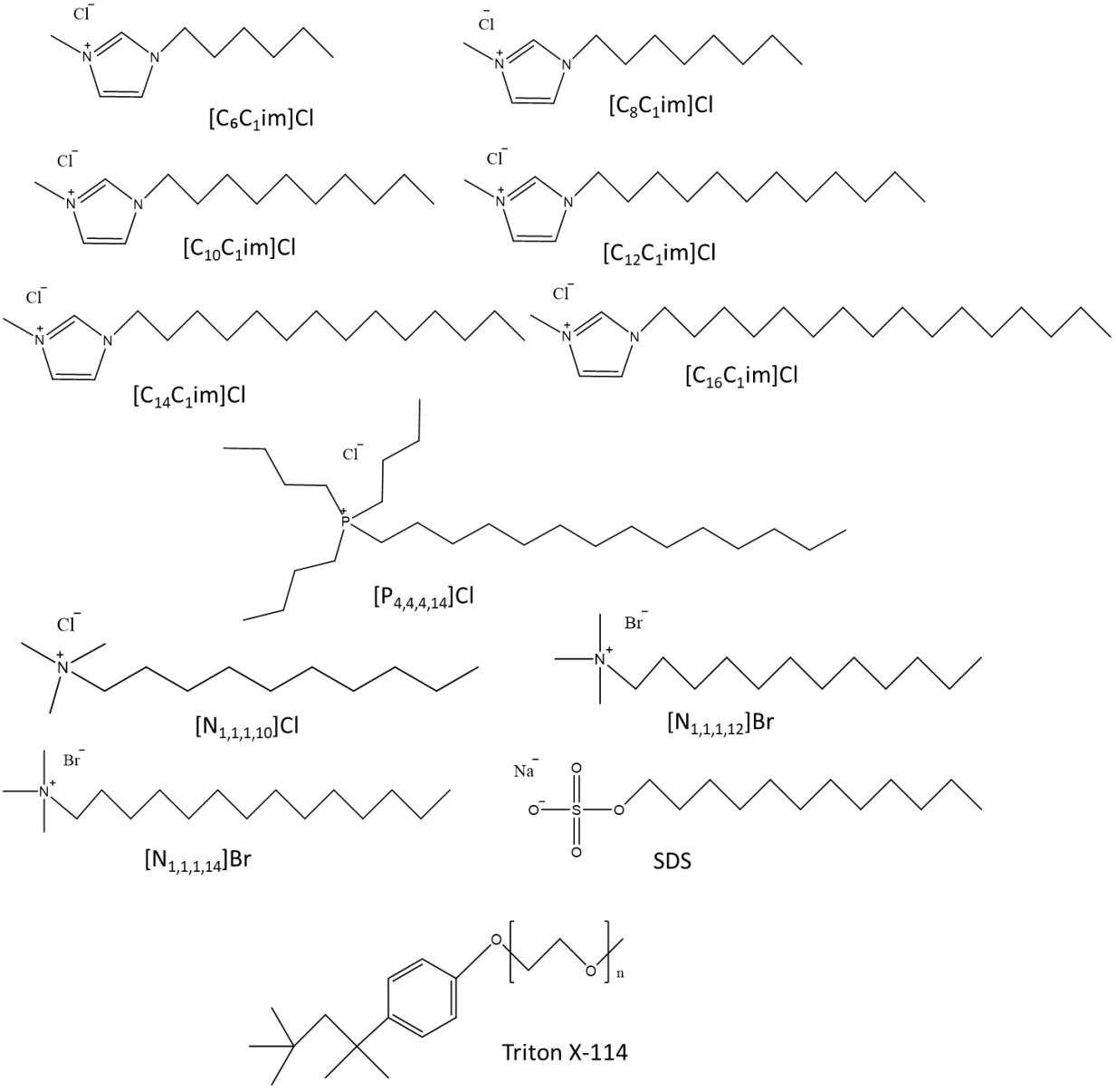


Figure A.1. Chemical structures of the tensioactive compounds (ILs and common surfactants) used in the screening of alternative solvents.

Appendix B

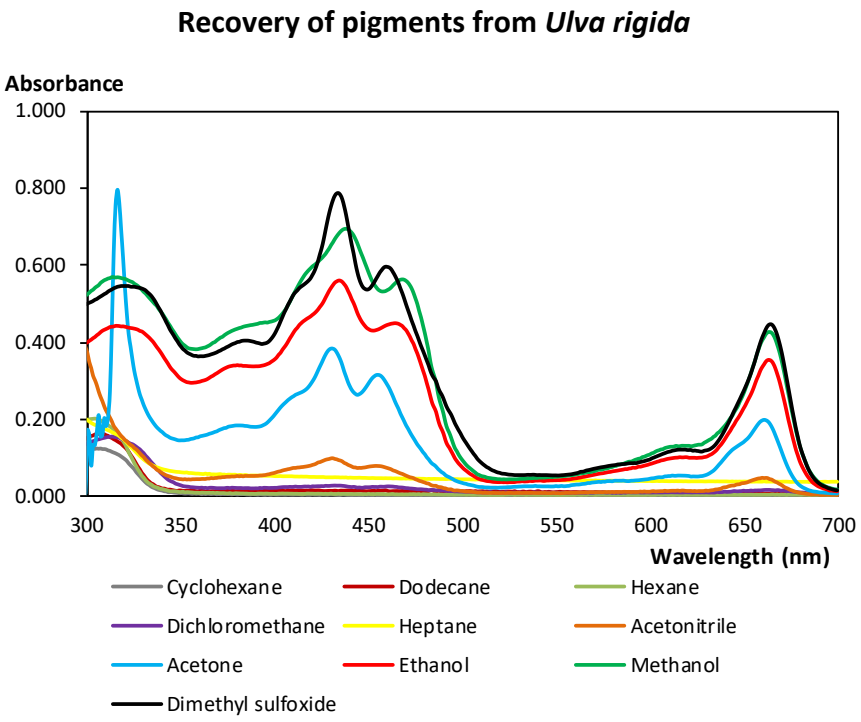


Figure B.1. UV-Vis spectra of the extracts obtained in the screening of organic solvents for the extraction of chlorophyll from *Ulva rigida*.

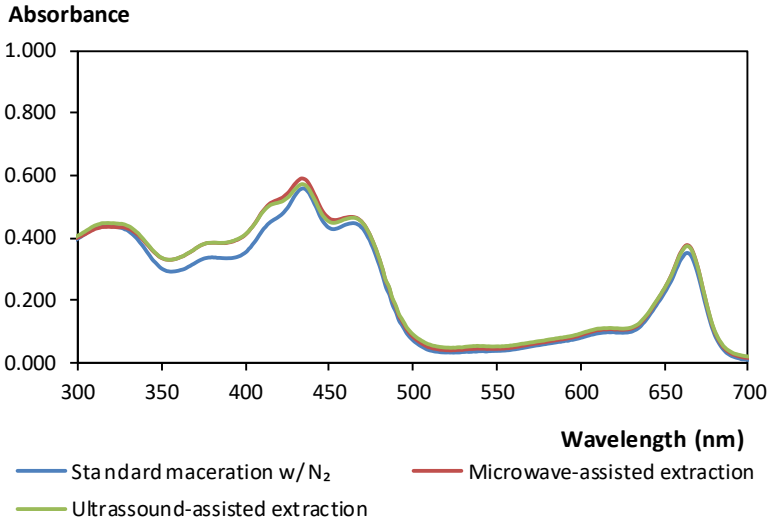


Figure B.2. UV-Vis spectra of the extracts obtained using ethanol in different approaches of extraction of chlorophyll from *Ulva rigida*.

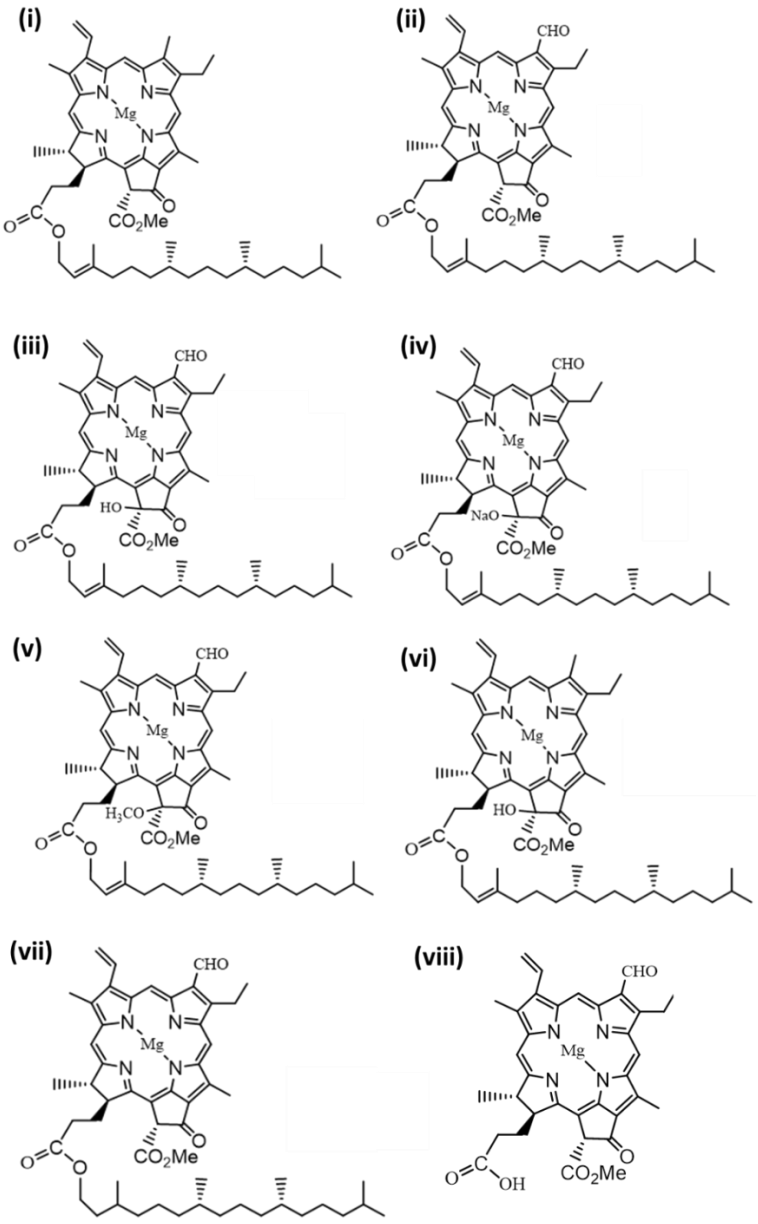


Figure B.3. Molecular structures of the extracted chlorophyll *a* and *b* as well as the proposed structures for the derivatives detected.

Table B.1. Chlorophyll concentration ($mg.L^{-1}$) and yield of extraction ($mg_{chl}.g_{fresh\ biomass}^{-1}$) obtained in the screening of organic solvents for the extraction of chlorophyll from *Ulva rigida*.

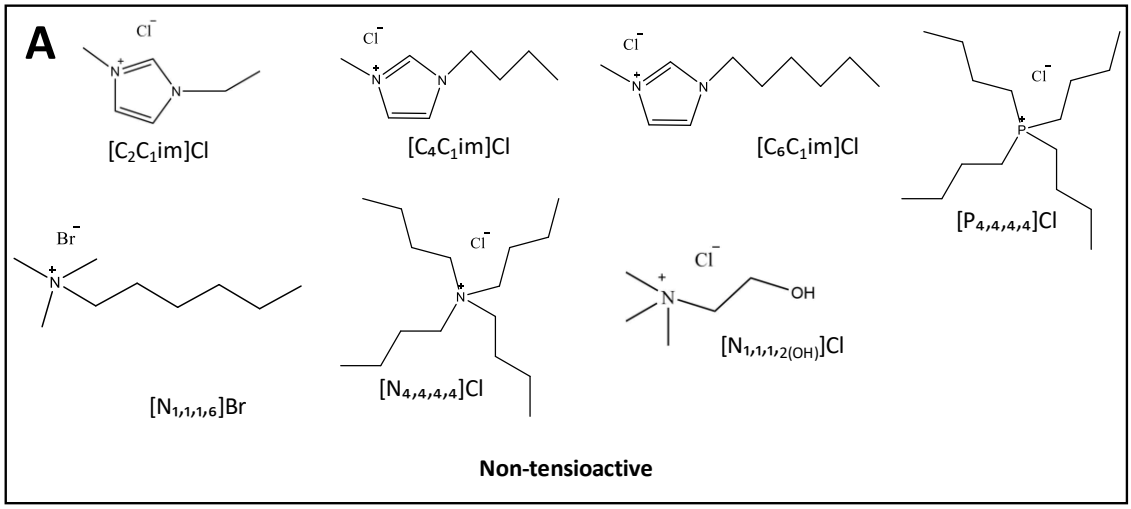
	Chlorophyll concentration ($mg.L^{-1}$)	**Standard deviation of chlorophyll concentration**	**Yield of extraction ($mg_{chl}.g_{fresh\ biomass}^{-1}$)**	**Standard deviation of the yield of extraction**	**% total chlorophyll extracted**
Cyclohexane	0.009	0.013	0.001	0.001	< 0.1
Hexane	0.299	0.346	0.030	0.035	1.6
Dodecane	0.236	0.333	0.023	0.033	1.3
Dichloromethane	0.462	0.192	0.044	0.020	2.4
Heptane	1.377	0.205	0.136	0.021	7.4
Acetonitrile	1.585	0.064	0.150	0.011	8.1
Acetone	6.463	0.865	0.637	0.077	34.4
Ethanol	12.636	0.192	1.243	0.006	67.1
Methanol	15.380	0.128	1.518	0.007	82.0
Dimethyl sulfoxide	16.178	0.589	1.599	0.086	86.3

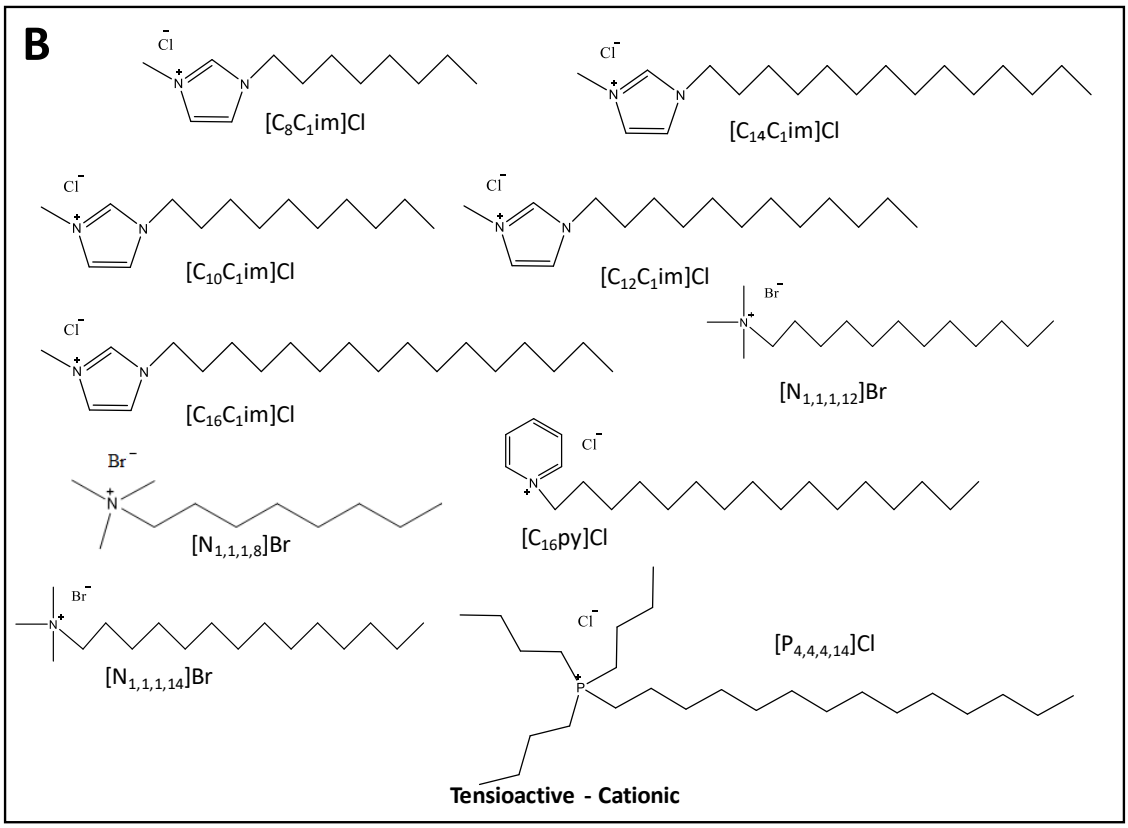
Table B.2. Chlorophyll concentration ($mg.L^{-1}$) and yield of extraction ($mg_{chl}.g_{fresh\ biomass}^{-1}$) obtained using ethanol in different approaches of extraction of chlorophyll from *Ulva rigida*.

	Chlorophyll concentration ($mg.L^{-1}$)	**Yield of extraction** ($mg_{chl}.g_{fresh\ biomass}^{-1}$)	**% total chlorophyll extracted**
Standard maceration w/ N_2	12.636 ± 0.192	1.243 ± 0.006	67.1
Microwave-assisted extraction	13.533 ± 0.384	1.33 ± 0.04	72.0
Ultrasound-assisted extraction	13.424 ± 0.179	1.30 ± 0.02	70.4

Appendix C

Recovery of chlorophyll *a* derivative from *Spirulina maxima*, its purification and photosensitizing potential





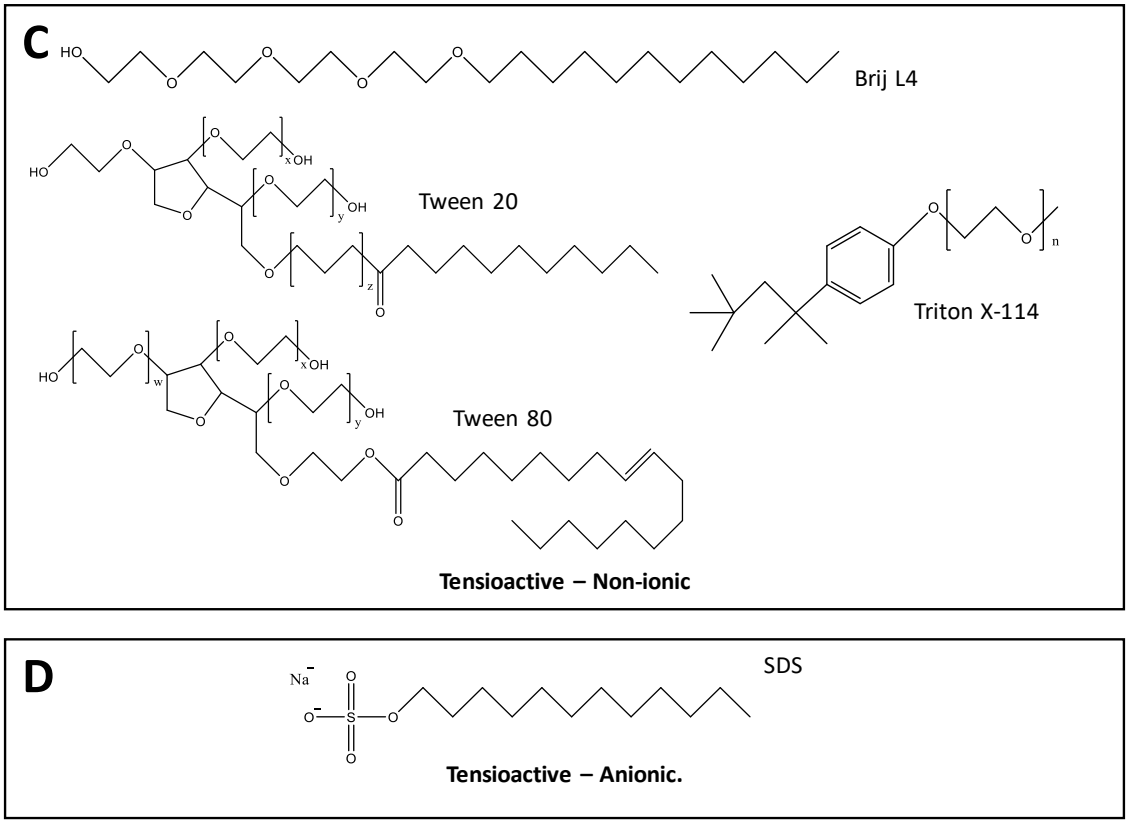


Figure C.1. Molecular structure of the ILs and common surfactants screened in this work: (A) non-tensioactive compounds, (B) cationic tensioactive compounds, (C) non-ionic tensioactive compounds, and (D) anionic tensioactive compounds.

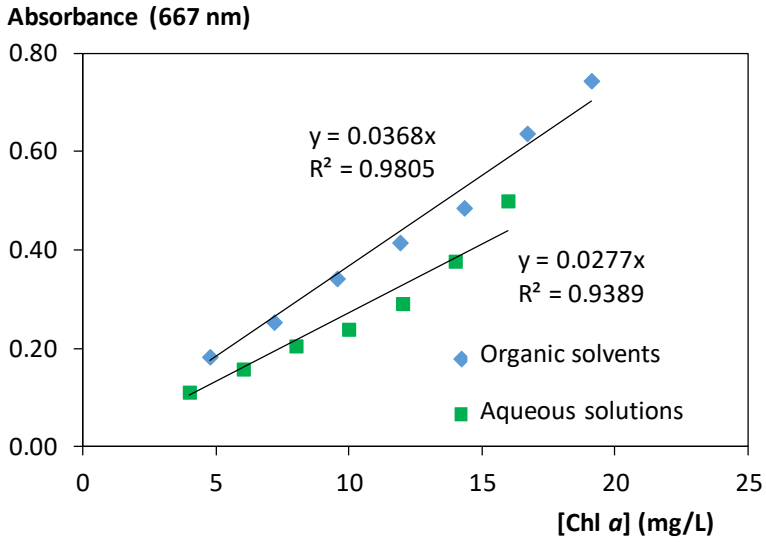


Figure C.2. Calibration curves experimentally determined and used to quantify chlorophyll in organic solvents and aqueous solutions using the Synergy HT microplate reader – BioTek.

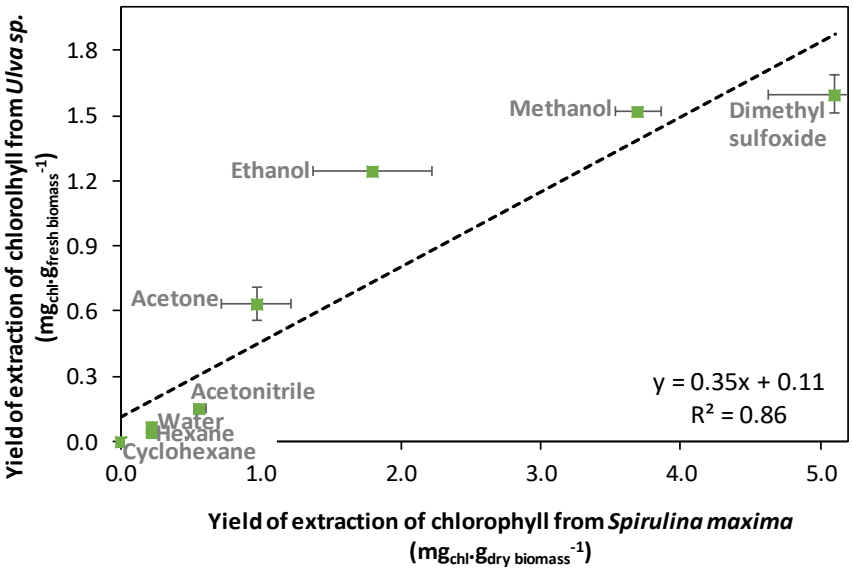


Figure C.3. Correlation between the yields of chlorophyll extracted from *Ulva rigida*[178] (Chapter 3.1) and *Spirulina maxima* using the same solvents.

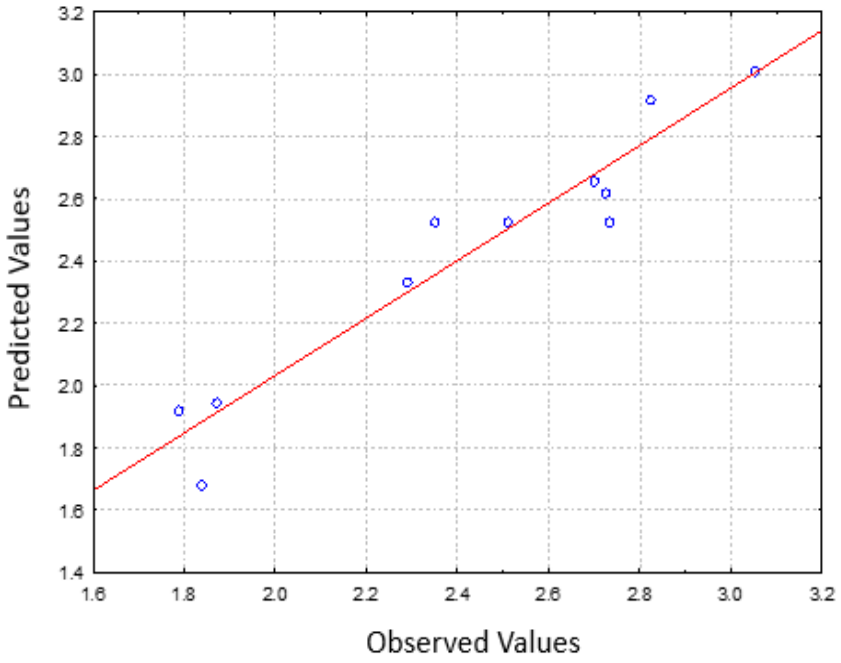


Figure C.4. Predicted *vs.* experimental values of the CCRD (2^2) regarding the yield of extraction of chlorophyll using methanol.

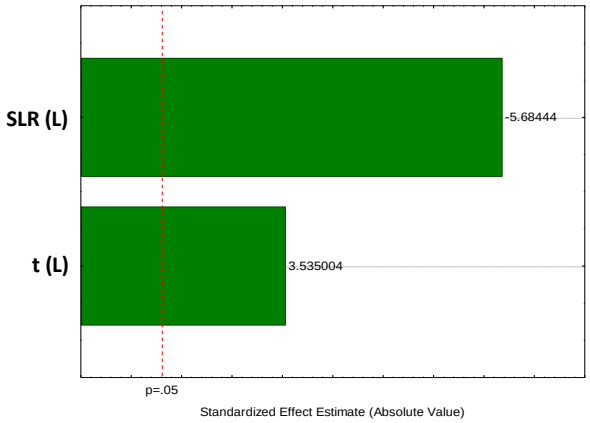


Figure C.5. Pareto Chart of the CCRD (2^2) regarding the yield of extraction of chlorophyll using methanol as solvent.

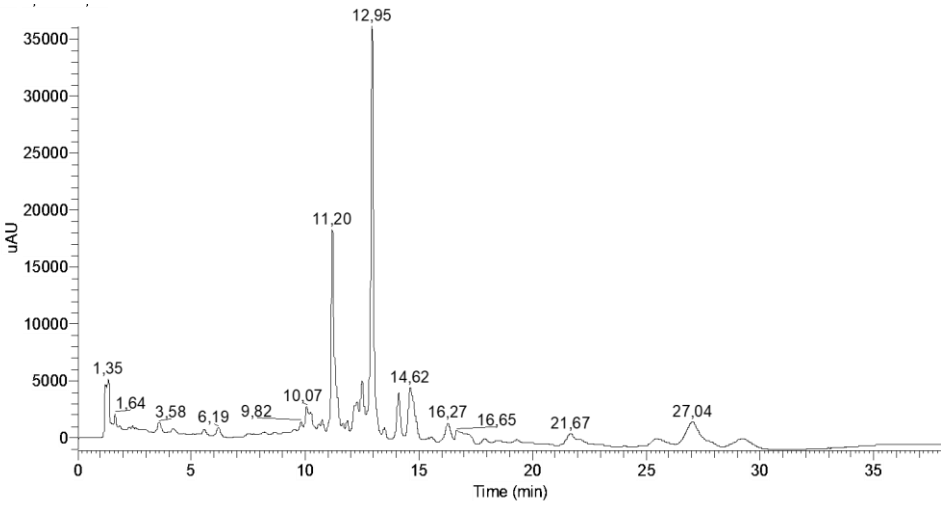


Figure C.6. UHPLC chromatogram of the methanol-based extract, recorded at 305 nm.

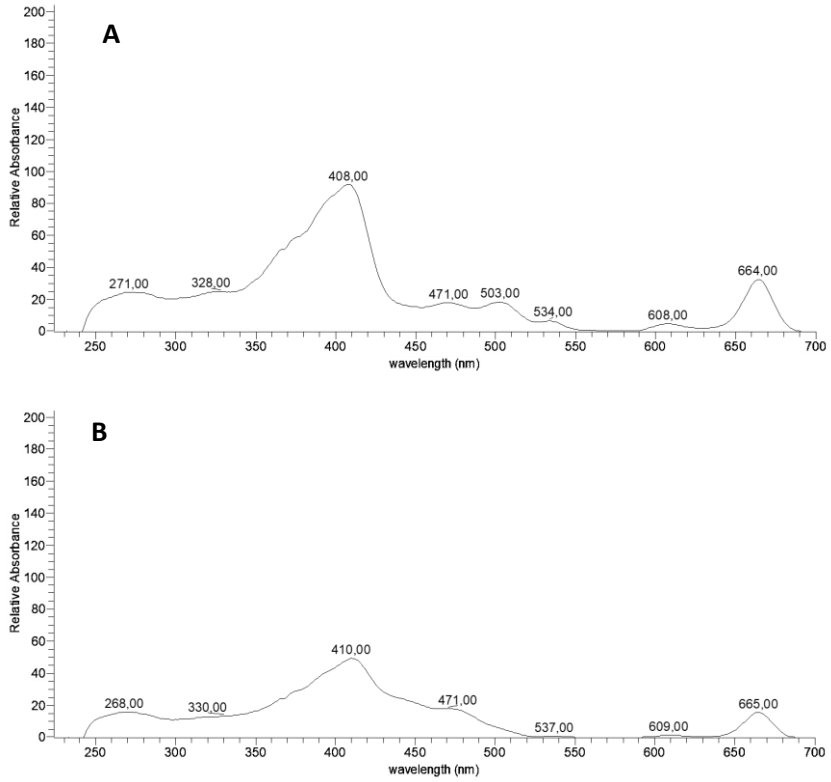


Figure C.7. UV-Vis spectra of the methanol-based extract considering the peaks at (A) 11.20 min and (B) 12.95 min.

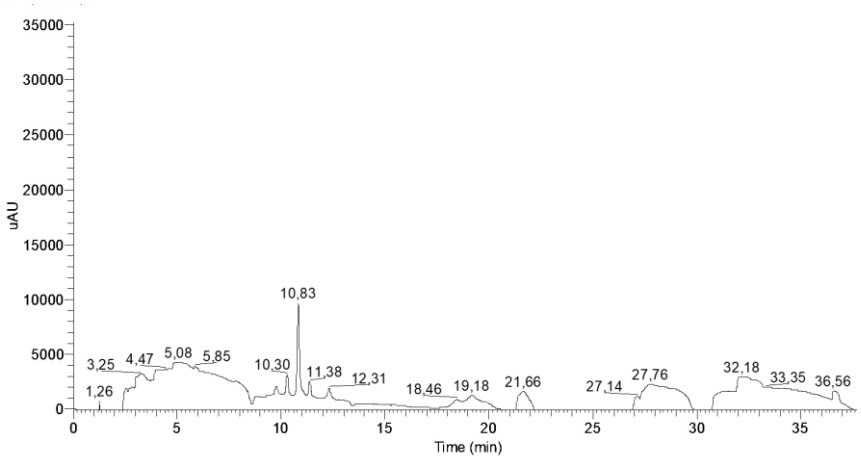


Figure C.8. UHPLC chromatogram of the 1st and 2nd fractions of back extraction (obtained from alternative extraction), recorded at 430 nm.

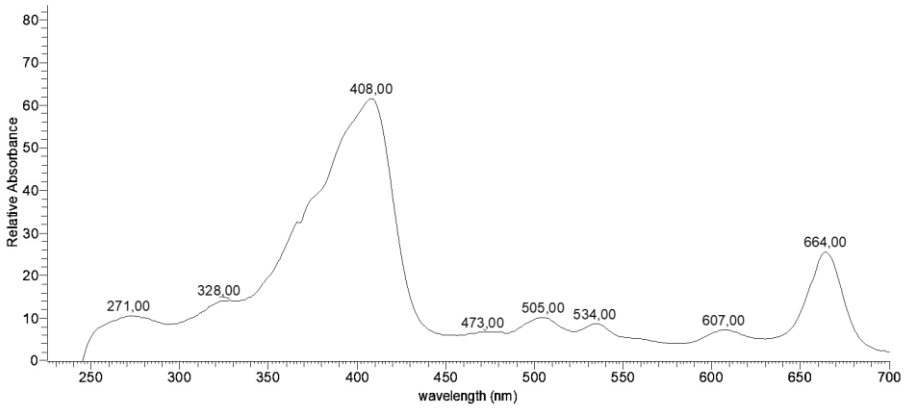


Figure C.9. UV-Vis spectra of the 1st and 2nd fractions of back extraction (obtained from alternative extraction) considering the peak at 10.83 min.

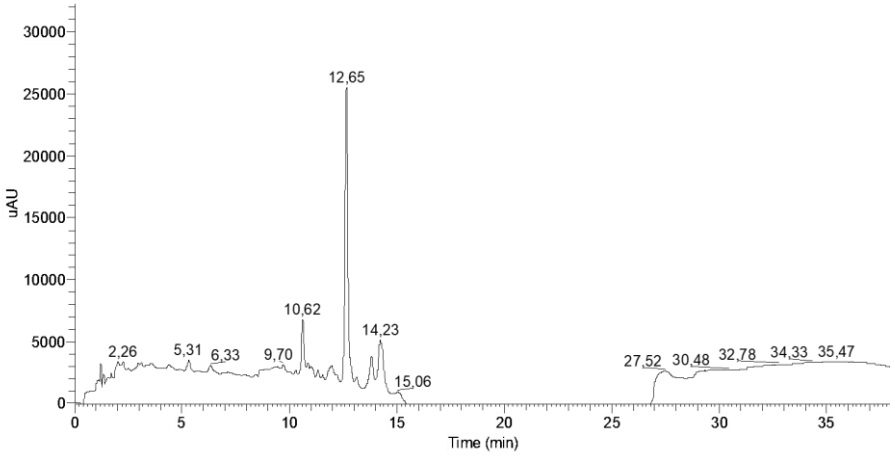


Figure C.10. UHPLC chromatogram of the 3rd fraction of back extraction (obtained from alternative extraction), recorded at 305 nm.

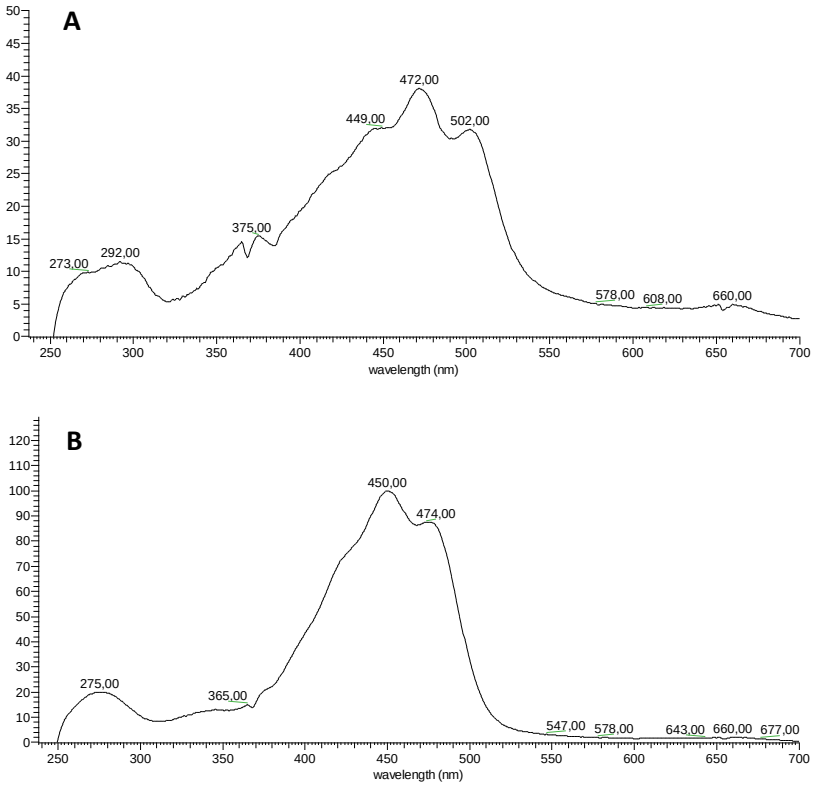


Figure C.11. UV-Vis spectra of the 3rd fraction of back extraction (obtained from alternative extraction) considering the peaks at (A) 10.62 min and (B) 12.65 min.

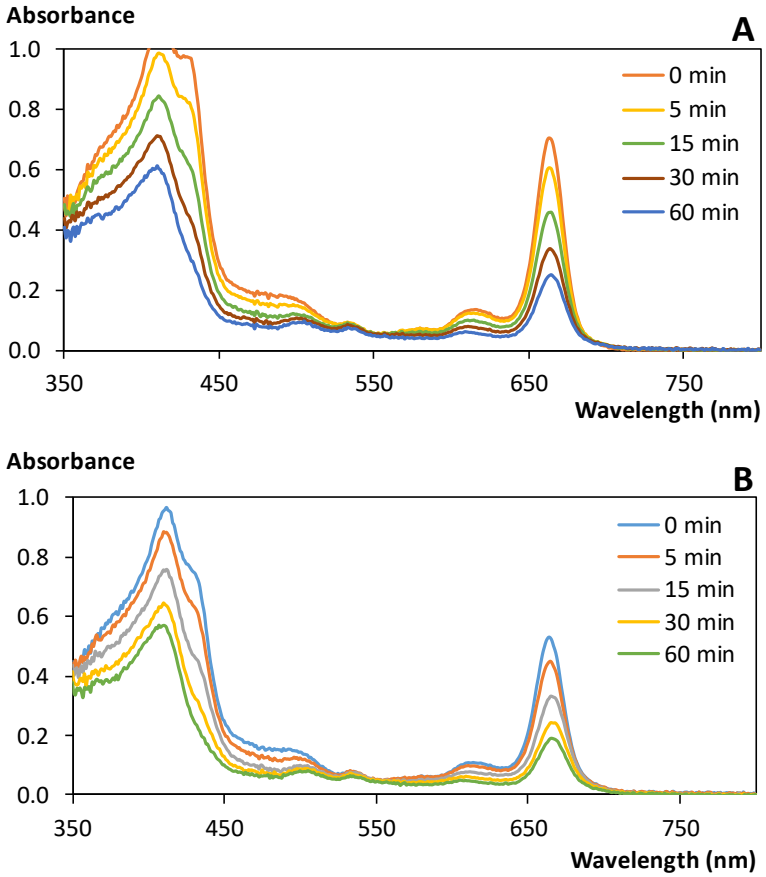


Figure C.12. UV-Vis spectra of the (A) methanol-based extract and (B) 1st and 2nd fractions of back extraction (obtained from alternative extraction) along the irradiation period in the photostability assays.

Table C.1. Real and coded values of the optimization process expressed by the yields of extraction chlorophyll extracted from *Spirulina maxima* by CCRD (2^2) using methanol as solvent.

	Design Matrix		Experimental conditions		Yield of extraction ($mg_{chl}.g_{dry\ biomass}^{-1}$)
Run	**Time**	**Solid-liquid ratio**	**Time** (min)	**Solid-liquid ratio** ($g_{dry\ biomass}.mL_{solvent}^{-1}$)	
1	-1	-1	28	0.030	2.698
2	1	-1	62	0.030	2.823
3	-1	1	28	0.055	1.837
4	1	1	62	0.055	2.287
5	-1.41	0	21	0.045	1.788
6	1.41	0	69	0.045	2.724
7	0	-1.41	45	0.024	3.052
8	0	1.41	45	0.059	1.869
9	0	0	45	0.045	2.350
10	0	0	45	0.045	2.731
11	0	0	45	0.045	2.510

Table C.2. Critical micellar concentration (CMC) values of the screened tensioactive compounds.

Tensioactive compound	CMC (mM)
$[C_8C_1im]Cl$	220[254]
$[C_{10}C_1im]Cl$	55[254]
$[C_{12}C_1im]Cl$	15[254]
$[C_{14}C_1im]Cl$	4[254]
$[C_{16}C_1im]Cl$	1.26[102]
$[N_{1,1,1,8}]Cl$	39.8[255]
$[N_{1,1,1,12}]Br$	15.6[256]
$[N_{1,1,1,14}]Br$	3.8[256]
$[C_{16}py]Cl$	0.96[257]
$[P_{4,4,4,14}]Cl$	4.69[255]
SDS	8[254]
Brij L4	n.d.
Triton X-114	0.168[258]
Tween 20	0.050[258]
Tween 80	0.010[258]

Appendix D

Extraction and fractionation of pigments from *Saccharina latissima* (Linnaeus, 2006) using an ionic liquid+oil+water system

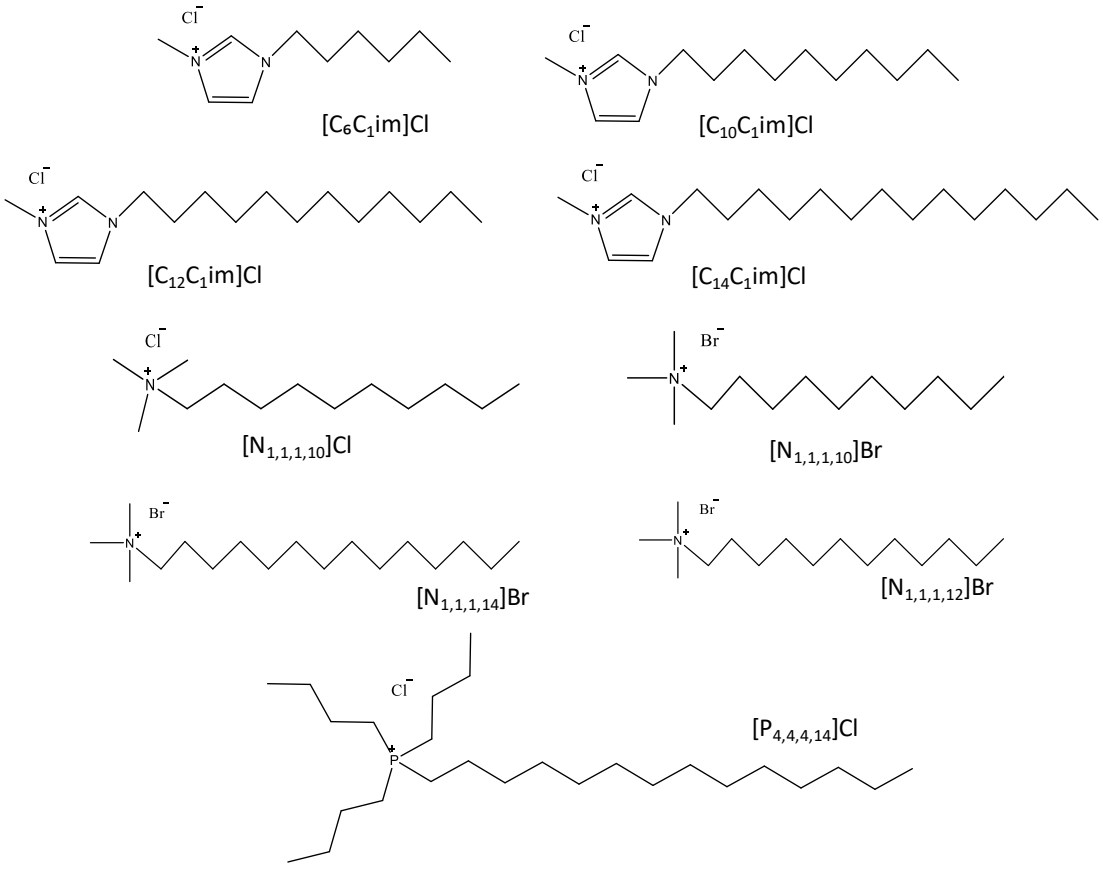


Figure D.1. Molecular structures of ILs screened in this work.

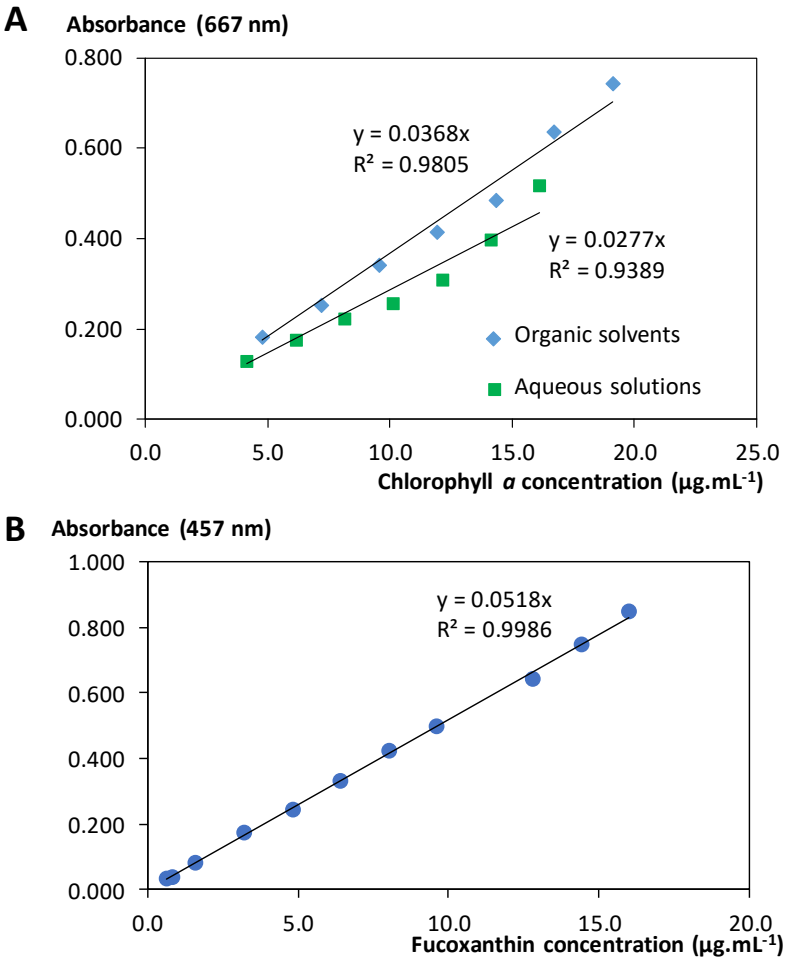


Figure D.2. Calibration curves experimentally determined and used to quantify chlorophyll in organic solvents and aqueous solutions and fucoxanthin in aqueous solvents using the Synergy HT microplate reader – BioTek.

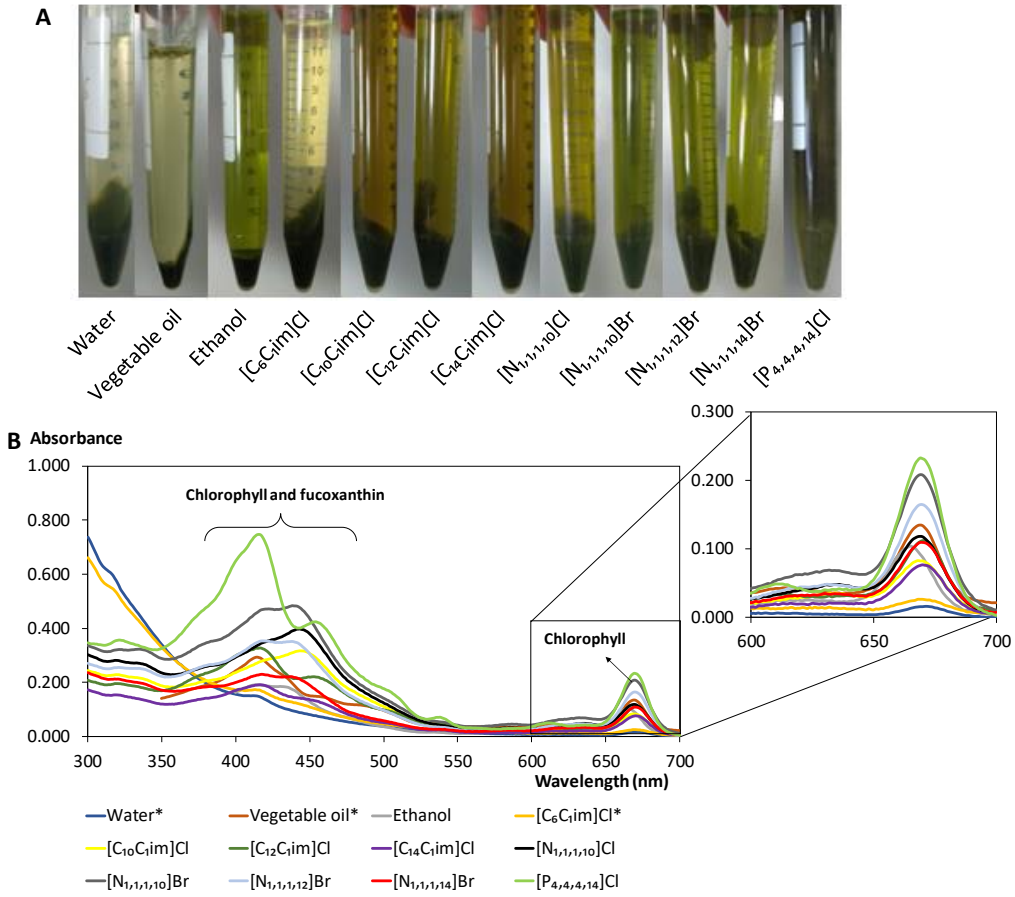


Figure D.3. (A) Photographs and (B) UV-Vis spectroscopy of the extracts obtained in the screening of solvents. In the absorption spectra (B), extracts with the mark * were not analysed with the same dilution factor.

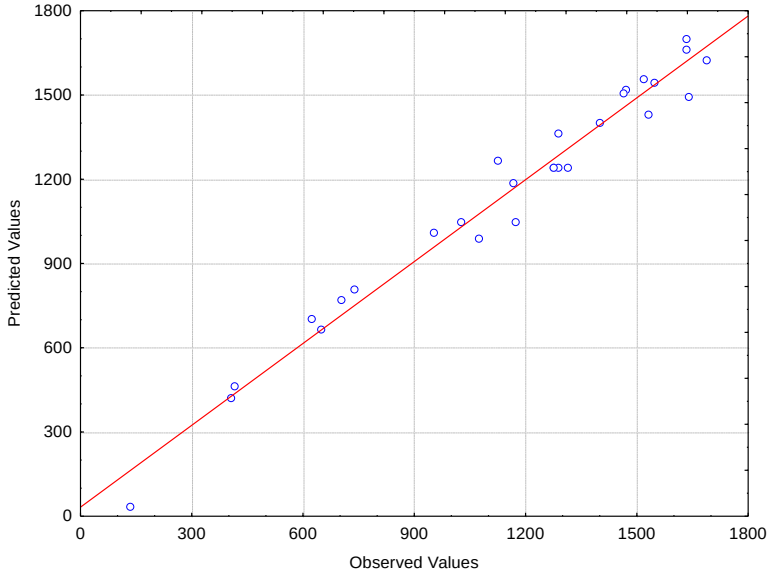


Figure D.4. Predicted *vs.* experimental values of the CCRD (2^4) regarding the yield of fucoxanthin ($\mu g_{fuco}.g_{dry\ biomass}^{-1}$) using systems with $[N_{1,1,1,10}]Br$.

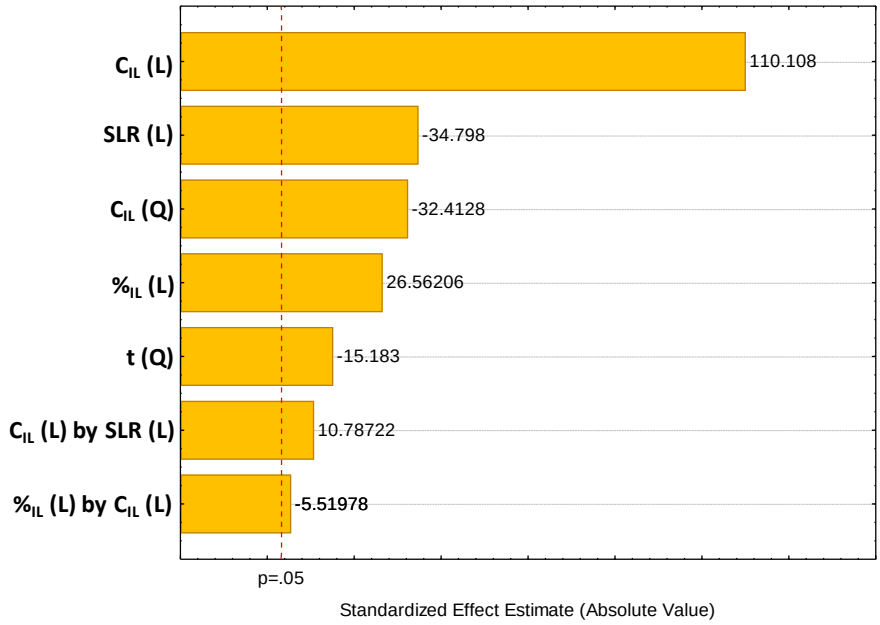


Figure D.5. Pareto Chart of the CCRD (2^4) regarding the yield of fucoxanthin using systems with $[N_{1,1,1,10}]Br$.

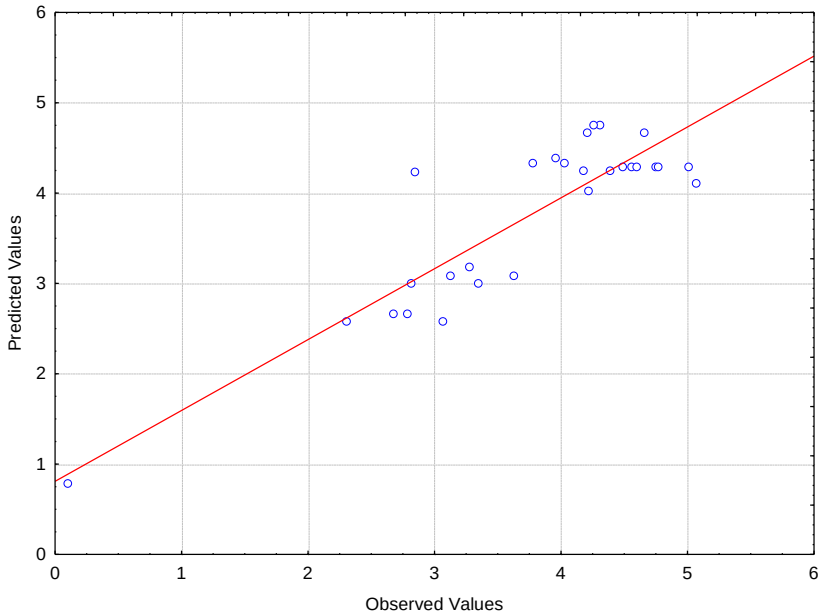


Figure D.6. Predicted *vs.* experimental values of the CCRD (2^4) regarding the yield of chlorophyll ($mg_{chl}.g_{dry\ biomass}^{-1}$) using systems with $[N_{1,1,1,10}]Br$.

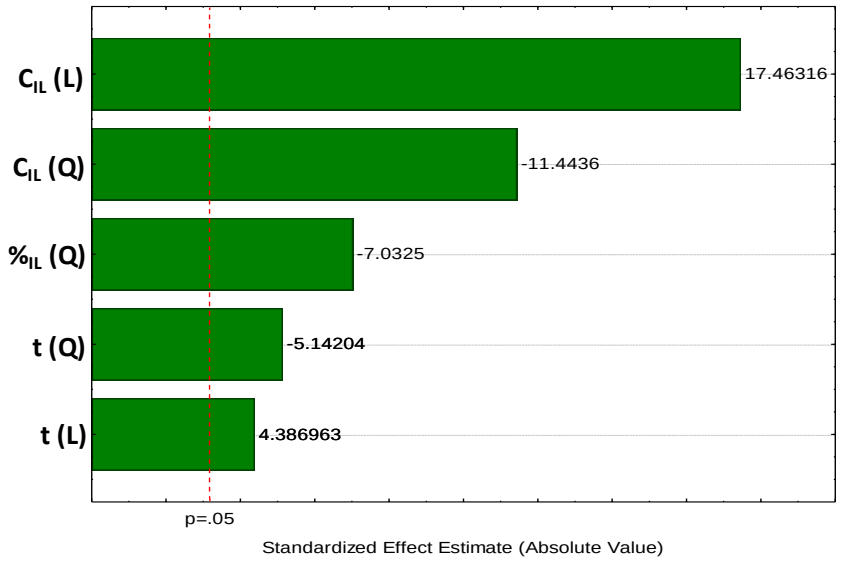


Figure D.7. Pareto Chart of the CCRD (2^4) regarding the yield of chlorophyll using systems with $[N_{1,1,1,10}]Br$.

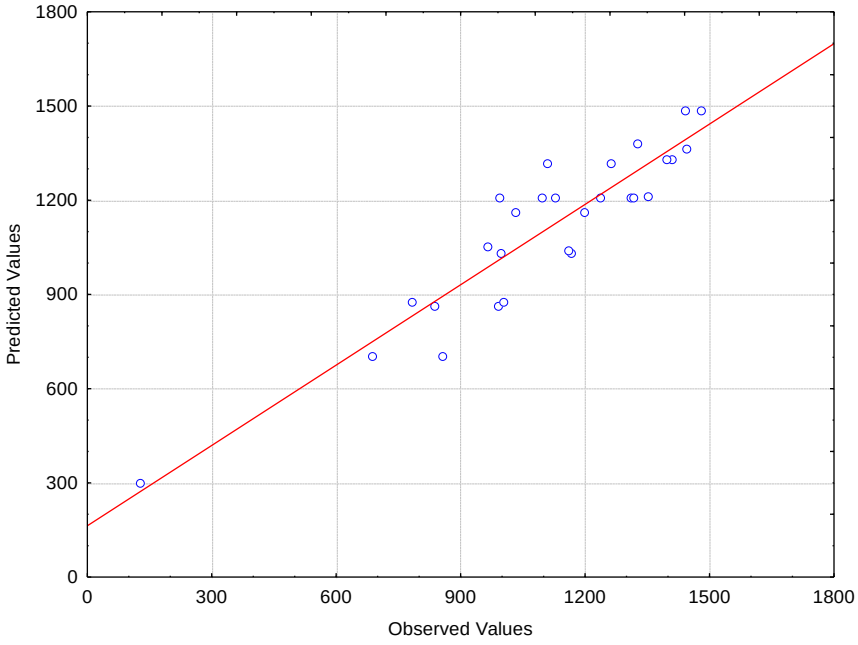


Figure D.8. Predicted *vs.* experimental values of the CCRD (2^4) regarding the yield of fucoxanthin ($\mu g_{fuco}.g_{dry\ biomass}^{-1}$) using systems with $[P_{4,4,4,14}]Cl$.

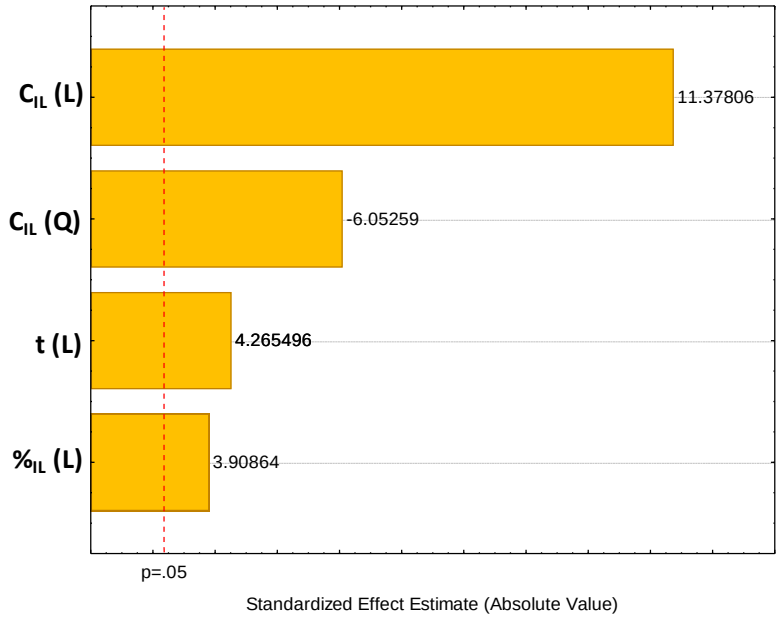


Figure D.9. Pareto Chart of the CCRD (2^4) regarding the yield of fucoxanthin using systems with $[P_{4,4,4,14}]Cl$.

Figure D.10. Photograph of the systems using as extractive conditions the central point (A) and the optimized point (B) considering the yield of extraction of fucoxanthin for systems using $[P_{4,4,4,14}]Cl$. (C) represents a system in the optimized point considering the yield of extraction of fucoxanthin however using water instead of aqueous solution of $[P_{4,4,4,14}]Cl$.

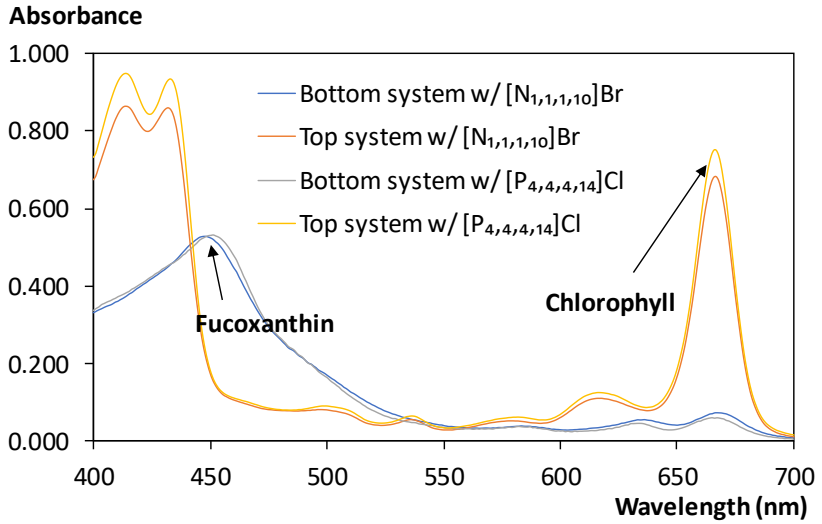


Figure D.11. UV-Vis spectra of the top and bottom phases for systems with $[N_{1,1,1,10}]Br$ and $[P_{4,4,4,14}]Cl$ in their respective best operational conditions from CCRD (2^4). Top (oil-rich phases) and bottom (IL-rich phases) were, respectively, analysed in a SHIMADZU UV-1700 PharmaSpec Spectrometer (using a dilution factor of 26) and in a Synergy HT microplate reader – BioTek (using a dilution factor of 4).

Table D.1. Real and coded values of the optimization process expressed by the yields of extraction fucoxanthin and chlorophylls extracted from *Saccharina latissima* by CCRD (2^4) using systems with $[N_{1,1,1,10}]Br$.

Run	$\%_{IL}$ (%)	C_{IL} (mM)	SLR ($g_{dry\ biomass}.mL_{solvent}^{-1}$)	t (min)	Yield of fucoxanthin ($\mu g_{fuco}.g_{dry\ biomass}^{-1}$)	Yield of chlorophyll ($mg_{chl}.g_{dry\ biomass}^{-1}$)
1	-1 (48)	-1 (150)	-1 (0.010)	-1 (20)	737.6	3.066
2	1 (72)	-1 (150)	-1 (0.010)	-1 (20)	1025.8	2.781
3	-1 (48)	1 (350)	-1 (0.010)	-1 (20)	1519.7	4.388
4	1 (72)	1 (350)	-1 (0.010)	-1 (20)	1634.6	3.774
5	-1 (48)	-1 (150)	1 (0.023)	-1 (20)	417.0	2.304
6	1 (72)	-1 (150)	1 (0.023)	-1 (20)	624.3	2.676
7	-1 (48)	1 (350)	1 (0.023)	-1 (20)	1401.1	4.175
8	1 (72)	1 (350)	1 (0.023)	-1 (20)	1546.4	4.027
9	-1 (48)	-1 (150)	-1 (0.010)	1 (40)	704.2	3.344

10	1 (72)	-1 (150)	-1 (0.010)	1 (40)	953.5	3.122
11	-1 (48)	1 (350)	-1 (0.010)	1 (40)	1471.5	4.654
12	1 (72)	1 (350)	-1 (0.010)	1 (40)	1634.6	4.306
13	-1 (48)	-1 (150)	1 (0.023)	1 (40)	405.1	2.818
14	1 (72)	-1 (150)	1 (0.023)	1 (40)	650.5	3.627
15	-1 (48)	1 (350)	1 (0.023)	1 (40)	1288.3	4.208
16	1 (72)	1 (350)	1 (0.023)	1 (40)	1464.2	4.255
17	0 (60)	0 (250)	0 (0.017)	0 (30)	1313.5	5.009
18	0 (60)	0 (250)	0 (0.017)	0 (30)	1276.0	4.486
19	0 (60)	0 (250)	0 (0.017)	0 (30)	1289.9	4.747
20	0 (60)	0 (250)	0 (0.017)	0 (30)	1276.0	4.558
21	-2 (36)	0 (250)	0 (0.017)	0 (30)	1174.2	2.846
22	2 (84)	0 (250)	0 (0.017)	0 (30)	1531.5	3.956
23	0 (60)	-2 (50)	0 (0.017)	0 (30)	133.4	0.103
24	0 (60)	2 (450)	0 (0.017)	0 (30)	1687.4	5.070
25	0 (60)	0 (250)	-2 (0.003)	0 (30)	1640.2	4.768
26	0 (60)	0 (250)	2 (0.030)	0 (30)	1073.4	4.594
27	0 (60)	0 (250)	0 (0.017)	-2 (10)	1125.9	3.281
28	0 (60)	0 (250)	0 (0.017)	2 (50)	1167.6	4.219

Table D.2. Real and coded values of the optimization process expressed by the yields of extraction fucoxanthin and chlorophyll extracted from *Saccharina latissima* by CCRD (2^4) using systems with $[P_{4,4,4,14}]Cl$.

Run	**$\%_{IL}$** (%)	**C_{IL}** (mM)	**SLR** ($g_{dry\ biomass}.mL_{solvent}^{-1}$)	**t** (min)	**Yield of fucoxanthin** ($\mu g_{fuco}.g_{dry\ biomass}^{-1}$)	**Yield of chlorophyll** ($mg_{chl}.g_{dry\ biomass}^{-1}$)
1	-1 (48)	-1 (150)	-1 (0.010)	-1 (20)	856.2	3.555
2	1 (72)	-1 (150)	-1 (0.010)	-1 (20)	989.7	2.709
3	-1 (48)	1 (350)	-1 (0.010)	-1 (20)	1032.3	3.199
4	1 (72)	1 (350)	-1 (0.010)	-1 (20)	1109.2	2.919
5	-1 (48)	-1 (150)	1 (0.023)	-1 (20)	687.8	3.323
6	1 (72)	-1 (150)	1 (0.023)	-1 (20)	837.6	2.856
7	-1 (48)	1 (350)	1 (0.023)	-1 (20)	1197.7	3.680
8	1 (72)	1 (350)	1 (0.023)	-1 (20)	1261.7	3.414

9	-1 (48)	-1 (150)	-1 (0.010)	1 (40)	1004.5	4.876
10	1 (72)	-1 (150)	-1 (0.010)	1 (40)	1167.6	3.505
11	-1 (48)	1 (350)	-1 (0.010)	1 (40)	1410.3	4.576
12	1 (72)	1 (350)	-1 (0.010)	1 (40)	1481.7	4.283
13	-1 (48)	-1 (150)	1 (0.023)	1 (40)	783.1	4.270
14	1 (72)	-1 (150)	1 (0.023)	1 (40)	997.2	4.040
15	-1 (48)	1 (350)	1 (0.023)	1 (40)	1397.9	4.670
16	1 (72)	1 (350)	1 (0.023)	1 (40)	1442.8	4.127
17	0 (60)	0 (250)	0 (0.017)	0 (30)	1130.0	3.420
18	0 (60)	0 (250)	0 (0.017)	0 (30)	1310.7	4.450
19	0 (60)	0 (250)	0 (0.017)	0 (30)	1237.1	4.235
20	0 (60)	0 (250)	0 (0.017)	0 (30)	1098.1	3.537
21	-2 (36)	0 (250)	0 (0.017)	0 (30)	964.9	3.905
22	2 (84)	0 (250)	0 (0.017)	0 (30)	1443.9	3.790
23	0 (60)	-2 (50)	0 (0.017)	0 (30)	126.5	2.876
24	0 (60)	2 (450)	0 (0.017)	0 (30)	1351.0	3.676
25	0 (60)	0 (250)	-2 (0.003)	0 (30)	993.8	3.076
26	0 (60)	0 (250)	2 (0.030)	0 (30)	1315.8	4.486
27	0 (60)	0 (250)	0 (0.017)	-2 (10)	1160.6	3.399
28	0 (60)	0 (250)	0 (0.017)	2 (50)	1327.4	4.870

Table D.3. Inputs of chemicals, water and electricity for the recovery of chlorophyll and fucoxanthin from 0.2 g of dry biomass of *Saccharina latissima*.

Input	**No IL reuse**	**IL reuse**
Seaweed grinding		
Electricity (W.h)	0.01	0.01
Extraction		
Sunflower oil (mL)	1.92	1.92
$[P_{4,4,4,14}]Cl$ (g)	1.54	0.28
Distilled water (mL)	8.60	8.60
Electricity (W.h)	11.96	11.96
Back-extraction		
Toluene (mL)	6.60	6.60
Electricity (W.h)	23.33	23.33

Table D.4. Price of the materials used in the process as well as market value of the products, phase proportions, and yields of extraction used for the calculation of production costs and subsequent calculations in economic analysis.

Item	**Price**	**Reference**
$[P_{4,4,4,14}]Cl$	409.3 $EU.kg^{-1}$	Ionic Liquid Technologies, Heilbronn, Germany
Water	1.09 $EU.L^{-1}$	Biosolve Process (Biopharm Services)
Sunflower oil	0.211324 $EU.L^{-1}$	Alibaba (as example)[259]
Toluene	10.3 $EU.L^{-1}$	Sigma-Aldrich (Cat No. 244511)
Chlorophyll (**Product Price ($\$_{prod}$)**)	63.86667 $EU.mg^{-1}$	Sigma-Aldrich (1116774)
Fucoxanthin (**Product Price ($\$_{prod}$)**)	12.16 $EU.mg^{-1}$	Sigma-Aldrich (F6923)
Mixture for extraction		
Phase	**Proportion**	
IL phase (aq.)	0.841667	
Oil phase	0.158333	
Yield of extraction (C_{prod})		
Chlorophyll	4.93 $mg_{chl}.g_{dry\ biomass}^{-1}$	
Fucoxanthin	1956 $\mu g_{fuco}.g_{dry\ biomass}^{-1}$	

Table D.5. Critical micellar concentration (CMC) values of the screened tensioactive compounds.

Tensioactive compound	**$CMC^{[Ref]}$ (mM)**
$[C_{10}C_1im]Cl$	55[254]
$[C_{12}C_1im]Cl$	15[254]
$[C_{14}C_1im]Cl$	4[254]
$[N_{1,1,1,10}]Cl$	70[254]
$[N_{1,1,1,10}]Br$	25.20[255]
$[N_{1,1,1,12}]Br$	15.6[256]
$[N_{1,1,1,14}]Br$	3.8[256]
$[P_{4,4,4,14}]Cl$	4.69[255]

Table D.6. Predicted results compared to the experimental values (real) obtained by the fitted model and the respective relative deviation (%) from the independent variables fixed at the optimum conditions for the yield of extraction of fucoxanthin using systems with $[N_{1,1,1,10}]Br$. V1, V2, and V3 represent the validation assays.

Assay	$\%_{IL}$ (%)	C_{IL} (mM)	SLR ($g_{dry\ biomass} \cdot mL_{solvent}^{-1}$)	t (min)	Yield of extraction of fucoxanthin ($\mu g_{fuco} \cdot g_{dry\ biomass}^{-1}$)		Relative deviation (%)
					Experimental values	Predicted values	
V1	84	400	0.017	30	1837.6	1876.0	-2.09
V2					1781.5		-5.30
V3					1889.9		0.73
						Mean of deviation	-2.21

Table D.7. Predicted results compared to the experimental values (real) obtained by the fitted model and the respective relative deviation (%) from the independent variables fixed at the optimum conditions for the yield of extraction of chlorophyll using systems with $[N_{1,1,1,10}]Br$. V1, V2, and V3 represent the validation assays.

Assay	$\%_{IL}$ (%)	C_{IL} (mM)	SLR ($g_{dry\ biomass} \cdot mL_{solvent}^{-1}$)	t (min)	Yield of extraction of chlorophyll ($mg_{chl} \cdot g_{dry\ biomass}^{-1}$)		Relative deviation (%)
					Experimental values	Predicted values	
V1	84	400	0.017	30	4.54	4.38	3.52
V2					4.46		1.79
V3					4.61		4.98
						Mean of deviation	3.44

Table D.8. Predicted results compared to the experimental values (real) obtained by the fitted model and the respective relative deviation (%) from the independent variables fixed at the optimum conditions for the yield of extraction of fucoxanthin using systems with $[P_{4,4,4,14}]Cl$. V1, V2, and V3 represent the validation assays.

Assay	**$\%_{IL}$** (%)	**C_{IL}** (mM)	**SLR** ($g_{dry\ biomass} \cdot mL_{solvent}^{-1}$)	**t** (min)	**Yield of extraction of fucoxanthin ($\mu g_{fuco} \cdot g_{dry\ biomass}^{-1}$)**		**Relative deviation (%)**
					Experimental values	**Predicted values**	
V1	84	350	0.017	40	1903.1	1937.7	-1.81
V2					1910.9		-1.40
V3					2053.0		5.62
Mean of deviation							-1.61

Table D.9. Production costs *per* mg of each pigment considering all recycling scenarios. Chl and Fuco stand for chlorophyll and fucoxanthin, respectively.

Recycling IL without H_2O												
	IL Recycled		**IL Recycled**		**IL Recycled**		**IL Recycled**		**IL Recycled**		**IL Recycled**	
	0%	**0%**	**20%**	**100%**	**40%**	**100%**	**60%**	**100%**	**80%**	**100%**	**100%**	**100%**
CoG.mg^{-1}	**Chl**	**Fuco**	**Chl**	**Fuco**	**Chl**	**Fuco**	**Chl**	**Fuco**	**Chl**	**Fuco**	**Chl**	**Fuco**
Capital	1.2E+00	2.9E-03	9.6E-01	2.4E-03	7.5E-01	1.9E-03	5.4E-01	1.4E-03	3.4E-01	8.5E-04	1.3E-01	3.3E-04
Material	7.2E-01	1.8E-03	5.9E-01	1.5E-03	4.7E-01	1.2E-03	3.4E-01	8.5E-04	2.1E-01	5.3E-04	8.2E-02	2.1E-04
Labor	3.5E-01	8.8E-04	2.9E-01	7.2E-04	2.3E-01	5.7E-04	1.6E-01	4.1E-04	1.0E-01	2.6E-04	4.0E-02	1.0E-04
Other	9.3E-02	2.3E-04	7.7E-02	1.9E-04	6.0E-02	1.5E-04	4.4E-02	1.1E-04	2.7E-02	6.8E-05	1.1E-02	2.7E-05
TOTAL	2.3E+00	5.9E-03	1.9E+00	4.8E-03	1.5E+00	3.8E-03	1.1E+00	2.7E-03	6.8E-01	1.7E-03	2.6E-01	6.7E-04
Recycling IL with H_2O												
	IL Recycled	**H_2O Recycled**	**IL Recycled**	**H_2O Recycled**	**IL Recycled**	**H_2O Recycled**	**IL Recycled**	**H_2O Recycled**	**IL Recycled**	**H_2O Recycled**	**IL Recycled**	**H_2O Recycled**
	0%	**0%**	**20%**	**100%**	**40%**	**100%**	**60%**	**100%**	**80%**	**100%**	**100%**	**100%**
CoG.mg^{-1}	**Chl**	**Fuco**	**Chl**	**Fuco**	**Chl**	**Fuco**	**Chl**	**Fuco**	**Chl**	**Fuco**	**Chl**	**Fuco**
Capital	1.2E+00	2.9E-03	9.4E-01	2.4E-03	7.3E-01	1.8E-03	5.3E-01	1.3E-03	3.2E-01	8.1E-04	1.1E-01	2.9E-04
Material	7.2E-01	1.8E-03	5.8E-01	1.5E-03	4.5E-01	1.1E-03	3.3E-01	8.2E-04	2.0E-01	5.0E-04	7.1E-02	1.8E-04
Labor	3.5E-01	8.8E-04	2.8E-01	7.1E-04	2.2E-01	5.5E-04	1.6E-01	4.0E-04	9.6E-02	2.4E-04	3.4E-02	8.6E-05
Other	9.3E-02	2.3E-04	7.5E-02	1.9E-04	5.9E-02	1.5E-04	4.2E-02	1.1E-04	2.6E-02	6.5E-05	9.1E-03	2.3E-05
TOTAL	2.3E+00	5.9E-03	1.9E+00	4.7E-03	1.5E+00	3.7E-03	1.1E+00	2.7E-03	6.4E-01	1.6E-03	2.3E-01	5.7E-04
Recycling only toluene												
	Toluene Recycled		**Toluene Recycled**		**Toluene Recycled**		**Toluene Recycled**		**Toluene Recycled**		**Toluene Recycled**	

	0%	0%	20%	100%	40%	100%	60%	100%	80%	100%	100%	100%
CoG.mg^{-1}	**Chl**	**Fuco**	**Chl**	**Fuco**	**Chl**	**Fuco**	**Chl**	**Fuco**	**Chl**	**Fuco**	**Chl**	**Fuco**
Capital	1.2E+00	2.9E-03	1.1E+00	2.9E-03	1.1E+00	2.8E-03	1.1E+00	2.8E-03	1.1E+00	2.7E-03	1.0E+00	2.6E-03
Material	7.2E-01	1.8E-03	7.1E-01	1.8E-03	6.9E-01	1.7E-03	6.8E-01	1.7E-03	6.6E-01	1.7E-03	6.5E-01	1.6E-03
Labor	3.5E-01	8.8E-04	3.4E-01	8.6E-04	3.4E-01	8.5E-04	3.3E-01	8.3E-04	3.2E-01	8.1E-04	3.1E-01	7.9E-04
Other	9.3E-02	2.3E-04	9.1E-02	2.3E-04	8.9E-02	2.3E-04	8.8E-02	2.2E-04	8.6E-02	2.2E-04	8.4E-02	2.1E-04
TOTAL	2.3E+00	5.9E-03	2.3E+00	5.7E-03	2.2E+00	5.6E-03	2.2E+00	5.5E-03	2.1E+00	5.4E-03	2.1E+00	5.3E-03
Recycling everything												
	IL & Toluene Recycled	**H_2O Recycled**	**IL & Toluene Recycled**	**H_2O Recycled**	**IL & Toluene Recycled**	**H_2O Recycled**	**IL & Toluene Recycled**	**H_2O Recycled**	**IL & Toluene Recycled**	**H_2O Recycled**	**IL & Toluene Recycled**	**H_2O Recycled**
	0%	**0%**	**20%**	**100%**	**40%**	**100%**	**60%**	**100%**	**80%**	**100%**	**100%**	**100%**
CoG.mg^{-1}	**Chl**	**Fuco**	**Chl**	**Fuco**	**Chl**	**Fuco**	**Chl**	**Fuco**	**Chl**	**Fuco**	**Chl**	**Fuco**
Capital	1.2E+00	2.9E-03	9.2E-01	2.3E-03	6.9E-01	1.7E-03	4.6E-01	1.2E-03	2.3E-01	5.8E-04	5.3E-04	1.3E-06
Material	7.2E-01	1.8E-03	5.7E-01	1.4E-03	4.3E-01	1.1E-03	2.8E-01	7.2E-04	1.4E-01	3.6E-04	3.3E-04	8.3E-07
Labor	3.5E-01	8.8E-04	2.7E-01	6.9E-04	2.1E-01	5.2E-04	1.4E-01	3.5E-04	6.9E-02	1.7E-04	1.6E-04	4.0E-07
Other	9.3E-02	2.3E-04	7.3E-02	1.8E-04	5.5E-02	1.4E-04	3.7E-02	9.2E-05	1.8E-02	4.6E-05	4.3E-05	1.1E-07
TOTAL	2.3E+00	5.9E-03	1.8E+00	4.6E-03	1.4E+00	3.5E-03	9.2E-01	2.3E-03	4.6E-01	1.2E-03	1.1E-03	2.7E-06

Table D.10. Return analysis of chlorophyll depending on the recycling percentage of all materials. C_{prod} (yield of extraction) and $\$_{biom}$ (cost of obtaining the biomass) was fixed at 4.93 $mg_{chl}.g_{dry\ biomass}^{-1}$ and 0 $EU.g_{dry\ biomass}^{-1}$, respectively. $\$_{prod}$ (market price) of chlorophyll is based on Sigma-Aldrich (1116774) being also considered prices 10 and 100-fold lower as well. α is a multiplier of production costs *per* g of dry biomass representing an increase and decrease by 10-fold, beside the base scenario.

	Recycling percentage	**$\$_{prod}$** ($EU.mg_{chl}^{-1}$)	***α***	**Production costs** ($EU.g_{dry\ biomass}^{-1}$)	**Return** ($EU.g_{dry\ biomass}^{-1}$)
No recycling	0 %	0.64	0.1	11.468	2.008
	0 %	6.4	0.1	11.468	30.405
	0 %	64	0.1	11.468	314.373
	0 %	0.64	1	11.468	-8.312
	0 %	6.4	1	11.468	20.084
	0 %	64	1	11.468	304.052
	0 %	0.64	10	11.468	-111.520
	0 %	6.4	10	11.468	-83.124
	0 %	64	10	11.468	200.844
Recycling	20 %	0.64	0.1	9.033	2.252
	20 %	6.4	0.1	9.033	30.649
	20 %	64	0.1	9.033	314.617
	20 %	0.64	1	9.033	-5.878
	20 %	6.4	1	9.033	22.519
	20 %	64	1	9.033	306.487
	20 %	0.64	10	9.033	-87.178
	20 %	6.4	10	9.033	-58.781
	20 %	64	10	9.033	225.187
	40 %	0.64	0.1	6.776	2.478
	40 %	6.4	0.1	6.776	30.874
	40 %	64	0.1	6.776	314.842
	40 %	0.64	1	6.776	-3.621
	40 %	6.4	1	6.776	24.776
	40 %	64	1	6.776	308.744
	40 %	0.64	10	6.776	-64.608
	40 %	6.4	10	6.776	-36.211
	40 %	64	10	6.776	247.757
	60 %	0.64	0.1	4.519	2.703
	60 %	6.4	0.1	4.519	31.100
	60 %	64	0.1	4.519	315.068

	60 %	0.64	1	4.519	-1.364
	60 %	6.4	1	4.519	27.033
	60 %	64	1	4.519	311.001
	60 %	0.64	10	4.519	-42.038
	60 %	6.4	10	4.519	-13.641
	60 %	64	10	4.519	270.327
	80 %	0.64	0.1	2.262	2.929
	80 %	6.4	0.1	2.262	31.326
	80 %	64	0.1	2.262	315.294
	80 %	0.64	1	2.262	0.893
	80 %	6.4	1	2.262	29.290
	80 %	64	1	2.262	313.258
	80 %	0.64	10	2.262	-19.468
	80 %	6.4	10	2.262	8.929
	80 %	64	10	2.262	292.897
	100 %	0.64	0.1	0.005	3.155
	100 %	6.4	0.1	0.005	31.551
	100 %	64	0.1	0.005	315.519
	100 %	0.64	1	0.005	3.150
	100 %	6.4	1	0.005	31.547
	100 %	64	1	0.005	315.515
	100 %	0.64	10	0.005	3.103
	100 %	6.4	10	0.005	31.499
	100 %	64	10	0.005	315.467

Table D.11. Return analysis of fucoxanthin depending on the recycling percentage of all materials. C_{prod} (yield of extraction) and $\$_{biom}$ (cost of obtaining the biomass) was fixed at 1.96 $mg_{fuco}.g_{dry\ biomass}^{-1}$ and 0 $EU.g_{dry\ biomass}^{-1}$, respectively. $\$_{prod}$ (market price) of fucoxanthin is based on Sigma-Aldrich (F6923) being also considered prices 10 and 100-fold lower as well. α is a multiplier of production costs *per* g of dry biomass representing an increase and decrease by 10-fold, beside the base scenario.

	Recycling percentage	**$\$_{prod}$** ($EU.mg_{fuco}^{-1}$)	***α***	**Production costs** ($EU.g_{dry\ biomass}^{-1}$)	**Return** ($EU.g_{dry\ biomass}^{-1}$)
No recycling	0 %	0.1216	0.1	11.468	-0.909
	0 %	1.216	0.1	11.468	1.232
	0 %	12.16	0.1	11.468	22.638
	0 %	0.1216	1	11.468	-11.230

	0 %	1.216	1	11.468	-9.089
	0 %	12.16	1	11.468	12.317
	0 %	0.1216	10	11.468	-114.438
	0 %	1.216	10	11.468	-112.297
	0 %	12.16	10	11.468	-90.891
Recycling	20 %	0.1216	0.1	9.033	-0.665
	20 %	1.216	0.1	9.033	1.475
	20 %	12.16	0.1	9.033	22.882
	20 %	0.1216	1	9.033	-8.795
	20 %	1.216	1	9.033	-6.655
	20 %	12.16	1	9.033	14.752
	20 %	0.1216	10	9.033	-90.095
	20 %	1.216	10	9.033	-87.955
	20 %	12.16	10	9.033	-66.548
	40 %	0.1216	0.1	6.776	-0.440
	40 %	1.216	0.1	6.776	1.701
	40 %	12.16	0.1	6.776	23.107
	40 %	0.1216	1	6.776	-6.538
	40 %	1.216	1	6.776	-4.398
	40 %	12.16	1	6.776	17.009
	40 %	0.1216	10	6.776	-67.525
	40 %	1.216	10	6.776	-65.385
	40 %	12.16	10	6.776	-43.978
	60 %	0.1216	0.1	4.519	-0.214
	60 %	1.216	0.1	4.519	1.927
	60 %	12.16	0.1	4.519	23.333
	60 %	0.1216	1	4.519	-4.281
	60 %	1.216	1	4.519	-2.141
	60 %	12.16	1	4.519	19.266
	60 %	0.1216	10	4.519	-44.955
	60 %	1.216	10	4.519	-42.814
	60 %	12.16	10	4.519	-21.408
	80 %	0.1216	0.1	2.262	0.012
	80 %	1.216	0.1	2.262	2.152
	80 %	12.16	0.1	2.262	23.559
	80 %	0.1216	1	2.262	-2.024
	80 %	1.216	1	2.262	0.116
	80 %	12.16	1	2.262	21.523
	80 %	0.1216	10	2.262	-22.385

	80 %	1.216	10	2.262	-20.244
	80 %	12.16	10	2.262	1.162
	100 %	0.1216	0.1	0.005	0.237
	100 %	1.216	0.1	0.005	2.378
	100 %	12.16	0.1	0.005	23.784
	100 %	0.1216	1	0.005	0.233
	100 %	1.216	1	0.005	2.373
	100 %	12.16	1	0.005	23.780
	100 %	0.1216	10	0.005	0.185
	100 %	1.216	10	0.005	2.326
	100 %	12.16	10	0.005	23.732

Appendix E

Ionic liquids as eluents in solid-phase extraction to purify pigments recovered from *Isochrysis galbana*

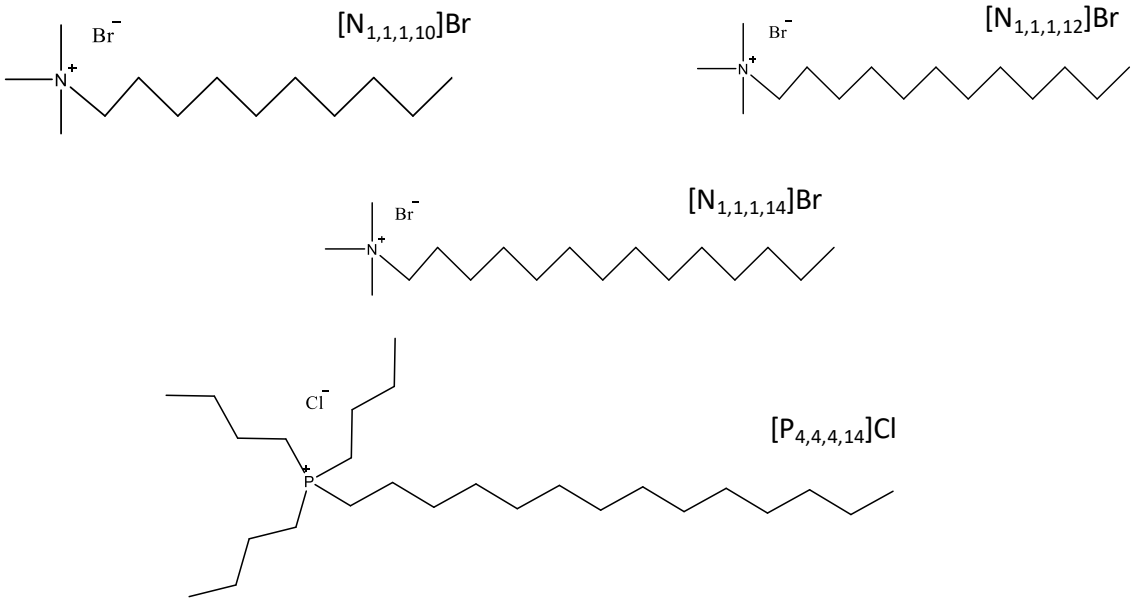


Figure E.1. Molecular structure and respective abbreviation of the ILs screened in this work.

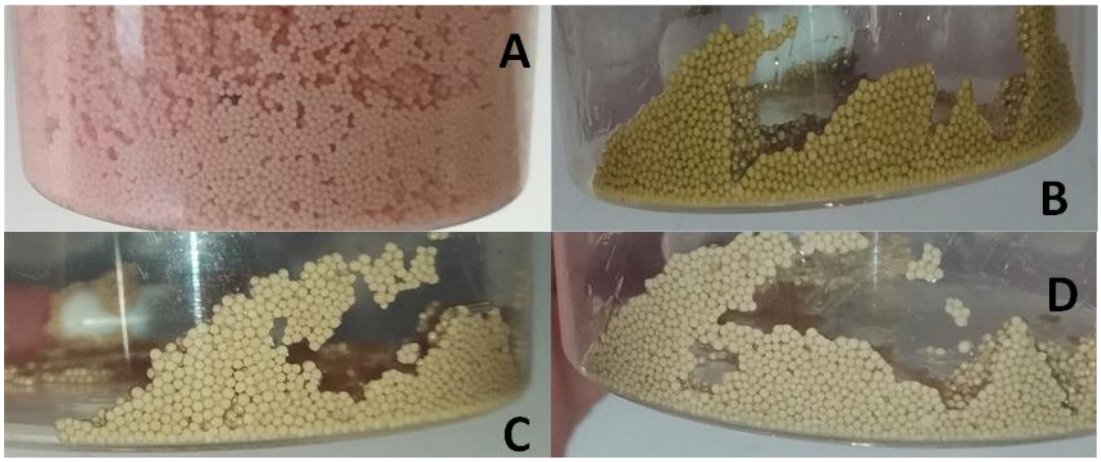
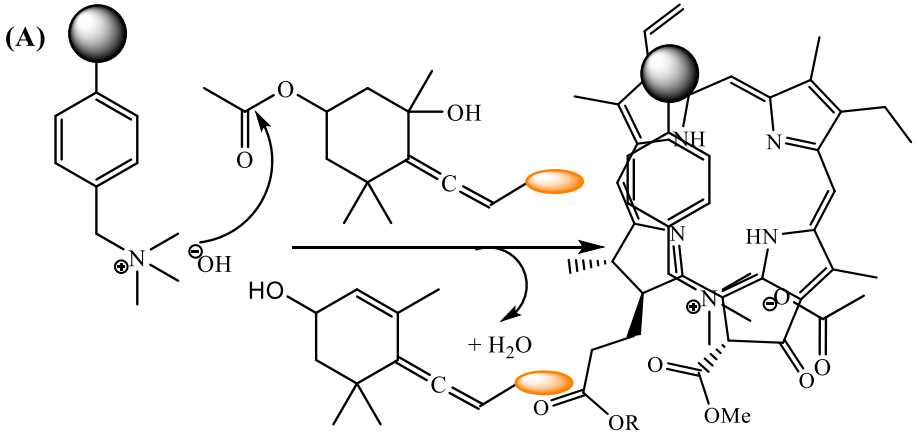
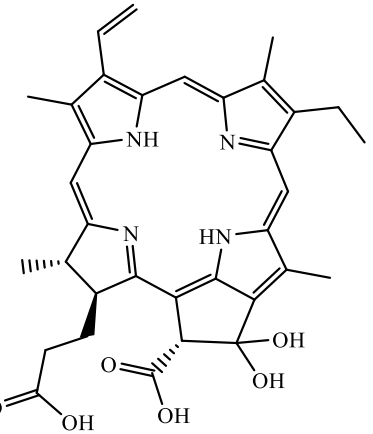


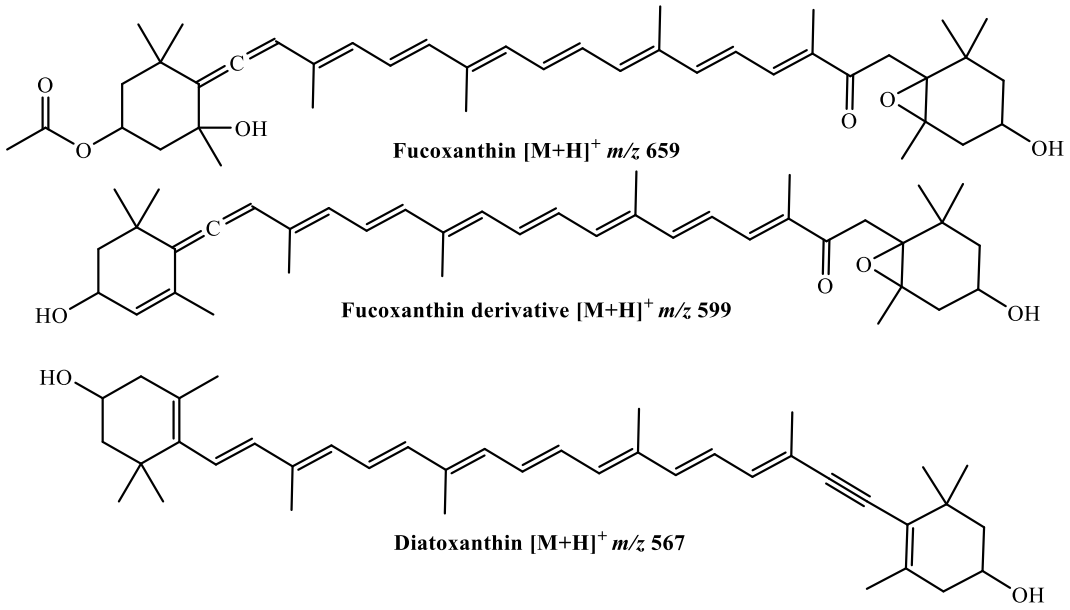
Figure E.2. Photographs of the resin AmberLite™ HPR900 OH: (A) resin before usage; (B) resin after chlorophyll adsorption and collection of the carotenoid extract; (C) resin after elution with aqueous solution of $[N_{1,1,1,12}]Br$ using the optimized conditions of elution by CCRD; and (D) resin after regeneration with solution of NaOH. These photographs are related to the assays performed in batch regime.



1) Pheophorbide *a* (R=H) $[M+H]^+$ *m/z* 593
2) Pheophytin *a* (R=Phytyl) $[M+H]^+$ *m/z* 871



Pheophorbide derivative $[M+H]^+$ *m/z* 597



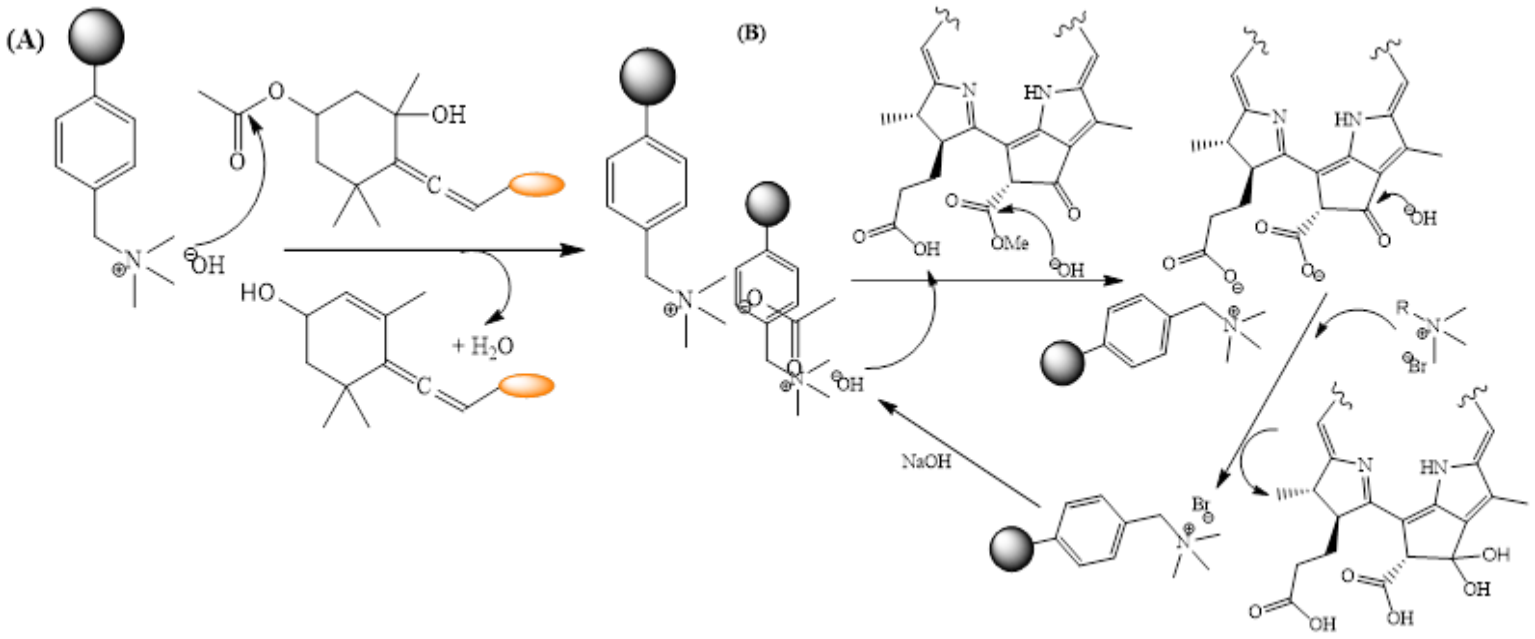


Figure E.3. Compounds chemical structures and proposed reactions between fucoxanthin (A) and pheophorbide (B) with the strong basic resin.

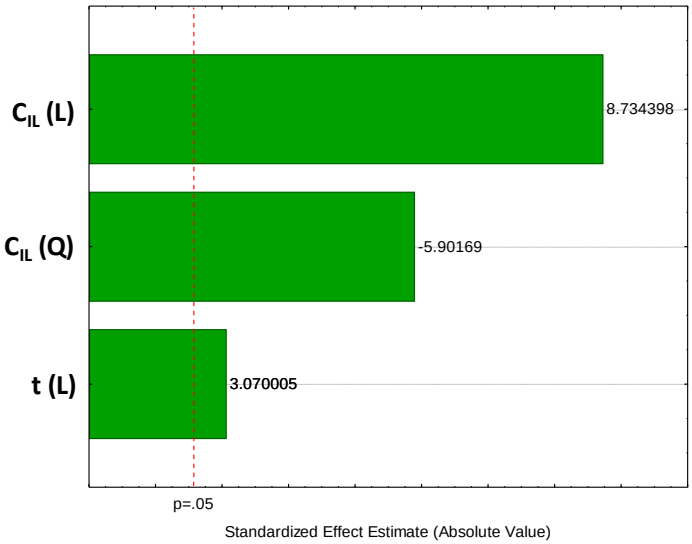


Figure E.4. Pareto Chart of the CCRD (2^3) regarding the chlorophyll recovery from the resin (%) using aqueous solutions of $[P_{4,4,4,14}]Cl$.

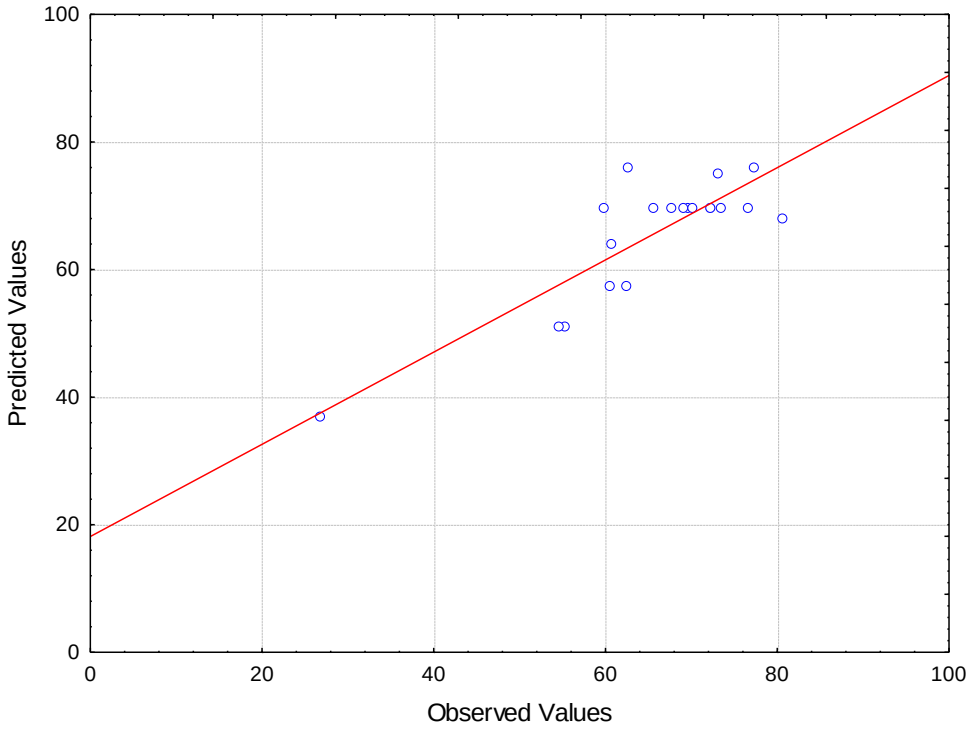


Figure E.5. Predicted *vs.* experimental values of the CCRD (2^3) regarding the chlorophyll elution from the resin (%) using aqueous solutions of $[P_{4,4,4,14}]Cl$.

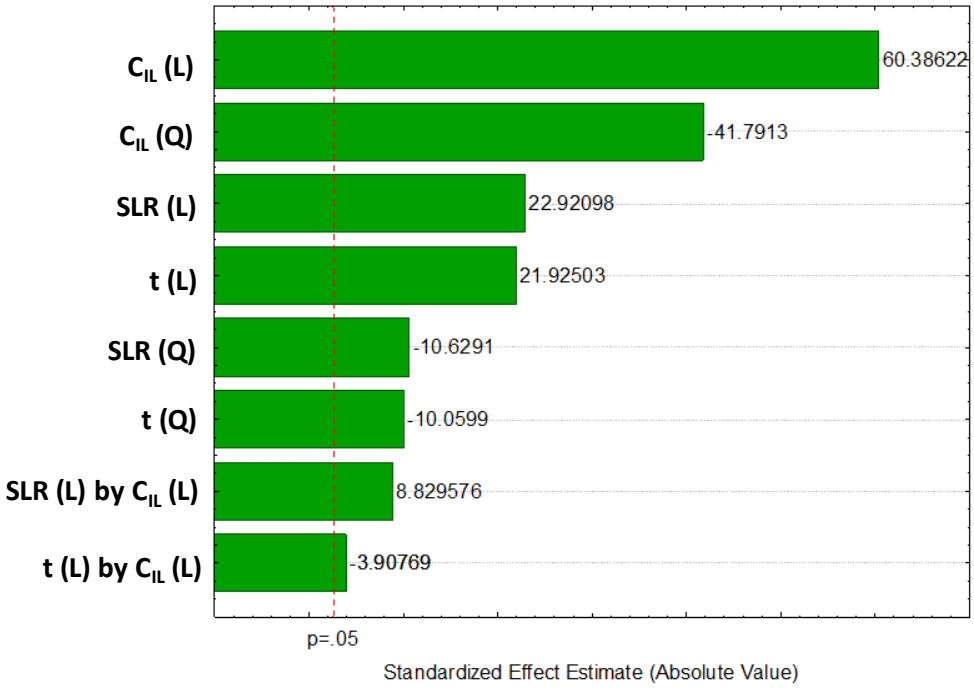


Figure E.6. Pareto Chart of the CCRD (2^3) regarding the chlorophyll recovery from the resin (%) using aqueous solutions of $[N_{1,1,1,12}]Br$.

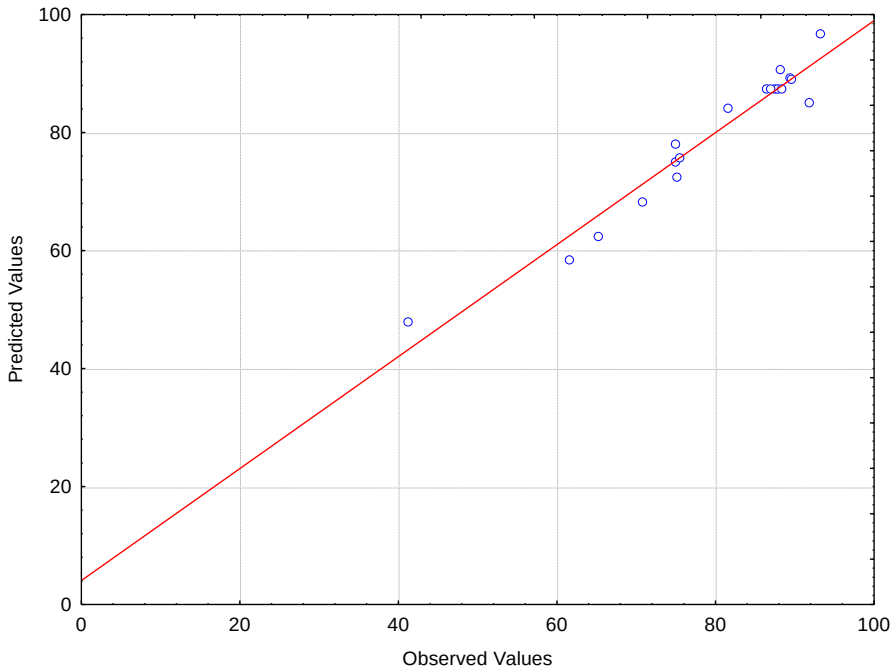


Figure E.7. Predicted *vs.* experimental values of the CCRD regarding the chlorophyll recovery from the resin (%) using aqueous solutions of $[N_{1,1,1,12}]Br$.

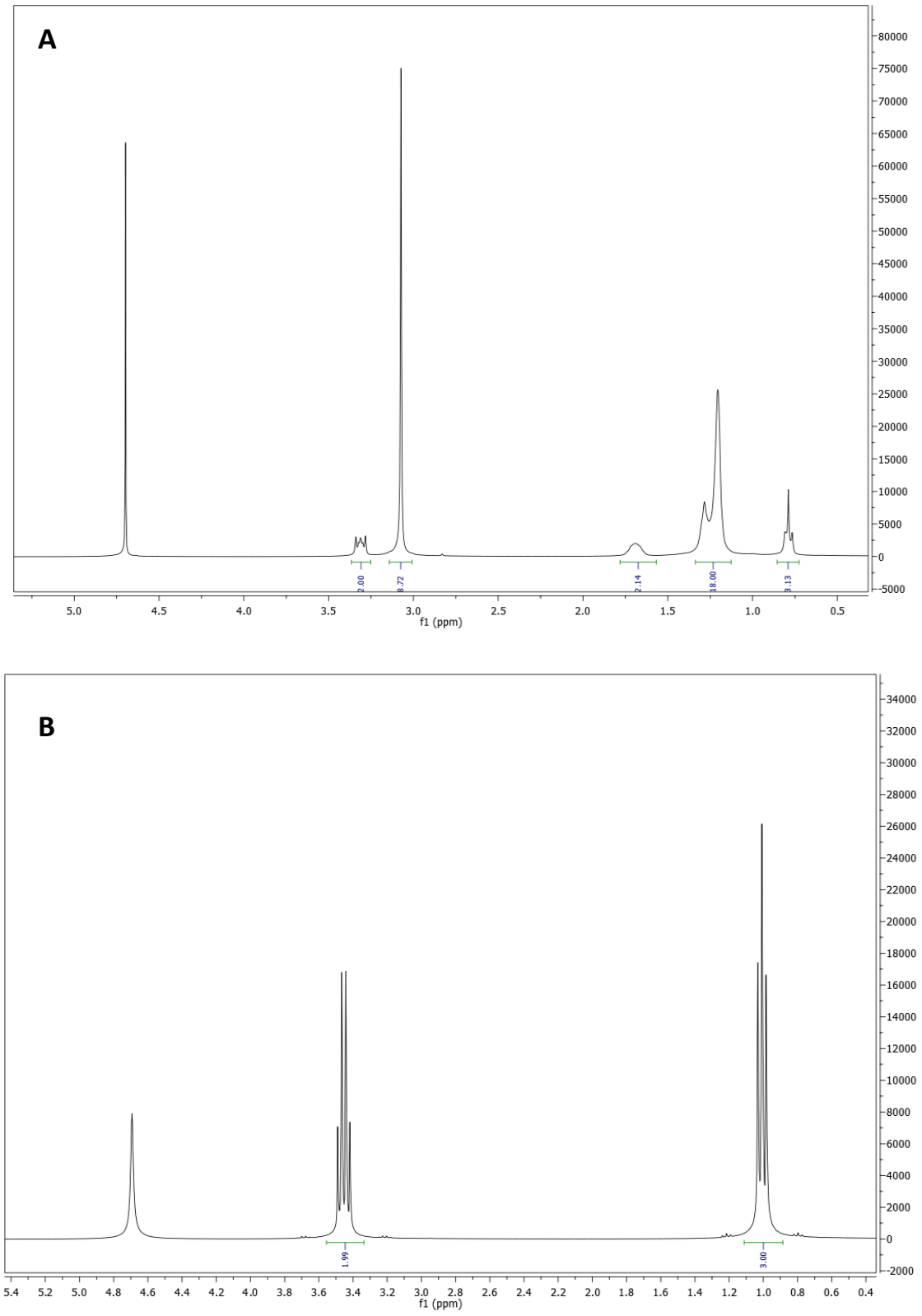


Figure E.8. ^{1}H NMR spectroscopy of (A) pure $[N_{1,1,1,12}]Br$ and (B) ethanolic fraction rich in chlorophylls (after the polishing step) dissolved in D_2O.

Table E.1. Critical Micellar Concentration (CMC) of tensioactive ionic liquids used to elute the chlorophylls.

Tensioactive compound	CMC[Ref] (mM)
$[P_{4,4,4,14}]Cl$	4.69[255]
$[N_{1,1,1,10}]Br$	25.20[255]
$[N_{1,1,1,12}]Br$	15.6[256]
$[N_{1,1,1,14}]Br$	3.8[256]

Table E.2. Real values used in the optimization process by CCRD (2^3) expressed by the chlorophyll recovery using aqueous solutions of $[P_{4,4,4,14}]Cl$ and $[N_{1,1,1,12}]Br$. In the SLR study, the mass of resin was the variable changed, using always 5 mL of ethanolic initial solution in the adsorption step, 15 mL of the eluent, and 5 mL of the regeneration solution (NaOH, 4 % (w:v)).

Run	SLR (g_{resin}. mL_{eluent}^{-1})	t (min)	C_{IL} (mM)	Chlorophyll recovery (%) using $[P_{4,4,4,14}]Cl$	Chlorophyll recovery (%) using $[N_{1,1,1,12}]Br$
1	0.030	19.0	130.0	55.3	61.6
2	0.070	19.0	130.0	54.6	65.1
3	0.030	41.0	130.0	60.5	70.7
4	0.070	41.0	130.0	62.4	75.1
5	0.030	19.0	370.0	59.7	75.0
6	0.070	19.0	370.0	69.5	88.1
7	0.030	41.0	370.0	62.7	81.6
8	0.070	41.0	370.0	77.3	93.3
9	0.016	30.0	250.0	70.2	75.0
10	0.084	30.0	250.0	73.4	89.4
11	0.050	11.5	250.0	60.7	75.4
12	0.050	48.5	250.0	73.0	89.6
13	0.050	30.0	48.4	26.8	41.3
14	0.050	30.0	451.6	80.5	91.9
15	0.050	30.0	250.0	72.2	87.9
16	0.050	30.0	250.0	67.6	86.4
17	0.050	30.0	250.0	65.5	87.4

18	0.050	30.0	250.0	69.1	87.9
19	0.050	30.0	250.0	72.2	88.3
20	0.050	30.0	250.0	76.5	87.0

Table E.3. Effect of the estimates for chlorophyll recovery optimized by the CCRD (2^3) using aqueous solutions of $[P_{4,4,4,14}]Cl$. Significant factors at the 95 % confidence level.

Factor	Effect	Standard error	Calculated *t* *	p-value
Mean/Interaction	70.55	1.60	62.05	≤ 0.001
t (min) - (X2)	6.52	2.12	3.07	0.027
C_{IL} (mM) – (X3)	18.55	2.12	8.73	0.000
C_{IL} (mM) – ($X3^2$)	-12.33	2.07	-5.96	≤ 0.001

*Degrees of freedom.

Table E.4. Predicted *vs.* experimental values (real) obtained by the fitted model and the respective relative deviation (%) from the independent variables fixed at the optimum operational conditions using aqueous solutions of $[P_{4,4,4,14}]Cl$. V1, V2, and V3 represent the validation assays.

Assay	t (min)	C_{IL} (mM)	Chlorophyll recovery (%) using $[P_{4,4,4,14}]Cl$		Relative deviation (%)
			Experimental values	Predicted values	
	X2	X3	Y	Predicted Y	
V1	48.48	370	81.0	78.4	3.3
V2			80.8		3.0
V3			76.9		1.9
				Mean of deviation	2.7

Table E.5. Effect of the estimates for chlorophyll recovery optimized by the CCRD (2^3) using aqueous solutions of $[N_{1,1,1,12}]Br$. Significant factors at the 95 % confidence level.

Factor	Effect	Standard error	Calculated *t* *	p-value
Mean/Interaction	87.47	0.27	319.75	0.000
SLR - (X1)	8.33	0.36	23.04	0.000
SLR – ($X1^2$)	-3.70	0.35	-10.67	0.000
t (min) - (X2)	7.99	0.36	22.03	0.000

t (min) - ($X2^2$)	-3.59	0.35	10.15	0.000
C_{IL} (mM) – (X3)	22.04	0.36	60.69	0.000
C_{IL} (mM) – ($X3^2$)	-14.88	0.35	-42.04	0.000
X1 by X2	4.21	0.47	8.87	0.000
X2 by X3	-1.86	0.47	-3.92	0.011

Table E.6. Predicted *vs.* experimental values (real) obtained by the fitted model and the respective relative deviation (%) from the independent variables fixed at the optimum operational conditions using aqueous solutions of $[N_{1,1,1,12}]Br$. V1, V2, and V3 represent the validation assays.

Assay	**SLR** (g_{resin}. mL_{eluent}^{-1})	**t** (min)	**C_{IL}** (mM)	**Chlorophyll recovery (%) using $[N_{1,1,1,12}]Br$**		**Relative deviation (%)**
				Experimental values	**Predicted values**	
	X1	**X2**	**X3**	**Y**	**Predicted Y**	
V1	0.070	48.5	370	96.7	100	-3.3
V2				98.1		-1.9
V3				96.3		-3.7
					Mean of deviation	-3.0

Appendix F

Sustainable strategy based on induced precipitation for the purification of phycobiliproteins

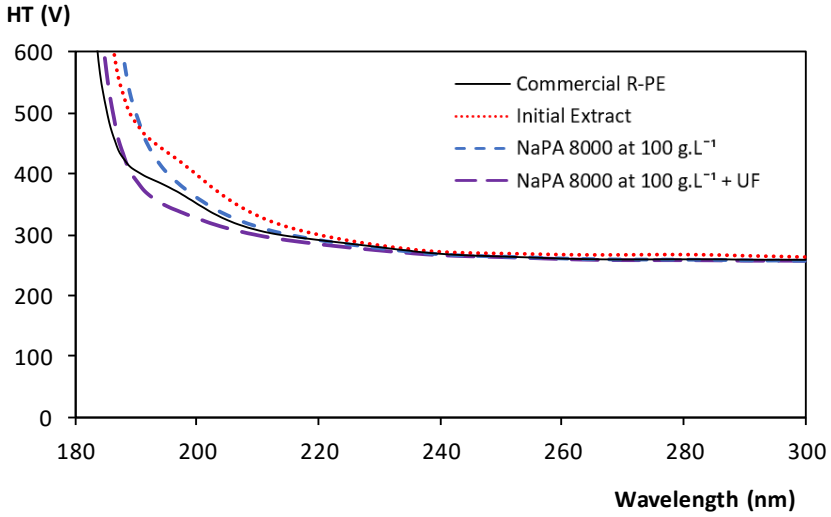


Figure F.1. High-tension voltage graph of the circular dichroism spectra displayed regarding the initial extract (dotted line), resuspended pellet after precipitation using NaPA 8000 at 100 $g.L^{-1}$ (smaller dashed line), and resuspended pellet after precipitation using NaPA 8000 at 100 $g.L^{-1}$ followed by an UF step (larger dashed line), and commercial R-PE from Sigma-Aldrich (solid line).

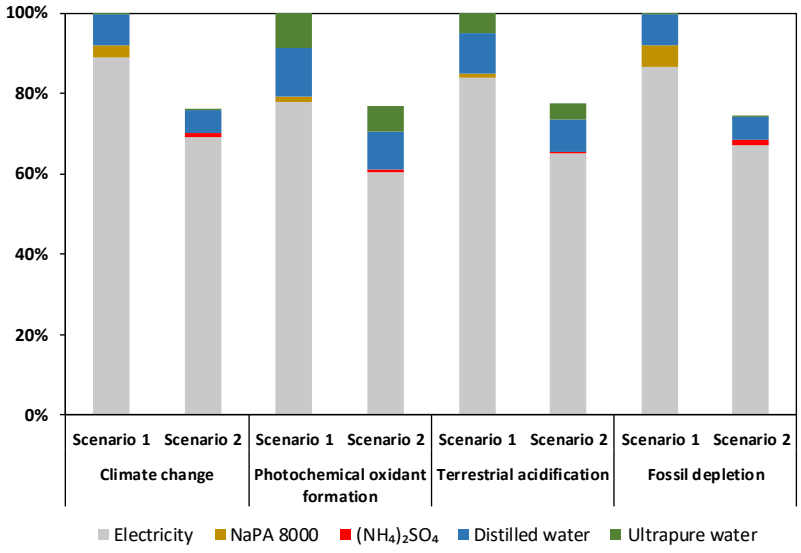


Figure F.2. Relative contribution of the inputs for the life cycle assessment results. Scenario 1 with NaPA 8000 and Scenario 2 with $(NH_4)_2SO_4$.

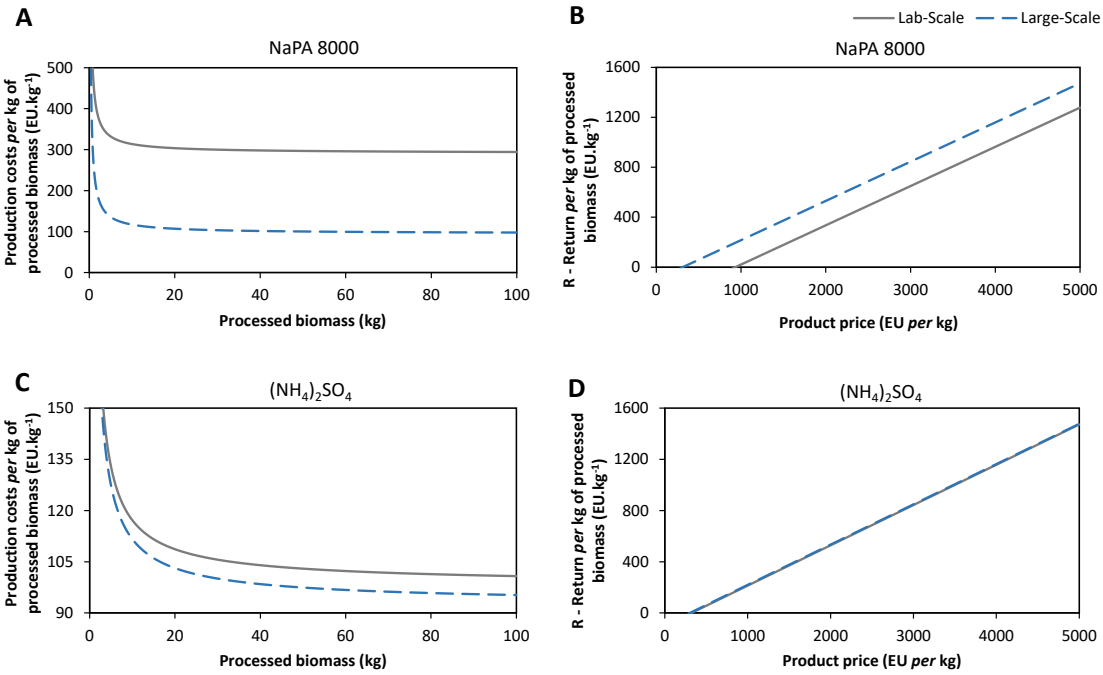


Figure F.3. Impact on the production cost of using laboratory and large-scale prices for the calculation of the CoG.kg^{-1} of biomass and the *Return*. Results for NaPA 8000 are presented in (A) and (B) for the CoG.kg^{-1} of biomass and *Return*, respectively. Results for $(NH_4)_2SO_4$ are presented in (C) and (D) for the CoG.kg^{-1} of biomass and Return, respectively. Solid lines are for laboratory prices and dashed lines for large-scale prices. Results presented in (D) show two lines that are almost overlapped, denoting the reduced impact on the prices of the materials in the Return for the case of $(NH_4)_2SO_4$.

Table F.1. Inputs of chemicals, water and electricity for obtaining 1 mg of R-PE in both scenarios under study. Scenario 1 using NaPA 8000 and Scenario 2 using $(NH_4)_2SO_4$ as precipitating agents.

	Scenario 1	**Scenario 2**
Inputs: Solid-liquid extraction		
Distilled water (mL)	1.39	1.10
Electricity (W.h)	2.91	2.31
Inputs: Purification		
NaPA 8000 (45 wt%)	0.39	-
$(NH_4)_2SO_4$ (g)	-	0.28
Distilled water (mL)	1.61	1.24
Ultrapure water (mL)	4.64	3.59
Electricity (W.h)	23.23	17.97
Outputs		
R-PE (mg)	1	1

Table F.2. Process and economic parameters for the construction of the economic model.

Note: For the recovery yield of each operation, as the experimental work obtained the overall recovery yield for the process, it was decided for all operations to have 100 %, while the precipitation will capture the overall recovery yield. For economic-related calculation this does not make any difference in the results. Duration shown is for the process to take place, additional time will be required for preparation and cleaning but that occurs simultaneously during previous operations. Size/volume/flow rate change accordingly for each scale analysed to meet the duration fixed. Price for large-scale NaPA 8000 is complicated to obtain as in Alibaba, it does not clarify molecular weight, so the average of three products was used as a reference in this work. Equations for cost calculations were constructed using prices in US dollars, and then they were changed to Euro by a factor of 1 US $ = EU 0.85 (consulted on July 31st, 2020).

Unit Operations	**Process Parameters**	**Equipment Cost**
Solid-liquid extraction	Solid-liquid ratio: 1:2 Recovery yield: 100 % Duration: 20 min	Stirred-Tank:

		$Cost\ [US\ \$] = Volume\ [L] \times 42.195 + 3035.2$
Centrifugation (Biomass Removal)	Recovery yield: 100 % Duration: 1 h	Centrifuge: $Cost\ [US\ \$] = 426720 \times \frac{Flow\ [L.h^{-1}]}{600}^{0.4}$
Mixing with precipitant	Recovery yield: 100 % Duration: 4 h NaPA 8000: 100 $g.L^{-1}$ $(NH_4)_2SO_4$: 200 $g.L^{-1}$	Stirred-Tank: $Cost\ [US\ \$] = Volume\ [L] \times 42.195 + 3035.2$
Centrifugation (induced precipitation)	Recovery yield: 100 % Duration: 1 h	Centrifuge: $Cost\ [US\ \$] = 426720 \times \frac{Flow\ [L.h^{-1}]}{600}^{0.4}$
Resuspension (dissolve precipitate)	Recovery yield: 100 % Duration: 1 h Volume: Same as before centrifugation	Stirred-Tank: $Cost\ [US\ \$] = Volume\ [L] \times 42.195 + 3035.2$
UF/Diafiltration	Flux: 4 $L.h^{-1}.m^{-2}$ Diavolumes: 5 (water) Recovery yield: 100 % Duration: 4 h	Ultrafiltration/diafiltration device: $Cost\ [US\ \$] = 91036 \times (Area\ [m^2])^{0.3741}$
Materials		
NaPA 8000	Lab-scale: EU 512 *per* kg Large-scale: EU 2.65 *per* kg	Lab-scale: Sigma-Aldrich (416029) Large-scale: Alibaba (Average of: shorturl.at/hij19; shorturl.at/loxKN; shorturl.at/agoLV)
$(NH_4)_2SO_4$	Lab-scale: EU 18.8 *per* kg Large-scale: EU 1.72 *per* kg	Lab-scale: Sigma-Aldrich (1012179050) Large-scale: Alibaba (shorturl.at/jADU8)

Consumables		
Vessel filters	Size: Depends on the vessel size, which in turn depends on the scale being analysed Reuses: 1	Vessel filter cost: $Cost\ [US\ \$] = 0.3058 \times (Tank\ Volume) + 45.334$
Ultrafiltration Membrane	Area: the area needed is calculated by considering the volume to process, diavolumes and flux Re-uses: 10	Ultrafiltration membrane cost: $Cost\ [US\ \$] = Area\ (m^2) \times (\frac{5259.046}{1.14})$

Table F.3. Hydrophilic-lipophilic balance of each copolymer used.

Copolymer	Hydrophilic-lipophilic balance	Reference
Pluronic PE 6800	29	[260]
Pluronic PE 6400	15	[260]
Pluronic 17R4	7.0–12.0	[261]
Pluronic PE 6200	7	[260]
Pluronic P123	7–9	[262]
Pluronic L81	1.0–7.0	[263]

Table F.4. Detailed data of the purity and yield (%) obtained in different fractions (*i.e.* initial extract, and resuspended pellets after precipitation using $(NH_4)_2SO_4$ at 200 $g.L^{-1}$, $(NH_4)_2SO_4$ at 200 $g.L^{-1}$ followed by an UF step, NaPA 8000 at 100 $g.L^{-1}$, NaPA 8000 at 100 $g.L^{-1}$ followed by an UF step, and lastly initial extract purified by an UF step) separately for R-PE and R-PC. Analysis performed by HPLC-DAD.

	Purity (%)			**Yield (%)**	
	R-PE	**R-PC**	**Phycobiliproteins (R-PE + R-PC)**	**R-PE**	**R-PC**
Initial extract	4.4 ± 1.0	3.2 ± 1.5	7.4	-	-
$(NH_4)_2SO_4$ at 200 $g.L^{-1}$	35.0 ± 2.4	18.5 ± 1.2	53.4	100.0 ± 2.6	81.1 ± 1.3
$(NH_4)_2SO_4$ at 200 $g.L^{-1}$ + UF	68.8 ± 5.0	20.7 ± 2.4	89.5	100.0 ± 8.7	57.77 ± 0.28
NaPA 8000 at 100 $g.L^{-1}$	50.5 ± 7.4	-	50.5	79.5 ± 3.6	-
NaPA 8000 at 100 $g.L^{-1}$ + UF	87.32 ± 0.90	-	87.3	79.5 ± 1.8	-
Initial extract + UF	29.6 ± 2.0	9.7 ± 2.0	39.4	100.0 ± 3.0	72.1 ± 3.0

Table F.5. Monte Carlo simulation.

NaPA 8000							$(NH_4)_2SO_4$						
	R-PE content	**Overall Recovery Yield**	**Materials Discount**	**Process Duration**	**0.01 kg CoG.mg^{-1}**	**100 kg CoG.mg^{-1}**		**R-PE content**	**Overall Recovery Yield**	**Materials Discount**	**Process Duration**	**0.01 kg CoG.mg^{-1}**	**100 kg CoG.mg^{-1}**
1	2.919256	-21.3404	4.918737	4.788512	107.5609	1.654121	**1**	4.079489	-11.389	5.409151	8.991537	60.03452	0.158467
2	4.508999	10.04028	8.110671	3.048164	71.86338	1.104721	**2**	3.485766	-18.187	4.88191	0.926288	62.8305	0.174363
3	8.995706	-6.68256	7.790545	4.32999	43.0251	0.656635	**3**	2.122141	-2.43167	1.266451	2.35279	24.82246	0.16506
4	0.357671	8.032359	1.852473	1.017459	214.4975	3.796332	**4**	4.818541	0.670222	3.771204	1.973736	28.90086	0.092795
5	2.811125	23.097	2.248828	9.28458	34.49502	0.501121	**5**	3.413123	0.896233	0.675157	7.80368	12.56139	0.102002
6	2.986782	-8.84413	2.562115	3.741296	46.44721	0.746189	**6**	2.305487	0.259269	6.798953	1.582637	107.2119	0.251882
7	4.254897	16.0651	1.657474	4.003682	16.48474	0.271795	**7**	4.049128	3.072738	5.887987	4.774642	53.33512	0.134735
8	0.969296	-9.77333	4.825936	1.677406	255.1114	4.055793	**8**	5.793949	-3.18374	0.654724	4.79678	6.301128	0.058994
9	4.996787	21.12177	9.154753	4.103114	65.42413	0.996518	**9**	3.130228	-5.88103	2.458095	1.660943	31.44447	0.132649
10	2.019293	-22.06	6.332978	4.951529	200.3208	3.057156	**10**	3.087326	-3.10698	4.383536	2.590287	54.78655	0.161204
11	3.932625	1.00694	3.621433	6.598253	44.65755	0.677123	**11**	1.652886	-12.7169	0.821905	4.222888	27.83115	0.23298
12	1.626066	2.186505	1.530524	4.554423	48.05582	0.784784	**12**	7.998362	-6.47998	6.005386	1.380348	29.34924	0.073157
13	2.299768	0.63265	1.772344	1.968373	36.07715	0.625442	**13**	3.182894	-6.80122	0.24902	3.62597	6.063326	0.103009
14	4.867334	-3.05434	4.300774	2.996511	42.20526	0.665347	**14**	7.141014	-0.77914	3.049103	1.270635	15.90398	0.058375
15	6.474509	9.036686	1.474525	3.064789	10.21288	0.175178	**15**	1.296841	0.458728	2.434238	4.090853	74.7469	0.310736
16	4.476277	6.700349	1.94772	2.861914	19.33522	0.323794	**16**	2.842384	1.705809	7.392262	7.942687	98.32262	0.22503
17	1.915185	-14.3345	3.593243	3.489476	106.9252	1.6908	**17**	0.88114	-1.41539	8.106655	3.002661	343.4289	0.748572
18	1.422753	1.760297	5.170452	8.373315	172.4953	2.561408	**18**	2.710633	-13.0347	3.925917	8.03223	68.45729	0.212345
19	1.478412	-22.71	1.254112	1.050432	55.19661	1.044702	**19**	1.208643	-5.79572	8.450102	1.414715	270.0001	0.576369
20	1.312504	-13.9578	2.691188	1.770627	114.0546	1.894733	**20**	1.617542	-9.01869	0.9297	0.80172	24.24437	0.215687
21	1.820316	-18.9199	1.321983	6.032743	54.2828	0.854956	**21**	4.911208	-18.657	1.73557	2.735733	17.45895	0.092196

22	8.128288	-7.05391	6.895129	0.906879	41.2523	0.647703	**22**	3.199848	0.05133	0.621226	0.968813	7.769924	0.094801
23	6.061083	-4.75683	2.78151	0.821	21.88458	0.368896	**23**	2.883809	0.929513	4.331142	3.478369	56.30058	0.166625
24	8.227182	4.486261	6.28416	2.496528	32.58473	0.50734	**24**	2.182779	-11.7545	5.916156	1.604074	112.8872	0.283554
25	3.654967	-20.4294	6.007288	2.928892	100.2561	1.558122	**25**	4.549988	-0.13071	6.034388	2.785519	49.15031	0.122428
26	0.851887	-18.0838	2.459103	3.27378	178.4751	2.901808	**26**	4.099018	1.751199	5.689409	3.523861	50.97042	0.130784
27	5.604332	-8.34719	0.316363	2.285233	4.106125	0.099372	**27**	0.747949	-14.6725	6.835219	3.028463	398.6776	0.936643
28	1.009872	-1.53502	2.333935	5.914558	120.554	1.865896	**28**	3.113445	-9.08038	2.294481	4.650562	33.25109	0.142749
29	1.367452	-4.90147	0.886026	1.070591	32.87886	0.672039	**29**	7.693773	-0.86869	3.091767	0.329346	14.67848	0.053659
30	1.647614	17.70917	1.287982	8.165076	39.61271	0.5857	**30**	0.792074	-20.3345	4.054288	2.047746	241.1509	0.742125
31	0.811481	-10.2026	4.384889	4.46773	290.3394	4.496302	**31**	8.631689	-0.63745	1.487183	2.918664	7.069864	0.041401
32	3.580409	2.449537	1.308692	2.148127	17.28074	0.310903	**32**	1.574655	-5.62485	2.913507	3.097057	75.67869	0.28305
33	1.022721	22.11243	2.49841	2.409597	89.31366	1.475566	**33**	1.906357	-19.587	2.821525	4.390044	73.80871	0.279483
34	4.719161	-5.43521	1.830384	5.267022	21.71312	0.344158	**34**	3.661908	-6.623	5.108066	1.055245	54.54137	0.147708
35	2.575289	-9.83813	4.055566	2.087755	81.63216	1.305706	**35**	8.151956	-7.15109	5.801251	0.228676	27.67598	0.070102
36	2.093729	-23.5813	3.639555	2.600061	113.6616	1.818344	**36**	9.040537	-22.6513	6.412388	3.795649	34.64358	0.083936
37	0.934594	4.253232	8.801966	4.43525	405.4569	6.168795	**37**	1.406889	-3.3586	4.789764	4.060435	133.8878	0.375223
38	3.150081	11.27542	2.715887	3.924842	36.29914	0.579092	**38**	2.454027	-24.3226	1.431467	1.718203	30.41362	0.188016
39	3.691457	11.79606	6.690566	1.85074	70.58763	1.101816	**39**	1.884122	-2.15981	1.572286	2.400068	33.89273	0.192928
40	2.542366	10.27533	5.049535	3.416792	80.66702	1.256641	**40**	6.072274	-0.75284	0.830823	5.01907	6.995746	0.056399
41	4.358756	15.30221	4.417075	0.244574	37.49619	0.60972	**41**	1.188168	-11.4586	5.320465	6.699321	197.9782	0.526186
42	6.244786	2.886577	6.655869	0.309733	45.33294	0.716411	**42**	3.253058	-0.68245	2.723741	4.616979	33.76065	0.130428
43	3.396627	-1.32596	1.565503	3.517215	23.62516	0.397063	**43**	6.679018	-22.2047	3.006699	2.010655	21.99062	0.08112
44	3.352006	3.021185	3.232055	4.766409	44.71876	0.697677	**44**	1.803683	1.176424	6.29958	1.512682	125.8479	0.306504
45	1.870727	-12.0469	6.991855	7.578917	207.0156	3.094689	**45**	1.229165	-12.6725	2.658634	1.568935	93.1332	0.373116
46	9.353996	-2.21867	4.502969	3.160042	22.75087	0.357173	**46**	7.568958	-14.4535	3.213902	1.40128	18.4881	0.065582
47	3.451436	-2.69991	6.253223	6.378553	87.80972	1.325341	**47**	3.615433	-3.77037	5.282112	1.914666	55.92675	0.148855
48	7.85884	7.70337	2.08954	3.02229	11.66107	0.193227	**48**	3.002313	-20.1235	4.689673	8.596895	79.69111	0.225515

49	1.505547	10.76887	1.544165	7.278854	51.80415	0.784451	**49**	3.070648	-5.93467	2.403785	1.088358	30.91901	0.133142
50	3.097665	13.45599	5.410806	1.887099	67.13155	1.058188	**50**	2.867606	-9.4062	4.115218	1.811888	58.83018	0.179563
51	1.088829	6.291349	5.775647	5.368653	228.9084	3.488006	**51**	3.495718	-11.3477	1.045503	3.777071	15.19888	0.110642
52	2.961133	-12.5906	2.847735	1.909342	52.43561	0.864244	**52**	1.753682	-5.98959	1.058403	0.947817	24.63023	0.196321
53	1.415204	-1.19928	5.918016	3.203402	193.0779	2.995343	**53**	1.054972	0.167237	4.22778	0.866539	145.6596	0.438291
54	1.83444	-6.0781	7.284049	2.472549	193.0371	2.989248	**54**	1.775564	-16.5648	5.677674	2.541804	142.8967	0.366719
55	5.277017	-25.3125	0.908628	4.974446	15.06011	0.252173	**55**	6.042994	-5.48652	0.63351	1.456799	4.649545	0.053935
56	3.469086	5.398844	6.602407	2.648928	80.33378	1.246849	**56**	0.911897	-11.0582	3.829339	1.739991	175.6805	0.559298
57	3.140631	7.116658	3.188513	6.124656	46.03031	0.704129	**57**	0.826612	-6.67955	7.900737	0.912198	371.5071	0.814976
58	2.415825	-16.2986	4.437789	3.444224	106.6417	1.669957	**58**	2.501943	2.74666	3.676989	3.701068	54.79484	0.177798
59	4.889873	5.011137	4.524009	5.046682	40.99702	0.630228	**59**	2.934638	0.096173	0.461017	1.108603	6.627453	0.101212
60	4.411409	8.413704	4.269241	6.20171	42.05824	0.639146	**60**	3.279821	-8.4704	2.442805	4.866516	33.29664	0.137249
61	0.982475	-26.167	5.072907	5.407729	361.4367	5.520163	**61**	2.290499	-7.63256	6.43049	4.529295	114.6495	0.277697
62	3.379169	-10.6942	1.418891	8.949709	30.00784	0.43484	**62**	5.924512	-19.0769	2.466689	2.191382	19.79699	0.082929
63	8.684915	3.517008	2.127003	2.18587	11.00579	0.185665	**63**	1.075141	-7.33222	3.877437	1.022254	142.7417	0.451792
64	1.015508	14.66209	4.906004	4.022963	188.724	2.925172	**64**	8.187347	-5.09938	3.075078	3.263028	15.26978	0.055193
65	0.514362	3.971967	2.058631	5.242696	195.1912	3.078243	**65**	4.475076	-2.08587	5.932049	2.130679	49.81477	0.125034
66	1.79042	-4.52133	3.611285	5.512546	103.2482	1.588087	**66**	2.153301	-6.07559	3.267223	1.182698	59.62025	0.209506
67	2.131212	15.55203	3.509407	8.01047	69.42443	1.033711	**67**	2.41495	-16.8189	7.487965	2.654788	138.1199	0.311277
68	3.95168	-6.23078	6.188398	2.754638	76.80752	1.194069	**68**	5.275612	-11.2771	4.145922	3.980852	34.0422	0.103151
69	0.231896	-7.15576	7.131762	2.162842	1513.976	23.51507	**69**	2.09289	-10.9886	6.021635	1.892408	119.0904	0.296635
70	0.616591	12.53931	8.137675	6.686301	527.2587	7.923055	**70**	2.605551	-11.8678	7.60855	1.856025	121.5351	0.271553
71	1.618334	-0.78747	5.787655	7.213415	171.4318	2.571537	**71**	4.442554	-9.34219	6.481338	0.397727	58.24993	0.139707
72	2.695723	11.47087	1.998934	3.591038	31.81694	0.522155	**72**	6.371888	-0.31348	0.972058	0.434257	5.694537	0.049685
73	2.108343	1.168252	3.838786	4.5004	84.83235	1.319537	**73**	1.855725	3.194373	2.764486	3.569674	56.31555	0.217014
74	6.55939	3.743502	2.880087	0.619973	18.70486	0.315242	**74**	3.422965	-18.2969	4.802212	4.831531	66.43768	0.185915
75	1.394237	-15.614	1.172076	1.486416	49.90862	0.939746	**75**	1.928653	-2.9531	6.268516	7.723106	130.3226	0.320733

76	3.524508	3.882526	5.54151	8.790235	72.58417	1.074278	**76**	0.328279	1.877741	6.619279	3.20974	732.9596	1.748193
77	7.272421	12.88478	6.019995	3.973703	32.6311	0.502631	**77**	3.523484	1.447388	0.789183	6.473319	12.34616	0.09719
78	2.025642	7.540682	1.649713	3.32758	37.00593	0.622196	**78**	8.090515	-13.6419	3.382803	3.071	18.57775	0.063432
79	1.436221	-18.596	5.436028	3.399281	225.8644	3.508978	**79**	7.595751	-11.5701	2.739623	7.406308	17.41383	0.065945
80	8.476605	-24.8389	6.396485	3.186439	49.77232	0.770117	**80**	3.165983	-10.1483	8.492476	1.928955	109.2736	0.233061
81	3.902996	-1.45321	1.151686	3.847013	16.09409	0.276021	**81**	1.14164	-14.8076	0.407069	3.334036	23.71119	0.320767
82	5.340034	14.90132	0.797584	2.027841	6.465838	0.128011	**82**	3.028113	-3.15508	4.310816	1.951314	54.47098	0.161886
83	5.443293	-5.04694	5.036467	2.795558	44.97487	0.705108	**83**	1.470036	3.733214	5.359135	1.682581	128.6432	0.339791
84	5.140093	-11.3232	8.749064	3.712568	89.5769	1.368782	**84**	1.892107	-0.45267	1.185902	1.075415	24.09631	0.174878
85	6.120137	-15.3632	3.135803	6.651648	31.73193	0.481804	**85**	5.377432	-6.30418	2.014192	3.054361	15.89077	0.075497
86	3.616473	-5.14057	3.15264	2.624454	43.18487	0.697406	**86**	1.914364	-15.6334	0.951143	2.695148	25.49646	0.205698
87	2.736311	5.642831	9.497294	2.536033	144.8179	2.223051	**87**	3.209381	-14.1072	4.745641	6.213063	67.70967	0.190585
88	3.644766	20.97558	1.356118	4.697989	15.85568	0.260084	**88**	2.477947	-4.40044	4.015585	3.496093	64.56673	0.199313
89	1.196276	-4.28798	2.706999	3.25664	113.3567	1.830761	**89**	4.982534	-16.0174	4.095636	5.115539	38.40439	0.11697
90	2.56657	-14.7205	7.761419	3.619219	167.9444	2.57532	**90**	4.496009	-13.1111	3.858613	6.291954	39.60931	0.124386
91	2.478829	-6.32909	2.225613	2.584478	46.13851	0.767688	**91**	1.034848	-6.39133	4.600808	5.482086	184.713	0.528478
92	4.617759	9.500387	7.370426	6.512426	66.19577	0.996574	**92**	3.450422	-7.87723	2.5941	9.347534	36.55786	0.141124
93	5.239013	5.493819	2.60197	2.820616	21.86251	0.357045	**93**	9.110611	-17.6723	3.427633	2.229335	17.28978	0.058742
94	5.768161	-9.54405	5.096391	0.440694	44.38269	0.712352	**94**	6.212419	-9.60735	4.448079	2.337711	29.5799	0.086351
95	4.3269	-8.01349	1.706895	8.445896	25.44184	0.374361	**95**	5.416582	0.239655	8.417757	5.314433	57.98623	0.125061
96	1.14239	16.23347	5.133281	4.21544	172.6569	2.666903	**96**	8.98912	0.286789	3.24051	3.433193	13.83332	0.0484
97	2.761163	-7.47675	7.89763	2.713948	141.7801	2.185965	**97**	2.19107	-9.24265	3.144138	4.809245	62.91404	0.222833
98	1.929475	-3.6391	6.141408	5.772304	155.425	2.357809	**98**	4.029762	-5.54044	5.595077	1.312021	53.73075	0.138771
99	8.191011	1.294801	1.480469	4.133754	9.239005	0.153187	**99**	0.433527	-0.93953	3.936591	5.644559	361.0753	1.122891
100	4.141814	-5.28745	6.497932	2.912586	75.98009	1.177531	**100**	5.521606	-10.5979	4.34258	2.408596	32.93133	0.097422
101	4.497215	20.33639	2.104146	4.858136	18.82758	0.299119	**101**	8.030991	-2.4038	3.196913	4.454663	16.04332	0.056352
102	0.802317	-15.2047	8.902539	7.047583	633.3757	9.492875	**102**	2.760246	-11.1673	8.141183	1.99622	121.7441	0.264309

103	4.061141	-14.5686	1.522879	3.817794	23.48021	0.392098	**103**	2.168026	-1.24478	3.455109	2.704021	61.05754	0.206112
104	4.763061	-16.5669	2.141678	3.770156	27.82568	0.452345	**104**	6.698581	-17.401	1.098803	5.496447	9.645934	0.064703
105	8.093993	-11.1988	1.728654	2.208966	11.8479	0.204641	**105**	7.228029	-4.18871	0.893267	7.476745	7.277309	0.051921
106	1.339119	4.629355	3.040882	2.223796	98.82442	1.610887	**106**	3.597379	0.193108	5.024745	5.055383	53.43102	0.146063
107	3.74885	9.793838	5.608321	6.28036	62.74184	0.948957	**107**	7.450245	-19.3909	3.338275	2.299223	21.10665	0.072855
108	5.999369	17.41645	0.877741	0.382896	5.454015	0.116443	**108**	0.627726	2.994792	6.972552	1.783742	393.2488	0.913537
109	3.844165	-0.13657	1.635901	1.828656	20.12879	0.354226	**109**	1.491188	-11.2375	4.930003	1.841863	137.8174	0.380431
110	2.168873	8.583023	1.786386	5.687895	39.41487	0.61901	**110**	8.824288	-25.222	3.55613	1.668715	20.2548	0.06742
111	6.166866	7.972868	2.838684	1.396274	18.9876	0.315974	**111**	3.980447	-8.34064	5.789204	2.199682	58.63682	0.148978
112	1.907177	-21.4929	6.725632	5.472565	223.4657	3.392941	**112**	5.441837	-17.7799	9.90622	0.605862	80.48671	0.161007
113	1.776371	-15.8694	5.567476	1.73478	175.5532	2.767086	**113**	5.547456	-11.0191	4.567533	6.925459	36.79822	0.105607
114	5.58962	-12.801	2.36411	3.865802	24.51211	0.39492	**114**	5.23762	-8.18508	4.513381	0.640313	34.15231	0.099012
115	2.843311	-27.2778	2.57765	5.8864	69.61124	1.074769	**115**	6.243019	-10.0077	2.789436	4.152165	19.71985	0.0753
116	5.378867	-0.46022	0.364385	3.895251	5.093007	0.101395	**116**	2.032937	-4.93437	2.805462	1.20316	53.79982	0.208554
117	3.254042	9.974822	1.192514	3.545074	17.0956	0.295454	**117**	2.801198	1.211745	7.735547	6.358623	103.2689	0.231164
118	3.796325	4.343965	3.319992	2.779554	38.42951	0.616891	**118**	3.719032	-14.7589	1.07858	1.353606	13.40793	0.103707
119	0.867086	-5.67956	4.458581	5.447141	262.5146	4.019414	**119**	1.249736	-10.454	2.380099	1.726028	80.59824	0.347278
120	3.825442	24.02453	3.015034	4.915958	29.40627	0.458994	**120**	2.629963	-9.73725	6.855754	2.018767	106.1793	0.248684
121	5.657171	-23.2278	0.838751	1.286361	10.16907	0.208028	**121**	2.541476	-5.59447	1.918161	1.169616	30.02345	0.151464
122	4.58438	-2.48201	8.342468	6.154558	86.43401	1.302765	**122**	4.705655	-23.6378	6.566387	4.071351	69.2083	0.165903
123	0.555783	22.58835	8.659961	0.704601	535.9067	8.3399	**123**	5.33861	1.114566	2.279515	1.366966	15.79261	0.070343
124	8.255989	-5.88392	7.843423	1.320356	45.56707	0.709223	**124**	5.880531	-7.29956	4.62223	5.328278	32.91146	0.09394
125	2.711543	2.838354	0.725662	0.942723	12.38719	0.2693	**125**	4.410221	-6.50344	7.134039	2.995426	64.02981	0.147532
126	6.044671	0.712164	5.33293	2.125209	39.41736	0.620371	**126**	1.195522	-16.9194	4.655845	2.486888	175.846	0.500582
127	2.08554	-11.3934	6.817202	4.394959	174.6032	2.671613	**127**	2.826275	-12.8255	4.259085	1.278059	63.72499	0.190839
128	1.271115	-1.49909	0.573496	4.5462	30.66948	0.548615	**128**	1.987826	-4.08037	3.410093	3.607886	68.94227	0.233838
129	0.890229	19.6102	2.349008	0.937389	95.55234	1.639624	**129**	8.472255	-1.17319	6.532932	2.423817	28.71779	0.068833

130	2.070969	19.83713	6.446847	1.078504	110.661	1.741002	**130**	4.222466	-5.01674	5.86001	1.443298	53.43608	0.134838
131	4.1925	-1.03779	6.266767	2.036704	67.94737	1.06207	**131**	4.583155	-14.9824	1.801247	2.969746	18.61481	0.095473
132	7.10321	4.202455	1.314522	5.219715	9.718278	0.157086	**132**	0.86622	-18.5247	7.534345	4.369505	401.8294	0.906188
133	2.206717	14.81489	8.076691	2.554195	138.3043	2.133063	**133**	4.140682	-18.0598	1.240057	2.176613	14.86422	0.100855
134	0.948312	-2.74789	0.425486	7.76403	44.573	0.668337	**134**	4.853439	-19.5161	1.837387	2.138915	18.43736	0.094485
135	1.892926	10.58049	6.364593	1.801164	132.7751	2.077946	**135**	8.358529	-15.582	1.43903	0.835805	7.694206	0.048412
136	4.983857	-3.46277	2.287064	6.619076	25.03129	0.38236	**136**	7.061373	0.595993	2.699016	5.75522	15.60831	0.060202
137	4.161552	13.82103	6.874335	9.224468	67.15559	0.992349	**137**	3.824368	-23.1269	5.084526	2.22282	64.72637	0.175771
138	1.470813	-11.0752	3.205481	2.466598	116.8752	1.889915	**138**	6.118427	-16.2614	5.257708	5.289861	39.65067	0.105941
139	1.661446	6.64425	2.947238	3.455005	77.43023	1.239162	**139**	2.214181	-7.3578	1.349382	9.189706	34.62201	0.191046
140	6.293086	-10.8838	1.011821	2.194913	9.366764	0.175519	**140**	2.229021	-25.6749	1.494796	1.922682	35.81934	0.213532
141	1.73266	-2.5296	6.636554	7.416957	186.3038	2.788656	**141**	6.291296	-28.0768	2.506037	7.187562	24.13259	0.0963
142	2.314773	-6.6948	1.925371	6.142922	48.16304	0.746298	**142**	0.396907	-12.129	6.050757	1.805704	638.909	1.587489
143	3.280809	5.052122	0.957959	6.268332	17.18871	0.272046	**143**	1.833628	-19.0092	3.058429	8.516906	88.69579	0.314225
144	2.048273	-5.58387	7.091796	5.903604	172.3674	2.606944	**144**	5.697324	-16.7628	0.574873	0.794464	4.846965	0.063854
145	7.002888	9.614461	1.411345	7.1338	10.48673	0.159608	**145**	1.400996	-26.5441	2.256208	3.682769	88.81446	0.388937
146	0.904704	-1.37164	2.513814	4.176669	137.6674	2.196008	**146**	5.481546	-7.59946	7.956463	2.237676	57.63412	0.126415
147	0.961812	5.345707	0.696164	3.223353	38.88189	0.734965	**147**	1.739696	-22.8332	0.915669	1.959367	28.52053	0.243735
148	0.776171	9.909579	0.644319	4.207698	46.28836	0.833989	**148**	3.872427	-4.42308	2.964551	1.332788	29.71462	0.111023
149	1.28435	6.800651	5.649549	1.593614	181.3236	2.859581	**149**	0.925993	-5.23555	4.034905	3.388326	174.8639	0.538429
150	3.981549	18.40451	5.676164	5.024607	53.76595	0.822016	**150**	3.901908	-13.9319	2.674375	3.011925	30.99981	0.122429
151	5.043145	-8.68612	2.17939	2.948066	23.20909	0.383776	**151**	5.766593	-24.1936	0.341349	6.867394	6.734522	0.076576
152	6.507405	1.122899	3.179009	5.295717	23.45012	0.363188	**152**	4.671726	-9.10809	7.239122	2.161872	62.67323	0.14324
153	2.977552	6.138287	5.000292	6.021463	73.81608	1.120971	**153**	0.649164	-14.2743	7.417222	5.603378	506.0871	1.151747
154	4.797587	7.218793	1.880858	6.672806	19.42116	0.297509	**154**	4.923953	-21.8532	6.913601	6.963236	69.65325	0.163852
155	4.672971	16.13873	4.321064	4.965537	36.28287	0.55899	**155**	1.291023	-11.6015	9.019948	6.014541	297.9171	0.625992
156	9.118103	-4.95366	1.764474	1.537198	9.598908	0.168581	**156**	6.165135	-6.73623	4.836999	0.740465	30.60948	0.085378

157	3.880986	-8.85762	4.848294	5.320319	66.14738	1.012161	**157**	0.711282	-15.7256	3.781612	1.082676	232.7841	0.747805
158	4.283025	11.72431	0.743848	2.364875	8.055261	0.158524	**158**	1.325036	-15.9645	4.872022	2.639089	164.1133	0.455883
159	3.147307	13.59755	1.98452	0.697143	24.24746	0.428443	**159**	0.947529	1.312474	2.03679	5.825449	90.91983	0.41518
160	7.96712	-0.70775	0.771254	8.046288	7.191262	0.106451	**160**	2.130368	1.379053	5.816708	6.135783	103.4699	0.263265
161	1.803897	3.107761	0.203782	5.158264	12.66513	0.238311	**161**	4.273865	-8.03062	3.482702	0.453106	32.22972	0.109107
162	1.060833	22.41383	6.781139	1.206294	221.6287	3.474622	**162**	7.269989	-4.5038	6.1431	1.576621	32.37328	0.079821
163	5.751732	-15.0479	1.75047	7.816265	21.25846	0.317448	**163**	0.851004	-0.06178	7.071834	1.06249	301.4542	0.694863
164	2.993312	18.09468	1.445837	5.462678	21.53409	0.343625	**164**	1.001932	-9.30635	8.046639	4.574261	331.3152	0.726841
165	3.287625	0.916806	4.155881	0.66616	55.51401	0.902495	**165**	1.951011	-1.48094	5.746622	8.400003	117.9311	0.302385
166	6.86267	-1.92133	2.118779	1.743329	14.66462	0.250125	**166**	5.078287	-9.83923	1.977256	2.284248	16.8195	0.081739
167	2.925724	-1.58103	3.391612	7.0716	58.96615	0.889148	**167**	4.24248	-0.35799	6.173146	4.995949	55.2749	0.136554
168	3.503781	-2.61463	4.20669	6.972326	60.37826	0.909788	**168**	7.475778	-15.4907	2.855734	0.989313	16.75321	0.064277
169	5.709522	0.385752	3.65773	4.427203	30.21122	0.471264	**169**	8.223443	-8.84918	1.152424	8.643848	8.359544	0.05066
170	5.065499	-9.90314	5.501272	2.618381	56.19092	0.878924	**170**	3.961301	-15.3493	0.686311	5.536641	11.48189	0.1012
171	2.661594	-4.71632	1.044774	7.197379	26.52207	0.404697	**171**	4.936731	-17.1805	5.976593	2.599034	54.47414	0.136299
172	4.451993	-0.18042	2.593219	2.341309	27.17758	0.448103	**172**	0.955138	-23.3217	1.628939	3.729743	93.5564	0.50707
173	1.263139	11.59716	5.745088	3.90683	182.0574	2.809987	**173**	1.761541	-9.90483	0.598588	3.806098	19.55829	0.20343
174	1.276361	-5.73343	6.049105	6.448491	239.8119	3.618811	**174**	0.660836	-11.4186	6.348605	3.579756	406.195	0.988483
175	4.571533	-6.2859	0.338629	3.338172	5.828213	0.123773	**175**	4.369072	-1.86522	1.132079	4.285616	11.94647	0.081298
176	6.148736	3.921737	3.074135	6.189931	23.66181	0.361891	**176**	3.682382	-8.27977	2.195	2.361266	25.15834	0.11375
177	0.918327	-2.26581	1.024673	3.781502	62.66408	1.091638	**177**	3.758158	-3.87123	8.850686	1.611793	89.13372	0.186794
178	4.149633	-0.58373	4.512192	4.374402	51.15003	0.792174	**178**	3.533494	-1.74529	8.617174	2.384894	90.7868	0.192681
179	9.253918	-5.98082	8.200725	1.260575	42.52023	0.660873	**179**	4.233689	-3.1275	5.121326	7.549205	49.33552	0.133648
180	3.989159	11.08199	3.954579	7.238874	42.74276	0.642375	**180**	2.322343	0.961933	3.127322	1.464385	49.42008	0.178307
181	0.817886	-4.57274	4.928715	6.532735	304.9367	4.608579	**181**	3.122858	-13.7228	9.225352	1.387866	124.9241	0.257371
182	2.701992	20.58079	0.950375	5.257564	16.69821	0.275334	**182**	4.09044	-13.1454	2.830554	3.175825	30.98825	0.117954
183	7.0182	17.31288	1.756612	1.045427	9.400364	0.167605	**183**	7.430455	-3.4579	4.534461	3.654306	23.96317	0.069159

184	2.726727	1.925938	0.653546	0.716171	11.09418	0.253264	**184**	2.943813	-2.92572	7.685132	4.041032	100.0439	0.223551
185	4.200388	-5.19327	4.361863	3.196812	51.14126	0.803769	**185**	2.85144	0.313506	3.906942	2.944159	51.53761	0.161759
186	3.358895	8.245704	3.675957	7.032444	48.94745	0.737911	**186**	5.152071	-10.9497	7.305185	7.362179	61.32347	0.140923
187	6.43658	1.585578	3.751655	2.530031	26.21686	0.419005	**187**	3.607504	2.82962	4.395081	1.633304	43.56163	0.128098
188	3.559501	-4.05673	3.527215	3.240408	48.6277	0.772429	**188**	3.991218	-6.18622	5.061099	0.897859	49.24546	0.134022
189	8.431545	4.577826	4.226649	2.764392	21.71422	0.343579	**189**	2.902038	-0.42761	1.685978	5.431272	25.54404	0.131719
190	4.48457	13.02158	2.317054	4.310695	21.86921	0.349598	**190**	7.251497	-7.78426	9.392369	2.794504	51.58495	0.105897
191	6.539203	0.263071	1.571511	4.814557	12.62381	0.20425	**191**	3.149112	-5.15246	7.816579	3.080355	96.61833	0.213914
192	8.632972	0.062898	2.340546	8.753743	14.77755	0.216904	**192**	0.85958	2.157132	6.096655	6.440739	266.6614	0.663771
193	7.079262	-8.51599	7.414696	2.658541	52.72972	0.814969	**193**	0.568366	-18.4102	1.460106	2.728763	128.8471	0.767365
194	1.188367	-8.40772	2.938059	2.830459	129.1412	2.089107	**194**	4.628572	0.872582	1.16548	3.289831	10.70088	0.073471
195	3.035634	10.2091	1.348627	4.065077	20.70598	0.346903	**195**	3.037411	-6.59133	3.752699	5.391996	52.24721	0.166913
196	5.186817	20.2019	3.126372	5.71941	23.61874	0.363661	**196**	6.357953	-8.6899	8.754293	8.126048	57.70962	0.123261
197	6.82609	-11.5901	3.601154	4.77992	29.46999	0.457798	**197**	4.540701	-8.43601	3.032516	2.47154	27.75309	0.101581
198	7.247001	7.913537	3.984462	1.881577	22.65314	0.363725	**198**	3.245555	-9.20698	3.800203	4.202942	49.97399	0.158938
199	1.027821	-16.1294	2.896318	3.75259	168.438	2.68499	**199**	5.327756	-6.97137	3.284389	2.550494	25.13032	0.087524
200	3.680425	7.059541	7.236759	7.372649	84.53628	1.265433	**200**	7.973214	-14.6243	4.988654	1.115892	26.90007	0.073781
201	3.009806	-12.4571	3.143186	2.157126	56.87043	0.925578	**201**	4.202521	-20.0488	5.568949	4.31724	63.25637	0.164144
202	0.497195	0.464601	0.854214	4.655717	101.7447	1.737526	**202**	4.151748	-14.4908	0.855079	0.874943	9.370809	0.08887
203	0.792328	8.692185	5.357729	3.81055	280.1408	4.337732	**203**	2.510587	-1.10178	3.170475	0.722181	46.61145	0.167307
204	4.659911	0.588122	4.621016	2.490558	44.77072	0.707219	**204**	2.526016	-11.5299	3.355625	3.912941	58.48897	0.200065
205	5.794366	-0.91236	3.031579	8.535608	27.47988	0.405915	**205**	6.962432	-22.5512	4.711455	1.704276	32.52947	0.09203
206	1.407093	-4.80872	6.346818	1.294469	213.7519	3.359621	**206**	0.835473	-10.7345	6.673011	3.758549	334.9656	0.796632
207	2.259014	6.958113	4.533267	1.349303	82.6733	1.324417	**207**	2.115714	-8.94686	5.528464	2.055896	106.0327	0.275692
208	1.167939	4.813857	3.239544	1.185825	117.7438	1.941595	**208**	6.260301	-7.69167	2.341398	2.510913	15.67393	0.067767
209	5.12578	-17.5927	5.319071	1.685896	59.76581	0.944783	**209**	1.668422	-11.7139	4.363021	6.577043	117.7831	0.346242
210	4.346421	9.729177	7.953272	7.608263	76.02582	1.136061	**210**	6.013929	-1.29073	0.287517	2.535015	2.812066	0.050411

211	4.431225	23.8094	4.195519	5.789042	34.86663	0.532529	**211**	2.018461	-10.6661	1.825998	3.961705	41.95606	0.210114
212	4.180518	-17.1203	4.144807	7.684836	62.15488	0.929132	**212**	4.253742	1.598935	4.180636	3.149469	36.47607	0.110135
213	4.969989	-24.4053	2.505166	3.482597	34.8596	0.563683	**213**	6.49104	-4.21116	3.395991	6.060018	22.01334	0.074241
214	2.858775	-9.14202	1.077849	5.502571	24.93444	0.403917	**214**	4.285189	-5.9039	2.145361	5.456608	22.46077	0.099745
215	3.727381	2.139494	4.257948	5.358988	52.85275	0.810954	**215**	3.91254	-11.128	7.270883	1.590336	76.54605	0.174413
216	4.806393	2.752634	0.829929	0.878492	7.879785	0.165722	**216**	3.106094	-2.45168	0.994991	4.234112	15.24456	0.112945
217	0.466984	-29.6404	2.110564	1.239978	329.248	5.68775	**217**	5.985074	-6.13084	1.048006	1.042349	7.202333	0.057644
218	3.531581	-22.8917	3.85755	1.51593	69.15705	1.118377	**218**	5.391466	-10.2818	0.881452	7.833374	10.54005	0.074938
219	3.430938	-10.5738	1.663548	0.633381	25.24535	0.460001	**219**	1.554965	-4.29121	1.372836	6.630102	44.06704	0.252179
220	6.056261	5.440476	3.704517	3.409989	26.66162	0.421377	**220**	6.80962	-0.61223	6.679753	6.122696	37.61428	0.089739
221	4.26287	-6.79508	2.013374	5.832057	26.95565	0.419982	**221**	1.009134	-7.06105	5.200437	1.340266	203.1689	0.545059
222	4.693765	14.42654	2.465823	4.513256	21.86264	0.346874	**222**	1.590046	-7.20894	6.73448	2.953345	169.1971	0.40018
223	3.486036	-12.6659	6.479484	2.427456	99.49696	1.548106	**223**	1.202883	-13.5967	3.087679	6.408454	122.4229	0.43521
224	0.298763	10.70095	4.278086	0.366585	557.9605	9.08332	**224**	2.423495	-28.9713	4.947228	4.163253	110.8971	0.305573
225	3.756177	17.60315	3.905871	0.789084	37.92233	0.618683	**225**	4.683897	-6.88204	4.819012	5.770508	42.99364	0.120076
226	1.317775	-7.37345	3.100204	2.990115	121.1199	1.946808	**226**	4.843606	1.486559	0.375111	1.849807	3.69499	0.060674
227	1.114225	-1.8384	5.974971	9.509052	265.8544	3.914409	**227**	1.115617	-15.8708	0.940142	1.31948	39.89861	0.34366
228	5.428031	-9.3173	5.382661	0.97198	49.94642	0.794528	**228**	1.257142	-8.09079	1.20892	5.008911	48.36486	0.309697
229	3.633815	13.37708	1.550157	2.403695	17.69019	0.308014	**229**	2.237362	-0.58703	8.431982	5.342165	141.8583	0.305758
230	7.694535	8.974353	4.175666	3.67688	22.65022	0.354695	**230**	5.562029	-16.3036	3.019177	2.583454	24.84781	0.091145
231	5.325109	9.436648	1.004617	7.988072	11.22434	0.166687	**231**	2.769199	0.544601	5.238132	5.470888	72.26444	0.193456
232	3.862151	8.078991	1.585218	1.628857	17.50043	0.311339	**232**	8.64205	2.919109	4.049539	6.64938	18.13343	0.055448
233	6.274342	12.46452	1.198267	8.307285	10.5078	0.154626	**233**	1.995915	-7.65848	2.08501	0.618509	41.65713	0.199412
234	0.719796	1.249248	2.418136	4.871888	164.3474	2.592105	**234**	7.123351	-13.6774	7.856881	6.076493	48.55331	0.107916
235	4.934289	-2.96794	2.788531	1.7076	26.84404	0.444943	**235**	0.605379	-15.2988	9.254837	2.911514	666.2931	1.376072
236	4.87626	1.376155	4.835735	4.480509	45.39697	0.700684	**236**	2.67903	-20.1824	2.621509	4.101826	49.21624	0.195327
237	3.061559	-14.8064	1.111046	4.290442	24.53708	0.41519	**237**	3.70068	-8.65516	7.645289	4.183063	84.47912	0.189247

238	3.078215	4.669721	1.252498	1.425477	18.18864	0.339093	**238**	2.399747	-10.0738	0.579568	3.942618	14.22067	0.149651
239	8.869743	-4.42996	2.232131	5.976422	13.74221	0.212729	**239**	8.124746	-0.95948	2.062275	4.696557	10.63224	0.048783
240	1.297043	5.73876	3.409102	1.315194	110.4886	1.809949	**240**	6.57633	-14.0971	3.270551	1.197404	21.46057	0.075359
241	2.637077	8.522006	2.763584	6.762881	48.1701	0.731529	**241**	6.718261	-2.89836	6.941979	1.886163	38.93839	0.090655
242	7.54317	10.89052	8.612462	8.140815	46.79306	0.697021	**242**	6.308804	-0.84327	1.317853	2.688217	8.634294	0.055318
243	2.159983	4.43502	1.990842	3.05833	42.22374	0.702177	**243**	7.750651	-2.18063	7.141868	8.783971	36.63338	0.085212
244	7.182301	-15.4528	4.341427	8.240412	36.99805	0.549724	**244**	4.454223	2.488302	4.378509	1.733861	35.32607	0.104092
245	2.533031	5.83507	0.597278	1.460026	11.25687	0.24868	**245**	3.46032	-23.2117	1.39375	1.654871	20.66643	0.130505
246	4.99299	-8.07309	2.673484	0.854988	26.76358	0.452884	**246**	4.486813	-11.6734	4.01933	3.516044	38.7794	0.119643
247	7.911109	21.66363	2.03597	2.789341	9.686691	0.161798	**247**	1.783445	-2.49965	3.843484	4.021692	85.06343	0.268909
248	5.029112	15.21344	0.97131	2.442715	8.310903	0.155003	**248**	5.352527	-1.98567	1.121595	4.433885	9.760383	0.066545
249	4.604832	5.19848	4.778801	5.746461	46.12018	0.702874	**249**	3.175468	1.417176	5.363966	3.272651	62.21138	0.164391
250	3.709006	-6.03536	6.564487	1.650412	85.52613	1.338226	**250**	5.643999	-17.9979	1.015041	8.441015	12.42768	0.080841
251	7.141628	-14.5025	6.705908	4.745511	52.75969	0.805472	**251**	6.440053	-23.5224	6.027271	1.908404	45.44237	0.11314
252	6.354558	21.08969	6.297817	3.58405	35.67896	0.550576	**252**	7.186694	-5.12266	7.040235	1.156554	37.55054	0.086748
253	3.698717	-9.96837	7.732666	3.08434	107.7176	1.657812	**253**	5.289343	0.465876	2.686286	5.957029	20.87108	0.080626
254	1.774061	-2.44484	4.446903	4.723588	121.4372	1.874379	**254**	1.725733	-13.4267	7.101762	3.115851	176.764	0.408203
255	2.984073	-17.2224	3.454112	0.460827	65.54719	1.086568	**255**	5.670579	-4.13136	1.154957	3.415638	9.196484	0.063304
256	3.745811	-17.6987	4.479451	2.096686	69.66131	1.107251	**256**	2.574396	-24.8085	0.299108	7.146814	14.77478	0.172999
257	3.234143	12.94468	1.778503	8.570617	27.25071	0.40003	**257**	0.919837	0.625179	2.430506	3.047071	102.3408	0.429658
258	4.921517	2.3547	3.266253	5.182164	31.23283	0.484101	**258**	9.1867	-3.02342	2.099608	3.636141	9.475216	0.043495
259	0.678921	-16.6647	3.169519	7.348108	298.3843	4.484281	**259**	5.495905	-11.8589	5.163035	5.912699	41.43972	0.111758
260	0.808805	0.871844	5.634684	2.5455	311.6532	4.872874	**260**	1.387583	-7.50764	1.338635	5.241099	47.60275	0.284452
261	3.12106	9.664721	6.428662	1.9691	82.29391	1.285577	**261**	8.729896	-7.26695	4.262989	2.778501	19.80395	0.059173
262	3.304844	-20.9613	7.172294	6.251014	136.7468	2.062925	**262**	5.824865	-7.99692	3.183252	4.607578	23.50235	0.082708
263	4.647794	6.33455	5.280729	6.707682	50.01977	0.754151	**263**	0.897553	-5.87317	2.710967	1.361101	119.5553	0.47355
264	1.214606	-23.4238	1.720826	3.355522	99.53109	1.665265	**264**	1.936683	-13.0236	4.242904	1.374473	93.01037	0.279116

265	5.453349	8.912204	3.481701	6.892463	28.44977	0.429917	**265**	7.671913	-17.7194	7.999582	4.730646	47.70837	0.104954
266	2.2973	12.59801	2.568928	9.391242	52.41587	0.762209	**266**	7.54821	-12.9026	1.767988	6.376456	12.09326	0.059763
267	4.303252	-5.63752	0.914868	1.307739	11.03577	0.220863	**267**	4.717905	-0.21924	4.431349	1.016613	34.3297	0.100537
268	1.890582	-16.4909	5.059777	7.869781	163.2232	2.434966	**268**	2.835311	-18.942	2.776546	4.296964	48.40389	0.18519
269	2.588288	-20.323	5.486896	3.72381	130.6213	2.022514	**269**	7.832248	-19.6428	4.00006	0.438457	23.23241	0.072265
270	5.311273	19.14429	1.382868	5.107926	11.43596	0.184927	**270**	2.550167	-9.43435	0.775551	4.132459	16.53744	0.144123
271	7.159405	-7.27062	4.964376	5.206183	35.80363	0.548176	**271**	4.171519	-7.75093	7.911437	0.927996	74.61987	0.163604
272	4.886155	-9.6724	5.815148	4.917107	62.77953	0.960186	**272**	6.924666	-6.5597	3.440911	2.819557	20.21628	0.068386
273	2.189486	5.535612	0.892444	5.844228	23.87182	0.385613	**273**	3.579375	-10.3489	3.690792	7.318775	47.03915	0.151006
274	1.680801	-17.4062	1.931647	1.837303	68.74991	1.183101	**274**	6.629378	-0.40257	1.44922	2.844699	8.949443	0.053443
275	1.738199	1.879362	4.599599	2.585266	117.7421	1.858413	**275**	4.040622	-17.5527	3.785217	4.349041	44.35375	0.141335
276	1.567178	5.780976	7.531699	3.472665	202.678	3.113514	**276**	4.662228	-26.9499	4.583605	3.48831	51.58682	0.148033
277	2.091326	3.14584	4.710221	1.071246	96.63516	1.548944	**277**	1.443013	-3.22594	6.072895	6.68401	167.87	0.418739
278	0.392711	12.18451	2.829644	2.418906	289.9506	4.738129	**278**	7.886169	-10.9801	4.211686	3.693031	22.93201	0.068909
279	6.660095	15.62303	7.813592	2.332855	43.90789	0.678911	**279**	7.022351	-9.80252	1.732153	9.129434	13.37942	0.064581
280	0.448887	-12.5319	2.544716	4.415592	329.0662	5.221883	**280**	5.602823	-1.52728	2.726821	4.641101	19.8076	0.076459
281	4.008394	2.571594	2.402926	5.011604	28.97037	0.455821	**281**	1.223392	-11.2684	2.792544	6.794544	108.126	0.406386
282	1.106938	3.506236	6.911487	5.513848	276.2209	4.190828	**282**	0.933798	-9.00966	2.597126	9.591835	137.6645	0.530326
283	6.131952	10.63326	3.573053	4.13475	24.27862	0.380609	**283**	3.432822	-3.30304	3.200207	7.386851	40.06341	0.139044
284	1.255727	24.19831	6.617349	1.018109	179.2952	2.818446	**284**	7.622847	0.266032	7.451202	1.285489	35.38898	0.07971
285	1.623797	15.372	4.033811	2.60961	95.32836	1.515244	**285**	2.74432	-12.4692	0.817981	3.158931	15.58064	0.136868
286	1.912835	-1.62711	4.654624	1.793267	111.9099	1.780788	**286**	3.477995	-6.09982	1.539927	5.367013	21.00318	0.114714
287	1.073755	8.740264	1.438268	7.657023	71.29809	1.069413	**287**	2.985836	-12.0876	5.574009	7.106639	83.23161	0.216315
288	3.929494	-15.9623	4.688444	3.955545	69.17808	1.074776	**288**	4.595127	-22.7306	0.615778	7.575781	11.57448	0.099003
289	1.268938	-0.10232	7.008741	4.299321	252.5799	3.864887	**289**	3.115496	-5.78789	0.467171	1.123186	6.737162	0.10168
290	5.14952	14.49239	5.593595	0.621095	40.67022	0.647604	**290**	5.303115	-27.7239	2.244205	3.816565	23.84001	0.10462
291	2.874958	9.085558	1.218765	3.877325	20.19573	0.343932	**291**	1.37419	2.374737	2.661687	3.132639	73.31785	0.290189

292	2.37445	1.667767	0.928913	4.555496	22.22539	0.378303	**292**	1.52003	-6.93922	2.99341	0.672696	77.44526	0.288626
293	1.181019	-12.8772	3.42664	7.581331	173.8476	2.60328	**293**	2.534688	-17.0208	2.039604	0.905937	36.24122	0.175582
294	3.476099	-5.39359	7.605822	6.566188	108.8464	1.637672	**294**	3.768545	-7.41618	5.46777	4.821739	59.79007	0.156621
295	2.312301	-5.00596	2.738724	3.573962	60.26574	0.966992	**295**	3.08937	-14.7104	0.842657	6.001852	17.17784	0.132584
296	1.344406	-6.18776	1.858692	5.731259	79.05671	1.238298	**296**	0.618039	-12.2773	7.313513	1.506716	493.6928	1.121771
297	2.84971	-2.79595	1.459342	2.014106	25.46497	0.452856	**297**	2.283932	-1.33676	4.130052	2.369751	68.40246	0.208179
298	4.220325	-15.1357	2.722844	2.028573	36.73892	0.606686	**298**	4.764491	-12.1614	1.906658	1.264834	17.22524	0.08715
299	2.068572	25.34013	5.119988	5.887821	88.58067	1.346393	**299**	0.941531	-6.79305	2.160071	5.303634	103.4259	0.458076
300	2.031385	-14.8928	2.005268	8.492022	67.97606	1.00092	**300**	2.995078	-9.53482	2.934984	4.499019	43.12395	0.159353