Universidade de Aveiro Departamento de Química
2021

Bárbara Maria Correia Vaz

Desenvolvimento de processos *downstream* para a valorização de pigmentos: um passo em direção a uma biorefinaria marinha

Development of downstream processes for pigment valorization: a step towards blue biorefinery

Bárbara Maria Correia Vaz

Desenvolvimento de processos *downstream* para a valorização de pigmentos: um passo em direção a uma biorefinaria marinha

Development of downstream processes for pigment valorization: a step towards blue biorefinery

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre 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.

“Believe you can and you’re halfway there.”

o júri

presidente

Prof. Doutor Carlos Manuel Santos da Silva
Professor Associado do Departamento de Química da Universidade de Aveiro

Prof. Doutor Jorge Fernando Brandão Pereira
Professor Auxiliar do Departamento de Engenharia Química da Universidade de Coimbra

Prof. Doutora Sónia Patrícia Marques Ventura
Professora Auxiliar do Departamento de Química da Universidade de Aveiro

agradecimentos

Chegar até aqui foi uma completa vitória.

Primeiro que tudo tenho de agradecer à Lisa por me ter convencido de que estava no curso certo e que não valia a pena mudar.

Em segundo, tenho de agradecer do fundo do coração à Prof. Sónia por me ter dado a oportunidade de ir trabalhar com ela. Foi a primeira vez que me apercebi que o meu sonho de entrar em doutoramento poderia vir a ser uma realidade.

Em terceiro lugar, não posso deixar de agradecer à Margarida, admiro-a imenso por todo o trabalho, dedicação e empenho em tudo o que faz. Ensinou-me tudo o que sei, e esteve lá sempre para me apoiar.

A seguir, tenho também de deixar um grande agradecimento à Andreia, acompanhou-me desde o início nesta minha jornada da investigação e até hoje nunca se deixou de preocupar comigo e está lá sempre para tudo o que eu preciso.

Agradeço também à Mariam por me ter acompanhado e apoiado nesta última etapa da dissertação.

Também não posso deixar de agradecer às minhas amigas, às Patrícias, à Daniela e à Carolina, e ao meu namorado. Só eles sabem o quão difícil foram estes 5 anos. Entre gerir o trabalho com os estudos, ter tempo social para os amigos e família, e fazer parte de um núcleo cultural, o tempo era sempre contado e nem sempre dava para tudo, e sem o apoio deles nada tinha sido possível.

Por último, agradeço à minha mãe e à minha irmã por me terem acompanhado em mais uma etapa da minha vida.

palavras-chave

Biorefinaria marinha, pigmentos, processamento *downstream*, solventes alternativos

resumo

As diretrizes da Agenda 2030 para o Desenvolvimento Sustentável foram muito claras. Novas estratégias precisam de ser encontradas para garantir um futuro sustentável. Desta forma, os recursos marinhos surgem como matérias-primas naturais e renováveis para integrar uma estrutura de biorefinaria marinha e uma abordagem de economia circular. Através da conversão de biomassa, uma produção integrada de biocombustíveis e compostos de valor acrescentado pode ser projetada, permitindo a recuperação de vários compostos com impactos ambientais mínimos e um crescimento económico sustentável. Pigmentos são compostos naturais utilizados em aplicações de elevado padrão devido à sua cor e ao seu alto interesse biológico, podendo ser encontrados em diversos recursos marinhos. No entanto, as técnicas convencionais para recuperá-los requerem o uso de metodologias complexas e demoradas que podem levar à degradação e/ou perda de estabilidade dos compostos, alto consumo de energia e baixos rendimentos de extração. Solventes alternativos, como soluções aquosas de líquidos iónicos e surfactantes, aparecem como opções mais sustentáveis que podem melhorar a estabilidade térmica e química dos pigmentos, obter rendimentos de extração mais altos, enquanto minimizam os impactos ambientais do processo. Neste trabalho, foram desenvolvidos dois procedimentos para recuperar diferentes pigmentos utilizando solventes alternativos, a fim de superar as dificuldades encontradas nos métodos convencionais. No primeiro trabalho, uma técnica de extração em fase sólida foi usada para fracionar clorofilas e xantofilas. A seguir, para eluir as clorofilas da resina AmberLite™ HPR900 OH, as soluções aquosas de líquidos iónicos revelaram-se solventes eficientes para valorizar simultaneamente xantofilas e clorofilas, e reaproveitar a resina em vários novos ciclos de purificação. O segundo trabalho teve como foco a extração do pigmento bacterioruberina com soluções aquosas de surfactantes a partir da *Haloferax mediterranei*, sendo a classe dos não iónicos a que apresentou maiores rendimentos de extração. Além disso, a purificação da bacterioruberina também foi abordada através de uma simples precipitação induzida com etanol como anti-solvente para recuperar proteínas como um segundo composto de elevado valor comercial. Em ambos os trabalhos foram desenvolvidos com sucesso procedimentos de fácil implementação e ambientalmente sustentáveis recorrendo ao uso de solventes aquosos, sendo as condições operacionais otimizadas para reduzir os impactos económicos e ambientais, e permitir alcançar rendimentos mais elevados.

keywords Marine biorefinery, pigments, downstream process, alternative solvents

abstract

The guidelines of The 2030 Agenda for Sustainable Development were very clear. New strategies need to be found to ensure a sustainable future. In this way, marine resources emerge as natural and renewable feedstocks to integrate a blue biorefinery framework and a circular economy approach. Through biomass conversion an integrated production of biofuels and value-added compounds can be designed, allowing the recovery of multiple compounds with lower environmental impacts and a sustainable economic growth. Pigments are natural compounds used in high-end applications due to their colour and high biological interest that can be found in several marine resources. However, the conventional techniques to recover them require the use of complex and time-consuming methodologies that may lead to the degradation and/or loss of stability of the compounds, high energy consumption, and low yields of extraction. Alternative solvents such as aqueous solutions of surfactants and ionic liquids (ILs) appear as more sustainable options that can improve thermal and chemical stability of pigments, obtain higher extraction yields, while minimizing the environmental impacts of the process. In this work, two procedures were developed to recover different pigments using alternative solvents, to overcome the bottlenecks found in the conventional methodologies. In the first work, a solid-phase extraction technique was used to fragmentate chlorophylls from xanthophyll. Then, to elute chlorophylls from AmberLite™ HPR900 OH resin, aqueous solutions of ILs revealed to be efficient solvents to valorize simultaneously xanthophyll and chlorophylls while reusing the resin in several new cycles of purification. The second work focused on the extraction of bacterioruberin pigment from *Haloferax mediterranei* with aqueous solutions of surfactants, being the non-ionic class the one that showed higher extraction yields. Moreover, the purification of bacterioruberin was also addressed by inducing a simple precipitation with ethanol as anti-solvent to recover proteins as a second added-value compound. In both works easy to implement and environmentally friendly procedures using water-based solvents were successfully proposed, being the operational conditions optimized to reduce both economic and environmental impacts, and to reach higher yields.

Index

List of Abbreviations

ANOVA	Analysis of variance
BSA	Bovine serum albumin
CCRD	Central composite rotatable design
Chl	Chlorophyll
CMC	Critical micelle concentration
DNA	Deoxyribonucleic acid
F	F-value
fuco	Fucoxanthin
HPLC	High-performance liquid chromatography
IL	Ionic liquid
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
RSM	Response surface methodology
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SF	Supernatant fraction
SLR	Solid-liquid ratio
UHPLC-MS	Ultra-high performance liquid chromatography coupled to mass spectroscopy
UV	Ultraviolet
UV-Vis	Ultraviolet visible
YPC	Yeast, peptone and casamino acids media

List of Symbols

C_{IL}	Concentration of ionic liquid in water
C_{Surf}	Concentration of surfactant in water
m/z	Mass-to-charge ratio
n	Number of variables
^{o}C	Degree celsius
p	p-value
R^2	Coefficient of determination
rpm	Rotation per minute
t	Time
V	Volume
V1,V2,V3	Experimental values in response surface methodology for validation of the optimum conditions
W	Weight
wt %	Weight fraction percentage
X1,X2,X3	Independent variables in the response surface methodology
Y	Dependent variable in the response surface methodology

List of Chemicals

$((HOCH_2)_3CNH_2$	Tris(hydroxymethyl)-aminomethane
$[C_3mpip]Cl$	1-methyl-1-propylpiperidinium chloride
$[C_4C_1im]Cl$	1-butyl-3-methylimidazolium chloride
$[C_4mpyr]Cl$	1-butyl-1-methylpyrrolidinium chloride
$[C_4py]Cl$	1-butylpyridinium chloride
$[C_6C_1im]Cl$	1-hexyl-3-methylimidazolium chloride
$[C_8C_1im]Cl$	1-methyl-3-octylimidazolium chloride
$[C_{10}C_1im]Cl$	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-hexadecyl-3-methylimidazolium chloride
$[C_{16}py]Cl$	Hexadecylpyridinium chloride
$[N_{1,1,1,10}]Br$	Decyltrimethylammonium bromide
$[N_{1,1,1,12}]Br$	Dodecyltrimethylammonium bromide
$[N_{1,1,1,14}]Br$	Tetradecyltrimethylammonium bromide
$[N_{1,1,1,16}]Br$	Hexadecyltrimethylammonium bromide
$[N_{1,1,1,6}]Br$	Hexyltrimethylammonium bromide
$[N_{4,4,4,4}]Cl$	Tetrabutylammonium chloride
$[P_{4,4,4,14}]Cl$	Tributyltetradecylphosphonium chloride
$[P_{4,4,4,4}]Br$	Tetrabutylphosphonium bromide
$[P_{4,4,4,4}]Cl$	Tetrabutylphosphonium chloride
$[P_{8,8,8,8}]Br$	Tetraoctylphosphonium bromide
AOT	Dioctyl sulfosuccinate sodium salt
D_2O	Deuterium oxide
DTT	Dithiothreitol
Genapol® C-100	-
Genapol® X-080	Polyethylene glycol monoalkyl ether
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate monobasic
Merpol® A	-
Na_2HPO_4	Di-sodium hydrogen phosphate anhydrous
NaCl	Sodium chloride
NaOH	Sodium hydroxide
SDS	Sodium dodecyl sulfate
SDBS	Sodium dodecyl-benzenesulfonate
Triton™ X-114	Polyethylene glycol *tert*-octylphenyl ether

Tween® 20	Polyethylene glycol sorbitan monolaurate
Tween® 80	Polyethylene glycol sorbitan monooleate

List of Figures

List of Tables

CHAPTER 1. Introduction

1.1. Motivation

Many steps are being taken now towards a greener and more sustainable economy to meet The 2030 Agenda for Sustainable Development adopted in 2015 by all United Nations Member States.[1] The 17 Sustainable Development Goals (SDGs) and 169 associated targets developed based on the goals not achieved by the Millennium Development Goals intend to balance the three dimensions of sustainable development: economic, social and environmental.[1,2] This plan is designed to eradicate poverty and hunger, while developing strategies to combat inequality and protect human rights, to achieve peace and justice, to ensure the protection of natural resources, to improve health and education, and to create a sustainable economic growth, having all countries and stakeholders the mission to join forces and help achieve this end.[1,2]

The development of marine technology through a sustainable and efficient management of natural resources to achieve clean industries with reduced environmental impacts, while addressing waste minimization and recycling, combines three major pillars (SDG 9, 12 and 14) that the 2030 Agenda for Sustainability Development pretends to attain.[3] As oceans represent more than two-thirds of the Earth's surface,[4] their extensive biodiversity is a source of many natural bioactive compounds with huge industrial potential.[5]

For centuries, the use of ocean resources has been fundamental in societal development, mainly in the food sector such as the obtention of marine living resources like fish or evaporation of brine to collect salt.[6] More recently, a set of new bio-based products have surfaced to meet the ever-growing demand for natural alternatives to the synthetic compounds (present in food, cosmetics and pharmaceuticals) and fossil fuel resources.[7] Consequently, marine resources received much attention as natural, renewable and sustainable feedstocks with industrial potential to be a part of food, materials, chemicals, fuel, cosmetics, and medicines.[8,9] However, these resources were mostly studied for the production of biofuels, with studies showing that its production alone is not economically viable due to the high costs associated with the process.[10] To improve the process economics, a cost-effective integration of technologies is needed to get maximum value from raw materials.[11]

The biorefinery approach is designed to achieve an integrated production of biofuels and high-value co-products through biomass conversion.[12] It not only aims to maximize the economic value of raw materials (feedstocks) but also to avoid the production of waste streams by generating multiple products.[7,13] Furthermore, it allows the development of an industrial production with minimum negative impacts on the environment in order to achieve a sustainable economic growth.[14] All the products obtained from raw materials can be separated to be commercialized or can be the feedstock for other manufacturing operations as a way to obtain value-added products.[15] Even so, waste and leftovers, that are generated throughout the process, can still be used as by-products. If it is no longer possible to integrate them as inflows to another process, they can always be used for energy production. This cascade approach (Figure 1.1.1A) of valuing first the high quality biomass, and then to utilize the low quality biomass for energetic purposes, enables not only the minimization of waste streams preferentially to zero, but also increases resource efficiency, being part of the circular economy strategy.[15] In a circular economy the goal is to minimize waste and resource consumption by circulating and recycling the products for as long as possible using preferably renewable resources and non-toxic chemicals. The life cycle of the products is redesigned to maximize the use of biomass in order to save resources and

minimize environmental impacts.[16] In this way, a circular bio-based economy could be a path forward to ensure a sustainable future for our planet (Figure 1.1.1B).[17]

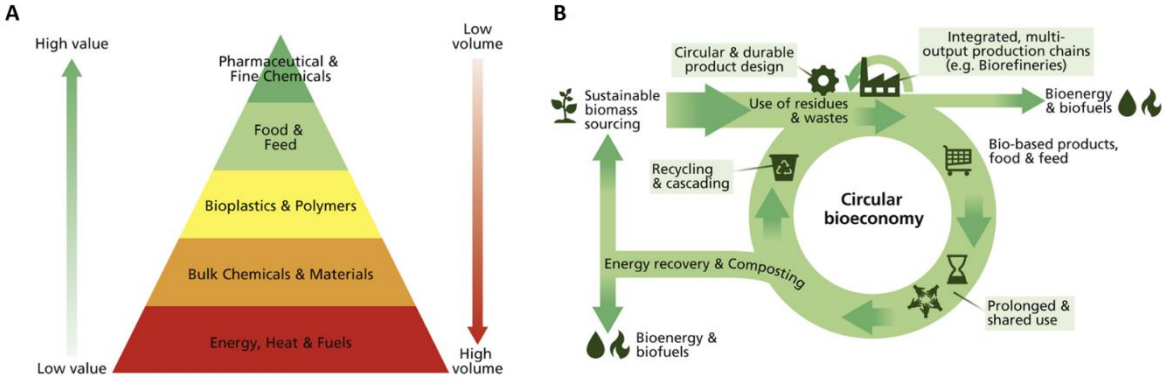


Figure 1.1.1. Schematic representation of the bio-based value pyramid considering the cascading use of biomass (A) and the circular bioeconomy and its elements (B). Adapted from Stegmann et al. (2020).[18]

Algae are well-known photosynthetic organisms that are mostly found in aquatic environments like ponds, lakes, wastewater, rivers, and oceans. They are responsible for the majority of the oxygen available, and approximately 40 % of global photosynthesis, being able to grow alone or together with other organisms under different conditions of light intensity, pH, salinity and temperature.[8,19] The classification of algae can be generally divided by colour as Phaeophyta (brown algae), Chlorophyta (green algae), and Rhodophyta (red algae), and by size as microalgae or macroalgae (also known as *seaweed*). Macroalgae are multicellular organisms visible to the naked eye, while microalgae are monocellular organisms only identified by a microscope.[8,19] Compared to other plants, microalgae have higher growth rates and can be cultivated using small amounts of water, nutrients and atmospheric CO_2. Since they can grow in degraded land without pesticides, there is no competition for land and food crops. Besides, microalgae can significantly reduce greenhouse gas emissions by being able to sequester the carbon dioxide from flue gases produced by industries and released to the environment, and excess carbon dioxide in the atmosphere.[20–22] A wide range of high-value compounds are produced, including lipids, carbohydrates, proteins and pigments.[23] Microalgae synthesize different types of pigments that can belong to one of three classes: chlorophyll (green colour), carotenoid (yellow/orange colour) or phycobiliprotein (red/blue colour).[19] Examples of their applications include use as vitamin precursors, additives and dyes in food, biomaterials, animal feeds, pharmaceuticals and cosmetics.[20] Carotenoids are natural pigments with vibrant colours varying from red to yellow that are easily found in nature, being present in several algae, bacteria, plants and fungi.[24] They are terpenoid pigments sensitive to light, oxygen and heat, that commonly share the same C_{40} backbone structure of isoprene units.[25] Their chemical structure divides them into two groups: carotenes, composed of carbonated chains, and xantophylls, composed of carbonated chains with at least one functional group containing oxygen (oxygenated hydrocarbon derivatives).[24] Carotenoids are also lipophilic compounds with light-absorbing characteristics necessary for photosynthesis,[25] with a variety of applications in medicine, as anticancer, immunomodulatory, anti-inflammatory, antibacterial, antidiabetic and neuroprotective, in food, as colorants, supplements and

antioxidants,[26] and in cosmetics products as protectors, tanners, creams, between others, due to their antioxidant and photoprotective properties.[27,28]

Chlorophylls, one of the most abundant pigments in nature, are found in algae, plants, and cyanobacteria in different chemical structures, being the most common a chlorin ring with four nitrogen atoms surrounding a magnesium ion in the center.[29] They are mostly lipid-soluble green pigments that play an important role in conversion of energy by capturing photons and transferring them to the photosynthesis reaction centers.[30] Their antioxidant,[31] antibacterial [32] and antimutagenic properties are significant for cancer prevention as anti-tumour agents,[33] and their photophysical and photochemical properties are the key for photodynamic and photothermal therapy, photocatalytic diagnosis, and also photoacoustic, magnetic resonance and nuclear medical imaging.[34] Furthermore, chlorophylls as well as carotenoids are also used in cosmetics and food industries.[35]

Other less explored marine resource, not present in the oceans or seawater such as haloarchaea, should also be considered alternative feedstocks to integrate the biorefinery framework. These extreme halophilic microorganisms, belonging to the *Haloferacaceae* and *Halobacteriaceae* families, phylum *Euryarchaeota*, Archaea domain, represent the major microbial population of hypersaline environments, being spread around the world in marshes, salt ponds or salt lakes.[36] They are mainly red-coloured aerobic heterotrophs that present metabolic adaptations due to the limited water available and high ionic strength, characteristics of extreme growing conditions.[37] These adaptations lead to the production of different added-value compounds with high (bio)technological interest to potentially be implemented on a large scale, namely enzymes, poly(3-hydroxybutyrate), polyhydroxyalkanoate, and carotenoids.[36] For the production of carotenoids, the use of halophyte microorganisms has two main advantages: first, they can growth under non-sterile conditions, since the high concentration of salt prevents contamination from other organisms; second, the accumulation of carotenoids can be easily increased by changing the culture medium conditions, like pH, temperature, oxygen concentration, light irradiance, and/or salt concentration. Several carotenoids can be found in haloarchaea microorganisms.[36] The most abundant is bacterioruberin, however lower quantities of carotene, lycopene, canthaxanthin, 3-hydroxy-echinenone, lycopersene, phytoene, phytofluene, and 2-isopentenyl-3,4-dehydrorhodopin, can also be identified.[38]

Bacterioruberin and its derivatives, monoanhydrobacterioruberin, bisanhydrobacterioruberin, and trisanhydrobacterioruberin, are C_{50} carotenoids that can be found in two different geometries, as *trans* or *cis* isomers (5-*cis*-bacterioruberin, 9-*cis*-bacterioruberin, 13-*cis*-bacterioruberin, 15-*cis*-bacterioruberin, and 5-*cis*, 9'-*cis*-bacterioruberin).[38] Bacterioruberin structure is composed of 13 pairs of carbon-carbon conjugated double bonds in their primary conjugated isoprenoid chain with four hydroxyl groups in the terminal ends. This higher number of conjugated double bonds compared to the carotenoids with 40 carbon atoms produced by most photosynthetic organisms, confers C_{50} carotenoids higher antioxidant capacities responsible for the protection of cells against harmful effects of radiation and osmotic stress, demonstrating enormous potential in food and pharmaceutical industries.[39] Other important biological functions are the improvement of cell membrane reinforcement and membrane fluidity due to the presence of the 4 hydroxyl groups in bacterioruberin structure.[37] Although there are still few studies on the applications of this pigment, its potential has already been proven in the field of biomedicine as antimicrobial,[40] as improver of sperm motility,[41] and in prevention of skin cancer or rehabilitation of skin tissue,[42] since it is able to repair human DNA strands.

Owing to the great potential of marine resources as natural sources of valuable bio-active compounds, efficient methodologies to recover them need to be developed under the guidelines of biorefinery and circular economy. These methodologies should follow three main steps for a sustainable development and exploitation of the biomass: aquaculture, harvesting, and downstream processes, as represented in Figure 1.1.2.[43]

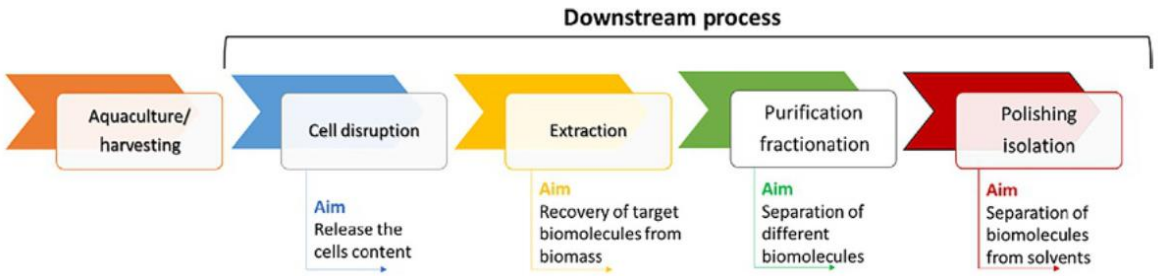


Figure 1.1.2. Schematic representation of the general steps of downstream processes.[43]

First, the biomass is obtained by cultivation/harvesting of cells where the focus should be on developing optimization approaches to maximize or induce the production of primary and secondary metabolites of organisms. Next, as part of the downstream process, the cell disruption and extraction steps include the rupture of the cell wall, and consequently, the recovery and solubilization of the intracellular biomolecules of interest, which can be performed simultaneously. Lastly, if necessary for the final application, a purification step should be applied to obtain higher purities of the compounds by separating them from each other, and an isolation of compounds from the solvents used (polishing step) to be able to reuse/recycling them.[43]

Industries are conventionally using decoction, maceration, infusion, digestion, percolation and soxhlet as extraction techniques involving the use of organic solvents. Besides requiring high volumes of solvent, these processes often face low yields of extraction, high energy consumptions and possible degradation of the compounds.[44,45] In recent years, non-conventional techniques like enzyme assisted extraction, microwave-assisted extraction, ultrasound-assisted extraction, supercritical fluid extraction, pressurized liquid extraction, and pulsed electric field assisted extraction, are being explored to efficiently extract the target compounds at lower costs, with higher yields and lower treatment times, and taking into consideration the sustainability of the procedure involved.[46] However, all techniques have limitations, and thus, the selection of the appropriate method to use should be based on several factors: biochemical properties of the compound, choice of solvent, extraction yield, time of the operation, reproducibility, denaturation or degradation of the compounds, simplicity and cost of the process, market value of the compounds, environmental effects and suitability of the operation to be applied industrially.[44,45]

The use of organic solvents causes problems related to workers exposure, toxicity, storage and flammability, which increases the urgency to find safer options.[45] Thus, the introduction of green solvents as alternatives to the traditional organic solvents to integrate downstream processes, either in the extraction or purification steps, has received much attention recently. In most cases, an improvement in thermal and chemical stability of pigments, higher extraction yields, and a reduction in environmental impacts was seen when alternative solvents as ionic liquids, eutectic solvents, surfactants and edible oils were applied.[47]

Ionic liquids (ILs) are salts with low charge density and low symmetry ions, which consequently lead to low melting temperatures (usually below 100 °C). Their usual composition of large organic cations and organic or inorganic anions with a possibility of multiple ions combinations, give them a label of "*designer solvents*". Given that, by tunning their properties some adaptation can be made to meet a specific end, namely their hydrophobicity and solution behaviour, their thermophysical properties, and their biodegradability and toxicological characteristics.[48] Other features of ILs usually include low to negligible vapour pressure and low flammability, high thermal and chemical stabilities, wide liquid temperature range, high ionic conductivity, and high solvation capacity for organic, inorganic and organometallic compounds.[49] Due to their distinctive properties, their use as extraction solvents can induce the cell wall lysis (ILs with long alkyl chains), and enhance the selectivity of the solvent to the target compounds depending on the IL chosen. However, besides the ILs potential, some controversy is found regarding economic viability and sustainability. Many ILs contain toxic and poorly biodegradable constituents that, associated with high production costs compromises their industrial applicability.[49] To overcome these issues three approaches should be considered. First, the use of aqueous solutions instead of pure ILs, in addition to reducing environmental and economic impacts, and decreasing the viscosity of the system, has already reported to be more efficient leading to higher extraction yields. Second, the choice of the solvent should not only be based on ions interactions to the compounds, but also in finding cheaper (Figure 1.1.3) and sustainable ILs for the same purpose. Lastly, a final step to isolate the compounds from the IL solution should always be considered to be able to reuse the IL in new cycles [43] or in functional materials to create valuable products.[50] Although this step is still poorly explored, some alternatives have been proposed consisting of back-extractions using organic solvents, evaporation of the solvents or compounds (when possible), precipitation with antisolvents, and through the use of macroporous material or anion-exchange resins.[48]

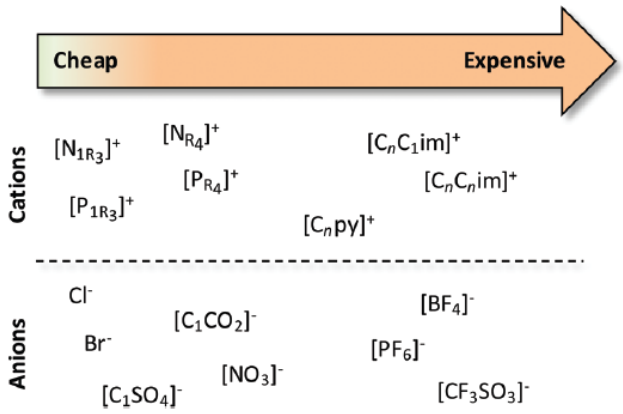


Figure 1.1.3. Schematic representation of the estimation of relative prices of ILs according to the cost of the raw materials used in their synthesis.[48]

Surfactants consist of another alternative class of solvents under investigation, widely used as a part of industrial and domestic day life in detergents, paper, cosmetics, motor oils, pharmaceuticals, and in more high-technology areas like electronic printing, magnetic recording, biotechnology, and microelectronics. Their

molecular structure generally consists of a lyophobic group with poor attraction to the solvent and a lyophilic group with a strong attraction to the solvent, thus being recognized by their amphipathic structure. When a molecule with this structure contacts with a solvent, the lyophobic group can alter the structure of the solvent, increasing the free energy of the system. As a result, the system tries to minimize the contact between the lyophobic group and the solvent. In the case of water, the lyophobic group breaks the hydrogen bonds of water molecules and structure them near the hydrophobic group by forming a single layer of surfactant molecules with their lyophobic groups oriented to the air minimizing the contact with water molecules.[51] Though, at the critical micellar concentration (CMC), surfactants interact with the cells releasing the intracellular content,[52] while starting to form aggregates called micelles, able to solubilize more hydrophobic compounds in water.[53] These interactions can be adjusted through the choice of the solvent, between cationic (positive charge), anionic (negative charge), non-ionic (non-ionic charge) or zwitterionic (positive and negative charges) surfactants. Regardless of the clear advantages of these solvents integrating extraction procedures, similar to ILs, some concerns are raised regarding their biodegradability and toxicity [51] that can be overcome by following the same strategies mentioned for ILs.

1.2. Scopes and objectives

So far, extraction, purification and polishing steps remain the major bottlenecks of downstream processes.[43] Despite the efforts of academia, some of the reported strategies to recover pigments are not economically viable, mostly because of the overall complexity of the process or due to low pigment recoveries, potentially complicating the scale-up of the process.[47]

Under this context, this thesis aims to:

i) develop efficient approaches to extract and purify different pigments using alternative solvents and methodologies;
ii) assess strategies to recover the solvents for their reuse in new cycles;
iii) envision the processes applicability in industry by proposing their final integrated design.

In this way, this thesis intends to contribute to the development of sustainable processes to integrate the blue biorefinery under the guidelines of a circular economy.

In short, this thesis is divided into 4 Chapters described below, plus Bibliography and Appendix.

Chapter 1: explains the motivation of this thesis, including the concepts of marine resources, circular economy, biorefinery, pigments, and alternative solvents. The scopes and objectives of this work were also incorporated;

Chapter 2: describes the recovery of pigments using alternative solvents through different techniques:

Chapter 2.1: encloses the purification of pigments from the microalgae *Isochrysis galbana* using solid-phase extraction processes and aqueous solutions of ionic liquids as chlorophyll eluents, based on the published article "Using aqueous solutions of ionic liquids as chlorophyll eluents in solid-phase extraction processes", Chemical Engineering Journal, 2022, DOI: 10.1016/j.cej.2021.131073.

Chapter 2.2: includes the extraction and purification of bacterioruberin from *Haloferax mediterranei* ATCC 33500 using alternative solvents, based on the article "Recovery of bacterioruberin and proteins using aqueous solutions of surface-active compounds", to be published soon.

Chapter 3: defines the final remarks and future perspectives.

Chapter 4: expresses the scientific contribution of this thesis, considering all the experience that served as the basis for its development.

Bibliography: details the references used throughout the work.

Appendix: includes the details of the experimental work done in Chapter 2.

CHAPTER 2. Recovery of pigments using alternative solvents

2.1. Using aqueous solutions of ionic liquids as chlorophyll eluents in solid-phase extraction processes

This chapter is based on the published manuscript:

Bárbara M.C. Vaz, Margarida Martins, Leonardo M. de Souza Mesquita, Márcia C. Neves, Andreia P.M. Fernandes, Diana C.G.A. Pinto, M. Graça P.M.S. Neves, João A.P. Coutinho, Sónia P.M. Ventura,* "Using aqueous solutions of ionic liquids as chlorophyll eluents in solid-phase extraction processes", Chemical Engineering Journal, 2022, DOI: 10.1016/j.cej.2021.131073.

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

Abstract

There is a need for handy and fast techniques to purify biomolecules, increasing their stability and value, because the separation units are current bottlenecks in downstream processes. Solid-phase extraction is a technique that enables the purification of a compound by its adsorption from a liquid matrix. The AmberLite™ HPR900 OH resin allows the separation of chlorophylls from complex extracts, however the recovery of the chlorophylls is not easy to achieve. An innovative procedure to elute the chlorophyll from AmberLite™ HPR900 OH, based on the use of aqueous solutions of surface-active ionic liquids is proposed in this work. The operational conditions were optimized, showing that the resin can be reused for at least five cycles without losing its efficiency and the chemical structures of the pigments recovered were identified.

Keywords: AmberLite™ HPR900 OH, solid-phase extraction, chlorophylls, carotenoids, resin reuse, surface-active ionic liquids, eluents.

Introduction

There is a growing demand for natural products associated with the increased awareness of human health and environmental concerns that may be addressed using compounds from natural sources instead of synthetic alternatives.[54,55] The concerns about climate change and the new policies to reduce the environmental impact of industrial processes and products reinforce this trend,[56] particularly when integrated into a smart and low-waste chain of different products or within a circular economy approach.[16,54,55,57] Thus, natural sources like microalgae biomass arises as a renewable and sustainable feedstock.[21] Under the concept of biorefinery, where the goal is to obtain high-value added products, biofuels and energy from biomass, microalgae emerge as an important and sustainable alternative to fossil fuels.[21] The main advantages associated to microalgae are related with their cultures in degraded land using only water and atmospheric CO_2 and also with their high growth

rates.[21] So, like macroalgae or cyanobacteria,[58,59] microalgae may also be considered a source of valuable bioactive compounds.[21]

Natural pigments are 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.[21,60,61] Two good examples are the chlorophylls and xanthophylls (oxygenated carotenoids) that are well-known pigments present in photosynthetic organisms such as plants, microorganisms, and algae.[60]

Along with the sustainable use of natural resources, obtaining and processing them should also be a concern.[16,57] Several works have proposed alternative approaches to extract these pigments without compromise their stability,[62,63] and studied the economic [63] and environmental [64] viability of these processes. Aqueous solutions of surface-active ionic liquids (ILs) and common surfactants have been proposed as solvents to extract chlorophylls [63,65] and carotenoids [64,66] from various natural matrices. Surface-active ILs can be very efficient in extraction processes due to their ability to create small pores in the cell membrane, allowing the release of intracellular compounds, (cell disruption step in Figure 1.1.2) and by increasing the solubility of hydrophobic compounds (like chlorophylls and carotenoids) in water through the formation of micellar structures [63,67] which are formed above the critical micellar concentration (Extraction step in Figure 1.1.2).

Although the extraction of these pigments is straightforward,[63] since chlorophylls and carotenoids have similar polarities and are usually present in the same cellular sites,[68] their co-extraction usually occurs at similar conditions. This means that an additional purification step is required to separate chlorophylls from carotenoids (Purification step in Figure 1.1.2). 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.[69] However, this purification step remains the bottleneck of the whole process,[70] because conventional techniques such as chromatography processes or saponification reactions are not only energy/time consuming but also require specific equipment and several steps to achieve the desired result.[71,72]

A simpler and reliable separation process for this purpose is the solid-phase extraction. This 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).[73–75] AmberLite™ HPR900 OH is a strong basic anionic resin that allows an effective and fast adsorption of chlorophyll, while the carotenoids remain in solution.[73–75] Its efficiency was already demonstrated for various extracts from green vegetables such as beans, broccoli, spinach, lettuce, and peas, among others.[74,75] 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 (Polishing step in Figure 1.1.2).

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, with the polishing step being accomplished by the use of aqueous solutions of surface-active ILs, to elute chlorophylls. The reutilization of

the resin was also carried during five cycles in batch and in continuous regimes to show the ability of this approach to extend the lifetime of the resin envisioning its industrial application.

Experimental section

Biomass

The microalga 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,[75] 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 based on ammonium family such as dodecyltrimethylammonium bromide, $[N_{1,1,1,12}]Br$ (99 wt% of purity, CAS 1119-94-4) and tetradecyltrimethylammonium bromide, $[N_{1,1,1,14}]Br$ (98 wt% of purity, 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. 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 A.1 in Appendix A. The deuterium oxide (99.9 atom % D 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. (2021).[63] 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 ^{o}C), during 30 min and protected from light exposure.[63] The samples were centrifuged in a Thermo Scientific Heraeus Megafuge 16R centrifuge at

4700 *g*, for 30 min at 4 °C.[63] The supernatant (initial extract) was collected and the biomass debris was discarded.

Chlorophyll adsorption and carotenoids recovery

The commercial resin AmberLite™ HPR900 OH was washed with distilled water following the procedure proposed by Larsen and Christensen (2005),[75] 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.[75] 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 analyzed for each initial solvent.

Chlorophyll elution and resin regeneration

Various solutions, reported in Table 2.1.1, were screened for their performance to elute chlorophylls from the resin, with ethanolic extracts being used in the adsorption step. Concentrations above the critical micellar concentration (CMC) were always used for the aqueous solutions of ILs (CMC values available at Table A.1 in Appendix A). For each case, the same conditions of chlorophyll adsorption were applied,[75] *i.e.* the resin was contacted with 10 mL of the regenerating eluent under stirring at room temperature (20–25 °C) during 30 min. After this period, the chlorophyll content of the solution was analyzed by UV-Vis spectroscopy.[63] Three replicates were made in order to decrease the error associated with each assay.

Table 2.1.1. Screening of eluents used to recover chlorophyll.

Type	**Eluents**	**Concentration**	**Ref.**
Alkaline	Sodium hydroxide	4 % (w:v) in water	73
Hot water	Distilled water at 80 °C	100 %	76
Organic solvents	Dichloromethane	100 %	77
	Acetonitrile	100 %	78
	Toluene	100 %	79
	Petroleum ether	100 %	80
Mixture of solvents	Formic acid, acetonitrile, and methanol	0.1 % : 69.93 % : 29.97 %	81
Aqueous solutions of ILs	$[N_{1,1,1,10}]Br$	250 mM in water	63,64
	$[N_{1,1,1,12}]Br$	250 mM in water	63,64
	$[N_{1,1,1,14}]Br$	250 mM in water	63,64
	$[P_{4,4,4,14}]Cl$	250 mM in water	63,64

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 analyzed 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 analyzed 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 2.1.2. The results were analyzed using the software Statistica® 12 according to the theory proposed by Dean et al. (1999) [82] and Rodrigues and Lemma (2014).[83] 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 2.1.2. 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).[63] 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. 2.1.1). Note that, during this work, it was used *chlorophyll recovery* instead of *eluted chlorophyll* since the chlorophyll content was analyzed 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,[84] diatoxanthin,[85] and fucoxanthin [86,87] (being also the diadinoxanthin the biologic precursor of both diatoxanthin and fucoxanthin),[88] 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 ($mg_{fuco}.L_{ethanolic\ extract}^{-1}$) calculated afterwards.

$$\text{Chlorophyll recovery (\%)} = \frac{\text{Chlorophyll in the collected fraction (mg)}}{\text{Chlorophyll in organic extract loaded into the resin (mg)}} \times 100 \qquad (2.1.1)$$

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 analyzed 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 adapted from Martins et al. (2021).[89] 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, up-scaled five times, 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.[73] The chlorophyll content of each solution collected from the solid-phase extraction cartridge was analyzed 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 colorless pellet was formed in the bottom of the flask and a green liquid in the top of the flask. This procedure was adapted from Mesquita et al. (2019).[62] 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.

Statistical Analysis

One-way ANOVA (Analysis of variance) was performed followed by Bonferroni post-hoc test to compare the efficiency between batch and continuous regime along five cycles of reuse of the resin. The results were expressed as the means ± standard errors of the mean. Statistically significant differences were determined considering an α of 95 % ($p < 0.05$).

Results and discussion

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 were later loaded in the resin.[74,75] However, after the adsorption of chlorophylls and the recovery of the carotenoids, no procedure was found to elute the chlorophylls from the resin. The scope of the present work was to develop a protocol that allows to elute the chlorophyll derivatives from the resin, without affecting their chemical structures and biological activities, and in this way, to extend also the resin's lifetime. Under this context, 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.[74,89] 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,[90] ethanol was used to progress the 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 [75] 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.[74] 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 A.2B in Appendix A).

According to the data sheet of the resin,[73] 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 2.1.1).

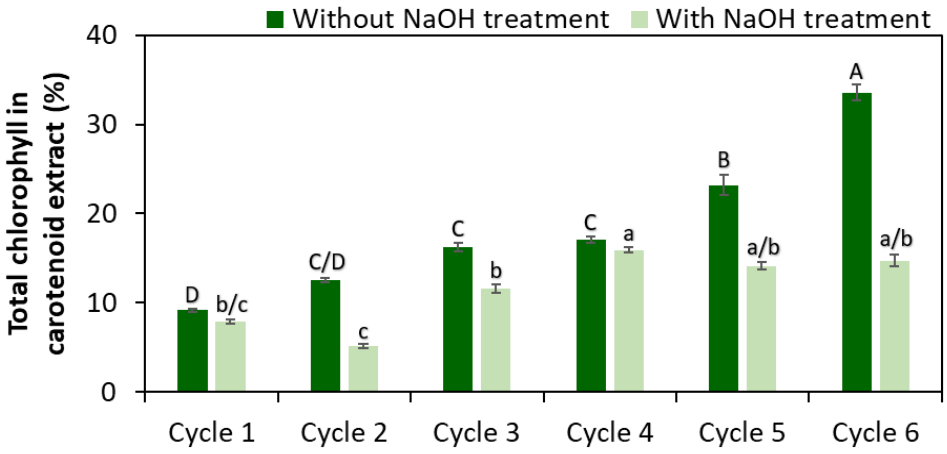


Figure 2.1.1. Percentage of the total chlorophyll present in the carotenoid extracts in several resin reuse cycles without and with NaOH treatment. Different capital letters represent statistically different values in the process without NaOH treatment ($p < 0.05$). Different lowercase letters represent statistically different values in the process using a NaOH treatment ($p < 0.05$).

The results in Figure 2.1.1 show that the contamination of chlorophyll in the carotenoid extract increases in each cycle ($p < 0.05$), 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 much lower and evolving towards an almost constant value (12–16 %) with the increase in the number of cycles of reuse, when the NaOH regeneration is carried (after cycle 4, there is no significant difference $p > 0.05$). 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 to extend the lifetime of the resin, the recovery of the chlorophyll pigments adsorbed is essential. Therefore, the performance of different eluents for an efficient recovery of the chlorophyll pigments was studied being the main results shown in Figure 2.1.2.

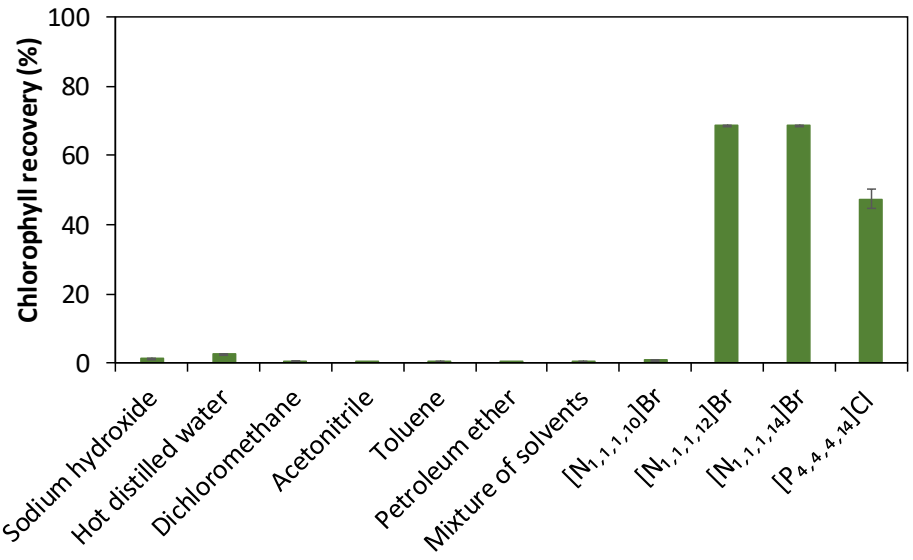


Figure 2.1.2. Screening of different solutions used to elute the chlorophyll from the resin. Aqueous solutions of ILs were screened at 250 mM and aqueous solutions of NaOH at 4 % (w:v).

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 for 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 2.1.2.

More appealing results were achieved when aqueous solutions of various ILs (at 250 mM) were investigated as eluents (Figure 2.1.2, right-side). The screened solutions of ILs were used before with success in the extraction of chlorophyll and carotenoids from biomass.[63,64] 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, see Table A.1 in Appendix A) providing the perfect environment to hydrophobic molecules.[63] 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 A.3 in Appendix A). 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 elution's of the resin, applying the same mass of resin and volume of eluent (fresh solutions of $[P_{4,4,4,14}]Cl$), were applied to achieve the maximum chlorophyll recovery from the resin (Figure 2.1.3). 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}). This analysis was just performed considering the IL $[P_{4,4,4,14}]Cl$,

since a similar response would be obtained with $[N_{1,1,1,12}]Br$. After, a more detailed analysis on the SLR 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 $[P_{4,4,4,14}]Cl$.

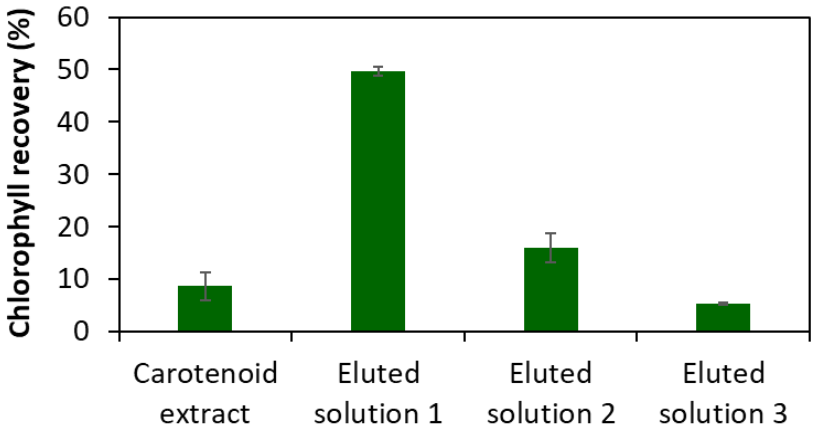


Figure 2.1.3. Percentage of chlorophyll recovered in the carotenoid extract and in three successive elution's of the resin, using $[P_{4,4,4,14}]Cl$ as eluent.

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 A.2 in Appendix A). The predicted values were expressed by the model provided by Eq. 2.1.2.

$$\text{Chlorophyll recovery (\%)} = 15.13349 + 0.29636(X2) + 0.28748(X3) - 0.00042(X3)^2 \qquad (2.1.2)$$

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 A.3 in Appendix A. 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 A.4 and A.5, respectively in Appendix A) 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. 2.1.2 was used to draw the response surfaces shown in Figure 2.1.4. As described in Eq. 2.1.2, 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 is not as energy intensive as ultrasound- and microwave-assisted extractions. The concentration of IL was completely optimized, achieving an optimum value around 350–400 mM (Figure 2.1.4). 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 A.4 in Appendix A), a very good result that suggests the high confidence and accuracy of the predictive model designed by the CCRD (2^3).

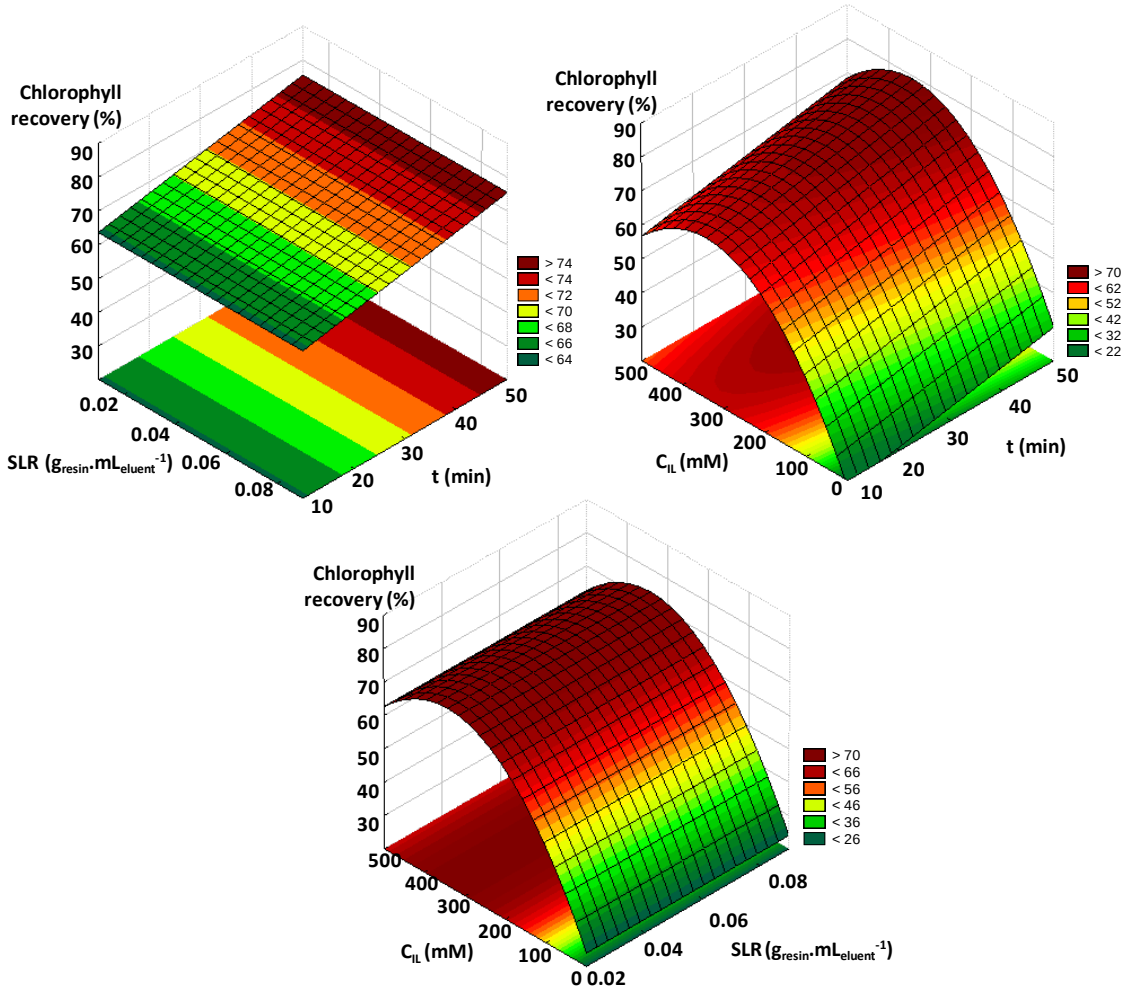


Figure 2.1.4. Response surface plots obtained for the CCRD (2^3) using an aqueous solution of $[P_{4,4,4,14}]Cl$ regarding: time of contact (t in min), IL concentration (C_{IL} in mM), and solid-liquid ratio (SLR in $g_{resin}.mL_{eluent}^{-1}$) in terms of percentage of the chlorophyll recovery from the resin.

Along with the aqueous solutions of $[P_{4,4,4,14}]Cl$, solutions of surface-active 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 $[N_{1,1,1,12}]Br$ as 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 (see Table A.2 in Appendix A). The data obtained in the Box-Behnken (Table A.5 in Appendix A) experiment was converted into a second-order polynomial equation with three independent variables (X1, X2, and X3), as described by Eq. 2.1.3.

$$\text{Chlorophyll recovery (\%)} = -15.30 + 322.06(X1) - 5751.61(X1)^2 + 1.17(X2) - 0.02(X2)^2 + 0.30(X3) + 0.62(X1 \times X3) \quad (2.1.3)$$

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 A.6 in Appendix A). The predicted and observed values were close to each other (Figure A.7 in Appendix A), making the model adequate. By applying ANOVA, the regression model was considered significant ($p < 0.05$, Table A.5 in Appendix A), 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 2.1.5, 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, see Table A.6 in Appendix A). 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 A.6 in Appendix A).

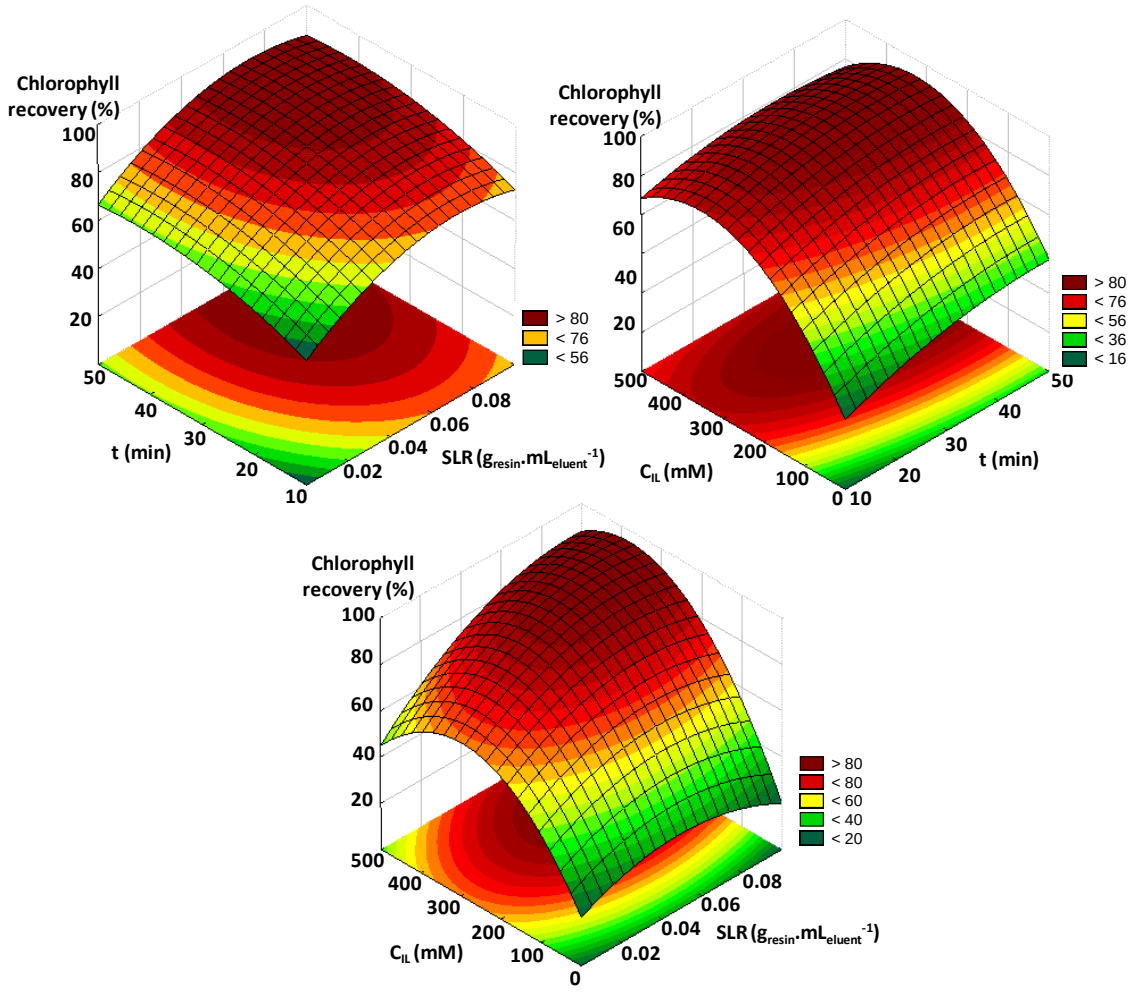


Figure 2.1.5. Response surface plots obtained for the CCRD (2^3) using an aqueous solution of $[N_{1,1,1,12}]Br$ regarding the time of contact (t in min), IL concentration (C_{IL} in mM), and solid-liquid ratio (SLR in $g_{resin}.mL_{eluent}^{-1}$) in terms of chlorophyll recovery (in percentage) from the resin.

Regarding the best conditions obtained for the aqueous solutions of the two ILs (summarized in Table A.7 in Appendix A), 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 $[P_{4,4,4,14}]Cl$.

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 considering the reuse of the commercial resin up to five times (Figure 2.1.6), 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 analyzed in terms of fucoxanthin content (Figure 2.1.6A). The concentration of fucoxanthin was kept constant from cycle 1 to cycle 5 (cycle 5 had the best performance), regardless the aqueous solution of IL used in the elution step, showing that the carotenoid fraction is not affected even considering five cycles reusing the resin. As depicted in Figure 2.1.6B, the three collected fractions were also analyzed in terms of chlorophyll when compared to the initial extract (before the solid-phase extraction).

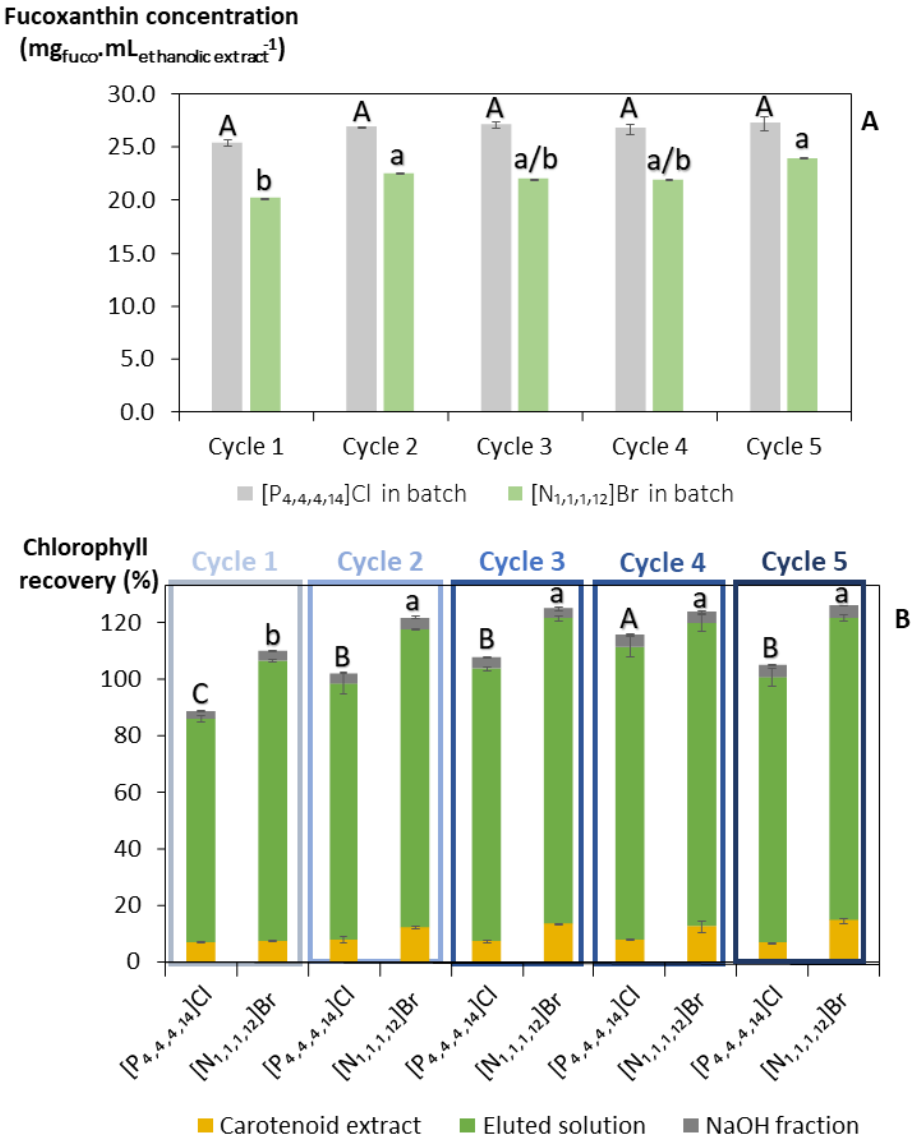


Figure 2.1.6. Comparison of the process using aqueous solution of [$N_{1,1,1,12}$]Br and [$P_{4,4,4,14}$]Cl as eluents at the optimized conditions previously selected along five cycles of reuse of the resin, considering (A) fucoxanthin concentration in the carotenoid ethanolic extract (first fraction collected from the resin); and (B) chlorophyll recovery in the different fractions collected from the resin. Note that different initial values in (A) are due to the need of preparing new initial extracts at the beginning of each experiment. Capital letters represent statistically results in the process mediated by [$P_{4,4,4,14}$]Cl ($p < 0.05$). Lowercase letters represent statistically results in the process mediated by [$N_{1,1,1,12}$]Br ($p < 0.05$).

It is important to note that values of chlorophyll recovery greater than 100 % in Figure 2.1.6B can be a result of the interference of slight amounts of the solvents from the previous steps present in the fraction being analyzed, due to some experimental difficulties to completely recover the liquid extracts from the resin leaving it dry for the next step, which may consequently change the behavior of the calibration curve. However, this only means that the recovery is complete, since the chlorophyll recoveries of the cycle 1 (for both ILs) are

lower compared to the other subsequent cycles ($p < 0.05$), *i.e.* the recycling approach guarantees better efficiency regarding the recovery of both classes of pigments under study. 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 slight increase in the content of chlorophylls in the extract of carotenoids. Additionally, for each IL individually, the behavior 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 A.2 in Appendix A and the UV-Vis spectra of the collected fractions in the first cycle for both ILs are depicted in Figure A.8 in Appendix A. In addition to the best elution performance of chlorophyll from the AmberLite™ HPR900 OH, surface-active ammonium-based ILs are commonly used in the industry due to its low price,[48] having lower associated environmental impacts when compared with other families of ILs.[91]

In the end and, after a deep analysis of all parameters optimized and results obtained, it was concluded that the ammonium-based IL provided the best results in terms of elution performance when compared with the phosphonium-based ILs. After the selection of the best eluent, the composition of the extracts at the different stages of the process was checked by UHPLC-MS. For that purpose, 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 analyzed, being the results obtained depicted in Table 2.1.3 (see also Figure A.3 in Appendix A). From the data collected using UHPLC-MS it was possible to confirm the presence of the chlorophyll derivatives (pheophorbide *a* and pheophytin *a*) in the initial extracts, and also the presence of the xanthophylls (fucoxanthin and diatoxanthin). Moreover, it was confirmed the ability 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 subsequently recover the adsorbed chlorophyll derivatives. Furthermore, the UHPLC-MS analysis confirmed minor 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 these results 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 A.3 in Appendix A).

Table 2.1.3. 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, although up-scaled five times, 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 analyzed, being the results depicted in Figure 2.1.7.

Figure 2.1.7. Comparison of the process using an 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. Capital letters represent statistically results in the process mediated by [$N_{1,1,1,12}$]Br in batch ($p < 0.05$). Lowercase letters represent statistically results in the process mediated by [$N_{1,1,1,12}$]Br in continuous ($p < 0.05$).

As previously observed for the batch process, the concentration of fucoxanthin (Figure 2.1.7A) was kept constant from cycle 1 to cycle 5, with no loss in efficiency in the collection of fucoxanthin (cycle 5 had better or equivalent performance compared to the previous ones). It can also be observed (Figure 2.1.7B) that, from

the second cycle onwards, the amount of chlorophyll in the different fractions analyzed 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 $\mu L.s^{-1}$ 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 ^{o}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 A.9 in Appendix A), 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 2.1.8. 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, for at least five cycles (number of complete cycles tested). The chlorophylls in the aqueous solution of IL are recovered using ethanol with 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 vacuum drying tecnique carried at low pressures and temperatures to avoid the degradation of the pigments.

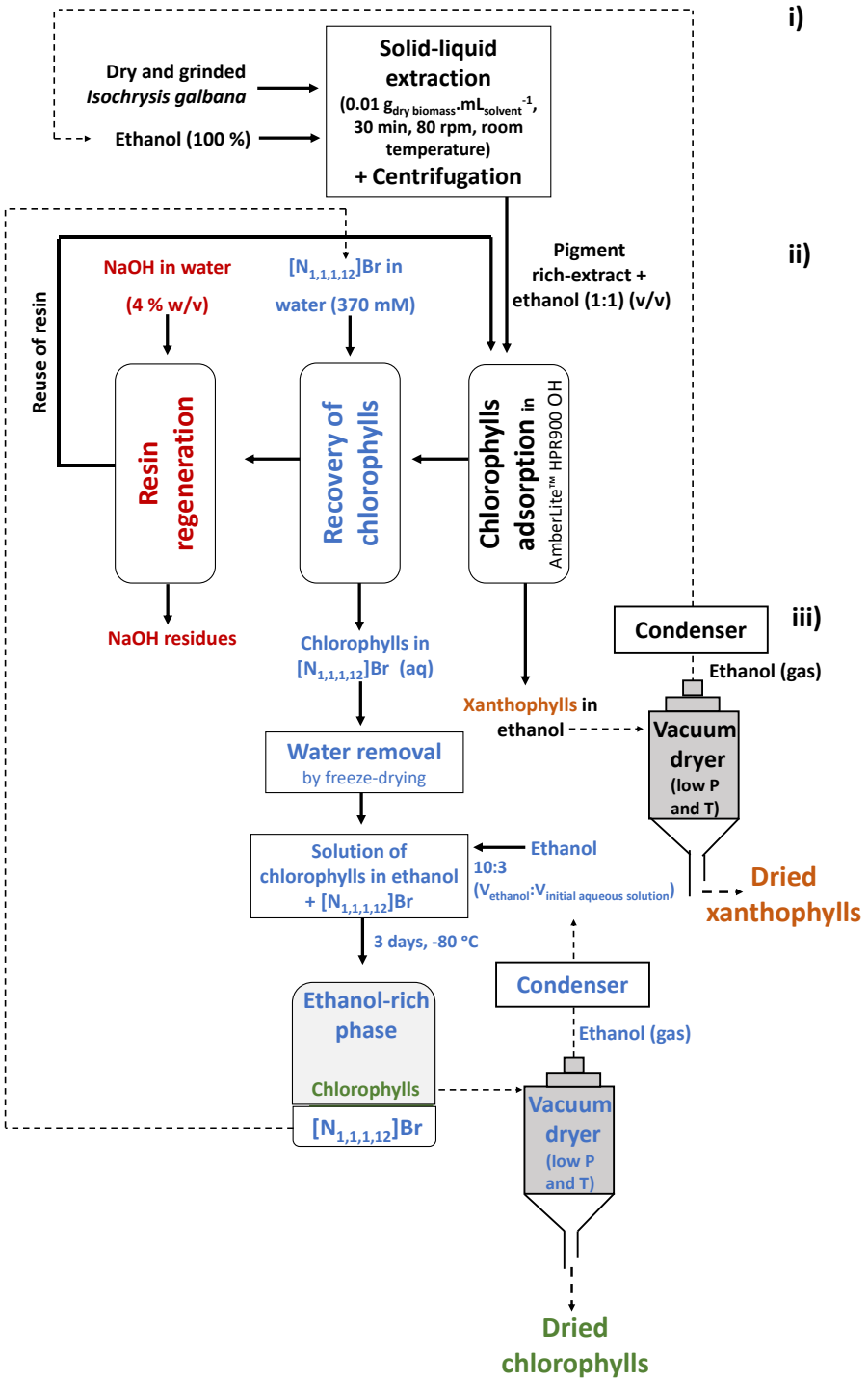


Figure 2.1.8. Schematic representation of the final process proposed in this work, where i) represents the solid-liquid extraction of pigments from the biomass; ii) the recovery of xanthophyll and chlorophyll through continuous process in column; and iii) the polishing of pigments and recovery of the solvents. Dashed lines were not experimentally tested, being just a proposal of what can be done.

Conclusions

In this study, the fractionation of chlorophylls and carotenoids was successfully achieved. Aqueous solutions of surface-active ILs have been demonstrated to be effective solvents in the elution of chlorophylls from a commercial anion exchange resin. The operational conditions of this step were optimized, reaching 97.0 ± 0.9 % of the initial chlorophyll loaded in the resin when $[N_{1,1,1,12}]Br$ was applied as a solvent in a concentration of 370 mM. Several cycles were tested to extend the lifetime of the resin AmberLite™ HPR900 OH, including a regeneration step with an aqueous solution of NaOH [4 % (w:v) in water]. As a result, the complete recovery of chlorophylls was achieved with no loss of efficiency after five cycles, suggesting the possibility of reusing the resin in more cycles. In order to evaluate the possibility to implement this process industrially, a test in continuous mode operation was performed. Some experimental limitations were found regarding the flow rate used in the continuous process, which turned out to be higher than necessary to accomplish complete chlorophyll adsorption and consequent recovery.

Furthermore, a polishing step and an integrated process were developed to obtain two purified fractions of chlorophylls and xanthophylls, while allowing the solvent recycling. Similar approaches could be applied to other biomolecules and biomass matrices, taking into account the economic viability of the process and associated waste production.

2.2. Recovery of bacterioruberin and proteins using aqueous solutions of surface-active compounds

This chapter is based on the manuscript:

Bárbara M.C. Vaz, Mariam Kholany, Inês P.E. Macário, Telma Veloso, Tânia Caetano, Joana L. Pereira, João A.P. Coutinho, Sónia P.M. Ventura,* "Recovery of bacterioruberin and proteins using aqueous solutions of surface-active compounds", to be published soon.

*Contributions: Bárbara M.C. Vaz acquired the experimental data. Bárbara M.C. Vaz and M.K. performed the data analysis. I.P.E.M. and T.V. performed archaea cultivation. Bárbara M.C. Vaz wrote the manuscript with substantial contributions from the remaining authors.

Abstract

The development of new or adapted processes towards a more sustainable economy is a growing industrial demand. The use of marine resources as natural and renewable feedstocks can be a way forward in that direction. Haloarchaea microorganisms are less explored marine resources that can be a promising source of valuable compounds. Their growth in extreme conditions gives bacterioruberin (major carotenoid found responsible for the pink color of hypersaline environments) the ability to model membrane fluidity, while decreasing water permeability. Therefore, the extraction of bacterioruberin using water-based solvents remains a challenge. In this work, the performance of several aqueous solutions of ionic liquids and surfactants in the extraction yield of bacterioruberin was investigated and compared with ethanol. Despite ethanol's better performance, the extraction of bacterioruberin with water-based solvents allowed the simultaneous release of proteins and thus, to envision a multi-product biorefinery, the non-ionic surfactants were chosen as the best. By a response surface methodology, the optimum operational conditions were achieved reaching a yield of extraction of 0.37 ± 0.01 $mg_{bacterioruberin}.g_{wet\ biomass}^{-1}$ and 352 ± 9 $mg_{protein}.g_{wet\ biomass}^{-1}$ when applying an aqueous solution of Tween® 20 at 182.4 mM as extraction solvent. In addition, higher purities of bacterioruberin were obtained, after performing a simple induced precipitation using ethanol as anti solvent to recover the proteins present in the initial extract. Lastly, a step for the polishing of bacterioruberin was also addressed to recycle the solvents enhancing the process loop closure for its implementation in the industry.

Keywords: *Haloferax mediterranei* ATCC 33500, bacterioruberin, solid-liquid extraction, surface-active compounds, induced precipitation, protein.

Introduction

Our continued reliance on a petroleum-based economy to satisfy the needs of the population cannot be sustained anymore. Mitigating climate change through a substantial reduction in greenhouse gas emissions is of the utmost importance. This requires the development of sustainable technologies and practices for a gradual transition to a renewable plant (bio-based) economy.[92]

Marine resources, like algae and cyanobacteria, gained significant interest as renewable feedstock to not only generate biofuels and bioenergy, but also to integrate bioactive medicinal products, food and materials,[8] being their sustainable use a significant part of The 2030 Agenda for Sustainable Development.[2] In this way, cost effective integration technologies based on marine resources should be developed considering the biorefinery framework. It aims for a complete valorization of all biomass fractions,[93] maximizing the value of raw materials. Besides increasing the economic viability of downstream processes for industrial implementation,[93] it avoids waste production following the circular economy principles.[94]

Halophilic microorganisms from the archaea domain are marine resources spread around the globe in hypersaline environments with approximately 35 % more salt than seawater. Although less explored, these are valuable sources of high demand products like proteins, poly(3-hydroxybutyrate), polyhydroxyalkanoate and carotenoids.[36] Proteins are complex molecules with an unique three dimensional structure composed of numerous combinations of the 20 different types of amino acids attached together to form long chains, where each sequence of amino acids determines the protein specific function.[5] Due to their multiple biological roles as regulatory proteins, transport facilitators, antibodies, structural maintenance, storage, toxins and contractile proteins,[95] their biotechnology interest is vast with applications in the textile, cosmetic, food and pharmaceutical industries,[96] and thus their purification is duly important. However, in halophilic organisms their role goes further. Halophilic proteins help these organisms to adapt to the extreme saline conditions by modifying their structure. The presence of acidic amino acids on the protein surface makes its surface more hydrophilic and flexible, capable of increasing the hydration of the protein, which prevents its aggregation and keeps the proteins functionally stable.[97]

On the other hand, carotenoids are natural pigments well known for their biotechnology applications and key properties in human health.[37,98] Their antioxidant properties keep the cells protected from reactive radicals preventing humans from premature aging, certain cancers, cardiovascular diseases and arthritis.[98,99] Besides, they can also be applied in the food industry as colorants and food supplements, and in the beauty industry as cosmetics.[99,100] Between the carotenoids produced by the haloarchaea group, namely β-carotene, salinixanthin, lycopene, bacterioruberin and its precursors, and phytoene, bacterioruberin represents the major carotenoid found.[36,37] Bacterioruberin can model the membrane fluidity acting as a water barrier and allowing the permeability of oxygen and other molecules needed for their survival in hypersaline environments, consequently increasing the membrane structure rigidity.[36,38,101] Of greater relevance, this C_{50} isoprenoid, composed of 13 conjugated double bonds and 4 hydroxyl groups, has higher antioxidant activity compared to other molecules conferring protection against intensive light, gamma irradiation and DNA damaging agents such as radiography, UV-irradiation, and H_2O_2 exposure.[36,38,101] These characteristics make bacterioruberin an appealing bio-active compound for its application in food and pharmaceutical industries.[39]

Despite the potential of bacterioruberin and halophilic proteins, extraction and purification procedures are still poorly explored. Since the exploitation of these microorganisms is in its initial stage, most of the reported literature focuses on exploring protein structures and its adaptations to extreme environments,[102–107] and on the optimization of bacterioruberin production and its possible applications, where in these studies bacterioruberin quantification is mostly accomplished after performing a solid-liquid extraction using volatile and toxic organic solvents.[39,41,101,108,109] Regarding the purification step of bacterioruberin, some works were found using thin

layer chromatography, or organic solvents.[39,110–115] However, despite thin layer chromatography being simple and efficient, industries require automated techniques. In this context, more efforts need to be made to develop efficient and sustainable approaches with scale-up potential.

Aqueous solutions of surfactants or ionic liquids (ILs) arise as alternative solvents to replace the conventional organic solvents. Surfactants and tensioactive ILs are amphiphilic molecules composed of a long hydrocarbon tail and a polar head group.[116] Due to their dual affinity they are able to self-organize and lower the surface tension between two immiscible phases,[117,118] becoming effective solvents in the extraction of either more hydrophilic compounds like proteins,[96,119–122] by interacting with phospholipids (present in the cell membrane) for the release of the intracellular content, and/or more hydrophobic molecules [63,64,122–124] such as bacterioruberin, by increasing their solubility in water. Furthermore, their use has several advantages, namely milder process conditions while using water as main solvent. It is not only a cheap solvent but it seems to be more adequate developing a facile cell disruption towards an halophyte microorganism, *i.e.* an archaea produced in saline media.[63,64] After the extraction of multiple compounds, usually a purification step is needed to obtain the appropriate purity of each compound considering a specific application. In this work, and considering the type of compounds to separate, an induced protein precipitation was applied. In a typical induced protein precipitation, the addition of a precipitating agent lowers the solubility of the proteins in water causing them to precipitate.[125] This allows for the separation of the solvent by concentrating the solute into a pellet in a more simple way. Behind its simplicity, it has industrial potential,[126] being recognized as having low carbon footprint and economic impact.[127,128]

In this work, an efficient process was developed to recover bacterioruberin and proteins from *Haloferax mediterranei* ATCC 33500. The process envisioned comprises three steps: (i) the cell disruption and consequent extraction of bacterioruberin and proteins using an aqueous solution of Tween 20, (ii) the recovery of proteins by induced precipitation with pure ethanol, and (iii) the polishing of bacterioruberin, allowing the solvent to be recycled. In the end, the final diagram of the process envisioning its industrial applicability is presented.

Experimental section

Archaea cultivation

Haloferax mediterranei ATCC 33500 was produced under controlled conditions to maximize the bacterioruberin production. For the biomass production, a YPC (Yeast, peptone and casamino acids media) culture medium was prepared with the following composition: peptone 1.0 ($g.L^{-1}$); casamino acids 1.0 ($g.L^{-1}$); yeast extract 5.0 ($g.L^{-1}$); NaCl 144 ($g.L^{-1}$); $MgSO_4 \cdot 7H_2O$ 21.0 ($g.L^{-1}$); $MgCl_2 \cdot 6H_2O$ 18.0 ($g.L^{-1}$); KCl 4.2 ($g.L^{-1}$); Tris-HCl 12 mM (pH 7.5); KOH 1M, and 0.5 M $CaCl_2$. The medium was sterilized at 121 °C prior to cell cultivation. A single colony was selected from the agar plate and inoculated into 25 mL culture medium in a 100 mL Erlenmeyer flask and cultured at 37 °C, 180 rpm under continuous light (4000 lux) for 72 h. To increase the cell mass concentration, 20 mL of this pre-inoculum was resuspended in 400 mL of fresh medium in a 1L Erlenmeyer flask and incubated under the same conditions (*i.e.* 37 °C, 180 rpm, 4000 lux) for 96 h. Archaea

growth was monitored by optical density determined at 600 nm. After the batch cultivation period the biomass was centrifuged in a Thermo Scientific Heraeus Megafuge 16R centrifuge at 18894 *g* for 15 min at room temperature (20-25 ºC). The supernatant was discarded, and the pellet was stored in the dark at -20 ºC until usage. Some variability was found in the data due to the use of different cultivation batches.

Chemicals

The series of 1-alkyl-3-methylimidazolium chloride ILs [$C_nC_1$im]Cl, as 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 114569-84-5), 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 purchased from IoLiTec. The ammonium-based ILs, namely tetrabutylammonium chloride ([$N_{4,4,4,4}$]Cl, 97 wt%, CAS 1112-67-0) and hexyltrimethylammonium bromide ([$N_{1,1,1,6}$]Br, 98 wt%, CAS 2650-53-5), were supplied by Sigma-Aldrich and Alfa Aesar, respectively. The phosphonium-based ILs, such as tetrabutylphosphonium chloride ([$P_{4,4,4,4}$]Cl, 95 wt%, CAS 2304-30-5), tributyltetradecylphosphonium chloride ([$P_{4,4,4,14}$]Cl, 95 wt%, CAS 81741-28-8), and tetrabutylphosphonium bromide ([$P_{4,4,4,4}$]Br, 95 wt%, CAS 3115-68-2), were provided by IoLiTec, and tetraoctylphosphonium bromide ([$P_{8,8,8,8}$]Br, CAS 23906-97-0) by Cytec. 1-methyl-1-propylpiperidinium chloride ([C_3mpip]Cl, 99 wt%, CAS 1383436-85-8), 1-butylpyridinium chloride ([C_4py]Cl, 98 wt%, CAS 1124-64-7), and 1-butyl-1-methylpyrrolidinium chloride ([C_4mpyr]Cl, 99 wt%, CAS 479500-35-1) were supplied from IoLiTec. Cationic surfactants, such as decyltrimethylammonium bromide ([$N_{1,1,1,10}$]Br, 99 wt%, CAS 2082-84-0) and hexadecyltrimethylammonium bromide ([$N_{1,1,1,16}$]Br, 99 wt%, CAS 57-09-0) were acquired by Tokyo Chemical Industry and Merk, respectively, while 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 by Alfa Aesar, and hexadecylpyridinium chloride monohydrate ([C_{16}py]Cl.H_2O, 99.0 % - 102.0 wt%, CAS 6004-24-6), by Sigma-Aldrich. Anionic surfactants as sodium dodecylsulfate (SDS, pharma grade, CAS 151-21-3) was provided by Panreac, and sodium dodecyl-benzenesulfonate (SDBS, technical grade, CAS 25155-30-0), and dioctyl sulfosuccinate sodium salt (AOT, 96 wt%, CAS 577-11-7), were provided by Sigma-Aldrich. Non-ionic surfactants like polyethylene glycol sorbitan monolaurate (Tween® 20, CAS 9005-64-5), and polyethylene glycol *tert*-octylphenyl ether (Triton™ X-114, lab grade, CAS 9036-19-5) were purchased from Acros Organics, polyethylene glycol sorbitan monooleate (Tween® 80, CAS 9005-65-6), Merpol® A (CAS 37208-27-8), polyethylene glycol monoalkyl ether (Genapol® X-080, CAS 9043-30-5) and Genapol® C-100 (CAS 61791-13-7) were purchased from Sigma-Aldrich. All molecular structures of the ILs and surfactants screened are depicted in Figure B.1 in Appendix B. Ethanol absolute (analytical reagent grade, CAS 64-17-5) used in solid-liquid extraction as control and in induced protein precipitation was supplied from Fisher Scientific. Sodium chloride (NaCl, 99.5 wt%, CAS 7647-14-5), potassium chloride (KCl, 99.5 wt%, CAS 7447-40-7), di-sodium hydrogen phosphate anhydrous (Na_2HPO_4, 99 wt%, CAS 7558-79-4), and potassium

phosphate monobasic (KH_2PO_4, 99.5 - 100.5 wt%, CAS 7778-77-0), used to prepare phosphate buffered saline, were purchased from Fisher Scientific, Chem-Lab, Panreac and Honeywell, respectively. To prepare the loading buffer for SDS-PAGE analysis, glycerol (99.88 wt%, CAS 56-81-5) provided from Fisher Chemical, bromophenol blue sodium salt (CAS 34725-61-6) supplied from Merk, dithiothreitol (DTT, 98 wt%, CAS 3483-12-3) purchased from NZYtech, and tris(hydroxymethyl)-aminomethane ($(HOCH_2)_3CNH_2$, 99 wt%, CAS 77-86-1) acquired from Alfa Aesar, were used. RunBlue Teo 20x Teo Tricine SDS and RunBlue SDS gel 4-12% 10cmx10cm were provided by Expedeon. BlueSafe used to stain the proteins and Full Range Rainbow Grisp were acquired by NZYtech and Cytiva.

Cell disruption and solid-liquid extraction

The cell disruption and solid-liquid extraction steps were performed simultaneously following an adapted protocol previously developed by us.[123] Briefly, this step was carried out in a shaker IKA Trayster Digital at room temperature (20 - 25 ºC) under a constant vertical rotation of 50 rpm, for 45 min, protected from light exposure. Several solvents including aqueous solutions of ILs and surfactants (cationic, anionic, and non-ionic) were screened at 100 mM to assess their capacity to release bacterioruberin (target compound) from the biomass. A control extraction using pure ethanol was also performed.

An additional screening of aqueous solutions of non-ionic surfactants at 250 mM was also carried out, being their critical micellar concentration (CMC) reported in Table B.1 in Appendix B. All tests were conducted at a fixed solid-liquid ratio (SLR) of 0.1 $g_{wet\ biomass}.mL_{solvent}^{-1}$, *i.e.* 0.1 g of wet biomass and 1 mL of the respective solvent. Lastly, a centrifugation step was performed at 16200 *g* for 15 min in a VWR Microstar 17 centrifuge, at room temperature (20 - 25 ºC). The resulting supernatant was recovered, and the biomass debris was discarded. All the assays were done in duplicate.

Optimization of the cell disruption/solid-liquid extraction step by a response surface methodology

A response surface methodology (RSM) was applied to simultaneously study different variables and to identify the most significant parameters and their interaction, which could lead to an enhanced yield of bacterioruberin extraction. A central composite rotatable design (CCRD - 2^3) was applied to the best solvent of the cell disruption/solid-liquid extraction in a total of 18 assays with 4 central points and axial points. The independent variables studied were the SLR ($g_{wet\ biomass}.mL_{solvent}^{-1}$), concentration of surfactant in water (C_{Surf}, mM) and time of extraction (t, min), being the dependent variable the yield of extraction of bacterioruberin (Yield of extraction, $mg_{bacterioruberin}.g_{wet\ biomass}^{-1}$). The results were statistically analyzed using the software Statistica® 7 and with a 95 % confidence level, following the theory proposed by Dean et al. (1999)[82] and Rodrigues and Lemma (2014).[83] The real values are presented in Table B.2 in Appendix B. Lastly, using the means of relative deviation (%) the optimum conditions determined were validated in triplicate.

Bacterioruberin quantification

The quantification of bacterioruberin was determined using a UV-Vis microplate reader (Synergy HT microplate reader – BioTek). The absorption spectra of the analyzed extracts were measured between 350 and 700 nm and the bacterioruberin content was determined by Eq. 2.2.1 using calibration curves previously obtained at 494 nm (R^2 = 0.9965) for ethanolic solutions and at 504 nm (R^2 = 0.9997) for aqueous solutions, Figure B.2A in Appendix B.

$$\text{Yield of extraction } (\text{mg}_{\text{bacterioruberin}} \cdot \text{g}_{\text{wet biomass}}{}^{-1}) = \frac{[\text{Bacterioruberin}] \times \text{Volume}}{\text{Weight}} \quad (2.2.1)$$

where "[Bacterioruberin]" corresponds to the concentration of bacterioruberin in the extract ($mg.mL^{-1}$), "volume" is the volume of solvent (mL) and "weight" is the weight of the wet cells tested (g).

Protein induced precipitation

The protein precipitation was performed using ethanol as precipitating agent added to the initial extract (extract collected after solid-liquid extraction). Different operational conditions, namely temperature (°C), volume of ethanol added (mL) and time for precipitation (min), were evaluated in a range of -20 to 30 °C, 0.5 to 4 mL and 2 to 30 min, respectively. The samples were centrifuged, and two different fractions were obtained, a pellet and a supernatant, using a Thermo Scientific Heraeus Megafuge 16R centrifuge at 4700 *g*, for 15 min. The protein recovery results were analyzed in terms of remaining protein content in the supernatant fraction to the protein content in the initial extract (in percentage, %). The pellet fraction from the optimized precipitation was resuspended in 2 mL of phosphate buffered saline (PBS) (1X, pH 7.4) for further analysis. The yield of precipitation ($mg_{protein}.g_{wet\ biomass}^{-1}$) was quantified using a calibration curve previously obtained with bovine serum albumin (BSA) as a standard protein at 280 nm (R^2 = 0.9976), Figure B.2B in Appendix B. Finally, the ethanol content of the supernatant fraction was evaporated to evaluate the bacterioruberin content after precipitation and to further pursue its polishing.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The resulting protein extract (pellet fraction resuspended in PBS) was analyzed by SDS-PAGE following the procedure of Laemmli (1970).[129] First, the sample was diluted (1:1) in loading buffer (4 % (w/v) of SDS, 20 % (w/v) of glycerol, 0.5 mM of Tris-HCl pH 6.8, 0.02 % (w/v) bromophenol blue, 3.1 % (w/v) of DTT) and incubated for 5 min at 95 ºC for denaturation to occur. Then, it was injected into the polyacrylamide gel along with the molecular weight marker (Full Range Rainbow Grisp) and left to run for 2 h at 110 V. Finally, to stain the proteins, an incubation with BlueSafe was carried out under mild agitation for 2 h.

Bacterioruberin polishing

The bacterioruberin polishing procedure used was adapted from the Chapter 2.1. First, the water content of bacterioruberin extract was removed by freeze-drying the sample. Then, ethanol was added in the proportion of 1:20 ($V_{bacterioruberin\ extract}$:$V_{ethanol}$) and the solution was kept for three days at -80 ºC to induce bacterioruberin release from the micellar complex present in Tween® 20. After such period two phases were formed, a viscous surfactant-rich phase formed at the bottom and an ethanol-rich top phase, to which the pigment migrated too. The bacterioruberin presence in the top phase was further evaluated by collecting a sample, removing the ethanol, and redissolving it in a controlled volume of 0.5 mL of ethanol. In addition, an ^{1}H NMR analysis was also performed to assess Tween® 20 content in the top phase using a Bruker AC 30 spectrometer (250 MHz) at room temperature. Deuterium oxide was used as solvent.

Results and discussion

Screening of surfactants and ILs as solvents

A large screening of aqueous solutions of surface-active compounds belonging to different classes (cationic, anionic, non-ionic) and other non-surfactant agents was performed at 100 mM to assess their ability to extract bacterioruberin (target compound), and these results compared with ethanol as control solvent. The surfactants tested were pre-selected considering works previously reported by us, where these were considered as the most efficient for carotenoids release.[122,123,130] In Table B.3 of Appendix B is reported the list of the 31 solvents tested and their capacity to extract bacterioruberin. Figure 2.2.1 shows the yields of extraction ($mg_{bacterioruberin}.g_{wet\ biomass}^{-1}$) in terms of the surfactants/ILs ability to induce the release of the target compound. Photographs of the obtained extracts are reported in Figure B.3 in Appendix B.

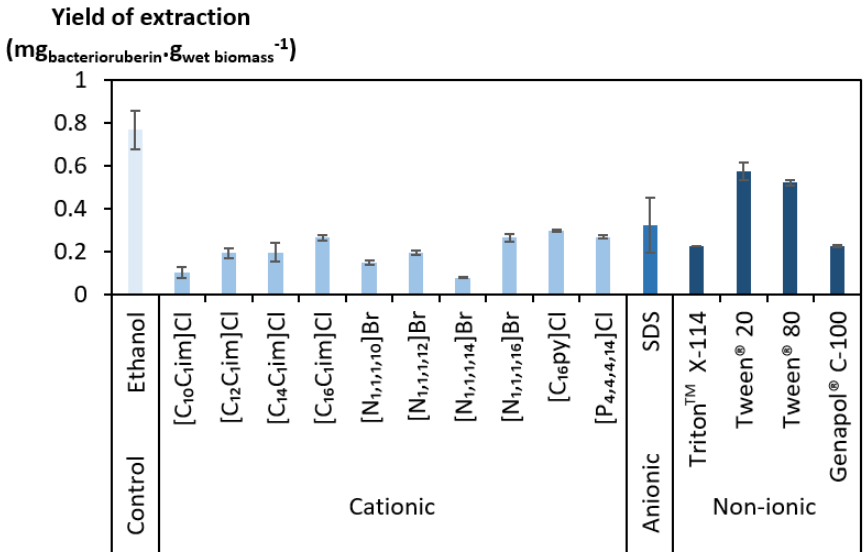


Figure 2.2.1. Yield of extraction of bacterioruberin using aqueous solutions of cationic, anionic, and non-ionic compounds at 100 mM with ethanol as control solvent at room temperature (20 - 25 ºC), under a constant vertical rotation of 50 rpm, for 45 min, protected from light exposure, and at a fixed SLR of 0.1 $g_{wet\ biomass}.mL_{solvent}^{-1}$.

The results presented in Figure 2.2.1 show that ethanol displayed a better yield of extraction when compared to the water-based solvents, which was not a surprise. Indeed, ethanol is recognized by several works for its high performance in pigment extraction and membrane solubilization.[63,131] Thus, and also due to the presence of bacterioruberin in the membrane cells, which confers a low water permeability,[36] the ethanol performance for the bacterioruberin extraction is expected to be better.

Regarding the performance of the water-based solvents, depicted in Figure 2.2.1 and Table B.3 in Appendix B, almost none of the anionic nor the non-tensioactive compounds were able to efficiently extract bacterioruberin. The low performance of the anionic surfactants can be attributed to the fact that both these types of tensioactive compounds and the phospholipids (components of the cell membrane) are negatively charged, creating repulsive forces.[122] On the other hand, the non-tensioactive ILs have low capability to disrupt the phospholipidic cell membranes due to their hydrophilic nature.[123] Contrastingly, all cationic compounds were able to release the intracellular material. This can be explained owing to their ability to form micelles above a certain critical micellar concentration creating the perfect environment for the solubilization of more hydrophobic compounds through attractive electrostatic interactions between the IL cation and the negatively charged head of the phospholipids.[124] Similar interactions can occur with the non-ionic surfactants,[123] being these the most effective class of solvents found for the release of bacterioruberin. However, at the conditions tested with a surfactant concentration of 100 mM, a high viscosity was noticed on most solutions obtained after extraction (see Table B.3 in Appendix B), which may be justified by the presence of a viscous component extracted from the biomass (new experiments are under development to characterize this viscous product). However, this increase in the viscosity is imposing some experimental constraints. To overcome this issue and better assess the extracting capability of the most effective class of solvents (in this case the non-ionic surfactants), a second screening at 250 mM of surfactant followed. These results are illustrated in Figure 2.2.2. The UV-Vis spectra of the resulting extracts can be found in Figure B.4 in Appendix B.

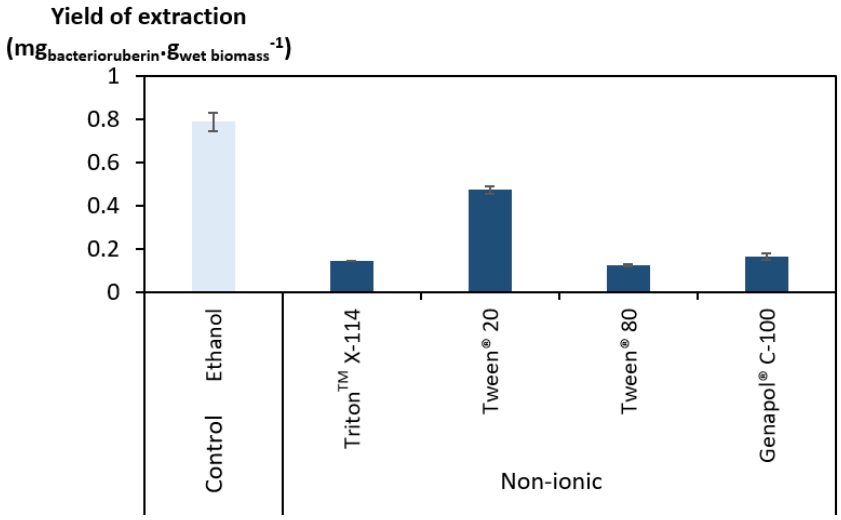


Figure 2.2.2. Yield of extraction of bacterioruberin using aqueous solutions of non-ionic compounds at 250 mM with ethanol as control solvent at room temperature (20 - 25 °C), under a constant vertical rotation of 50 rpm, for 45 min, protected from light exposure, and at a fixed SLR of 0.1 $g_{wet\ biomass}.mL_{solvent}^{-1}$.

Since the non-ionic surfactants tested are viscous liquids at room temperature, an increase in their concentration results in a more viscous aqueous solution. However, the viscosity of the final solutions (after bacterioruberin extraction) at 250 mM revealed to be substantially lower when compared to the same solutions at 100 mM. However, it must also be considered that a high concentration in the initial aqueous solution of surfactant also leads to viscosity issues. Therefore, a balance between these two extremes is a key factor in the success of this work. Taking this into account, from Figure 2.2.2 it is possible to reiterate that the highest performances were once again accomplished by Tween® 20 and ethanol. However, ethanol lacks the necessary capacity to simultaneously extract both the bacterioruberin (target compound) and the proteins (second valuable compound) present in the biomass envisioning a multi-product biorefinery (ethanol confers a protein yield of extraction of approximately 24 $mg_{protein}.g_{wet\ biomass}^{-1}$, while Tween® 20 confers a protein yield of extraction of approximately 352 $mg_{protein}.g_{wet\ biomass}^{-1}$). On the other hand, aqueous solutions of surfactants provide a milder environment that allows for the extraction of the different fractions of interest (proteins and pigments), both with applications related with food industry. As a result, the focus of the work was directed to the aqueous solutions of surface-active compounds, with Tween® 20 selected as the best solvent.

Optimization of the solid-liquid extraction by a response surface methodology

An optimization of the operational conditions was performed based on a central composite rotatable design (CCRD - 2^3) comprising three independent variables, namely SLR (in $g_{wet\ biomass}.mL_{solvent}^{-1}$, X1), time of extraction (t in min, X2) and concentration of surfactant in water (C_{Surf} in mM, X3). 18 assays with four central (level 0) and axial points (-1.68, and +1.68 levels) were studied in terms of bacterioruberin yield of extraction (in $mg_{bacterioruberin}.g_{wet\ biomass}^{-1}$) ranging from 0.111 $mg_{bacterioruberin}.g_{wet\ biomass}^{-1}$ (assay 5) to 0.331 $mg_{bacterioruberin}.g_{wet\ biomass}^{-1}$ (assay 9), Table B.2 in Appendix B. The fitted model expressed in Eq. 2.2.2, obtained using SS residual, by an analysis of variance (ANOVA), revealed a high predictability at a confidence level of 95 % with R^2 = 0.80912 and $F_{calculated} > F_{tabulated}$.

$$\text{Yield of extraction } (mg_{bacterioruberin} \cdot g_{wet\ biomass}{}^{-1}) = 0.147055 - 0.270072(X1) + 0.002002(X3) - 0.000005(X3)^2 \quad (2.2.2)$$

The response surfaces plotted in Figure 2.2.3 evidence a lack of influence by the extraction time. In this way, the minimum time tested was chosen to reduce the energy consumption of the process. On the other hand, Tween® 20 concentration seemed to display great influence in the extraction process, where an optimum value is reached at 182.4 mM, the minimum value tested for this variable. These results suggest that a higher yield of extraction could be achieved if the concentration of Tween® 20 was below 182.4 mM, however an increase in the tested range was unviable to perform due to the viscosity issues previously explained. Regarding the SLR, a positive influence in the predictive model is seen, being the best response obtained at 0.06224 $g_{wet\ biomass}.mL_{solvent}^{-1}$. These results can be confirmed by the data in Table B.4 in Appendix B, the Pareto Chart (Figure B.5 in Appendix B) and the predicted vs. observed values graph (Figure B.6 in Appendix B), showing

that the solid-liquid ratio and the concentration of Tween® 20 in water were the effects that most influenced the model's response regarding the bacterioruberin yield of extraction. Therefore, after finding the optimal operational conditions (8.04 min, 182.4 mM and 0.06224 $g_{wet\ biomass}.mL_{solvent}^{-1}$), a validation of the model was done. With a deviation of 11.6 %, a 0.37 ± 0.01 $mg_{bacterioruberin}.g_{wet\ biomass}^{-1}$ yield of extraction of bacterioruberin was obtained (Table B.5 in Appendix B). Furthermore, at these conditions, a protein yield of 352 ± 9 $mg_{protein}.g_{wet\ biomass}^{-1}$ was obtained.

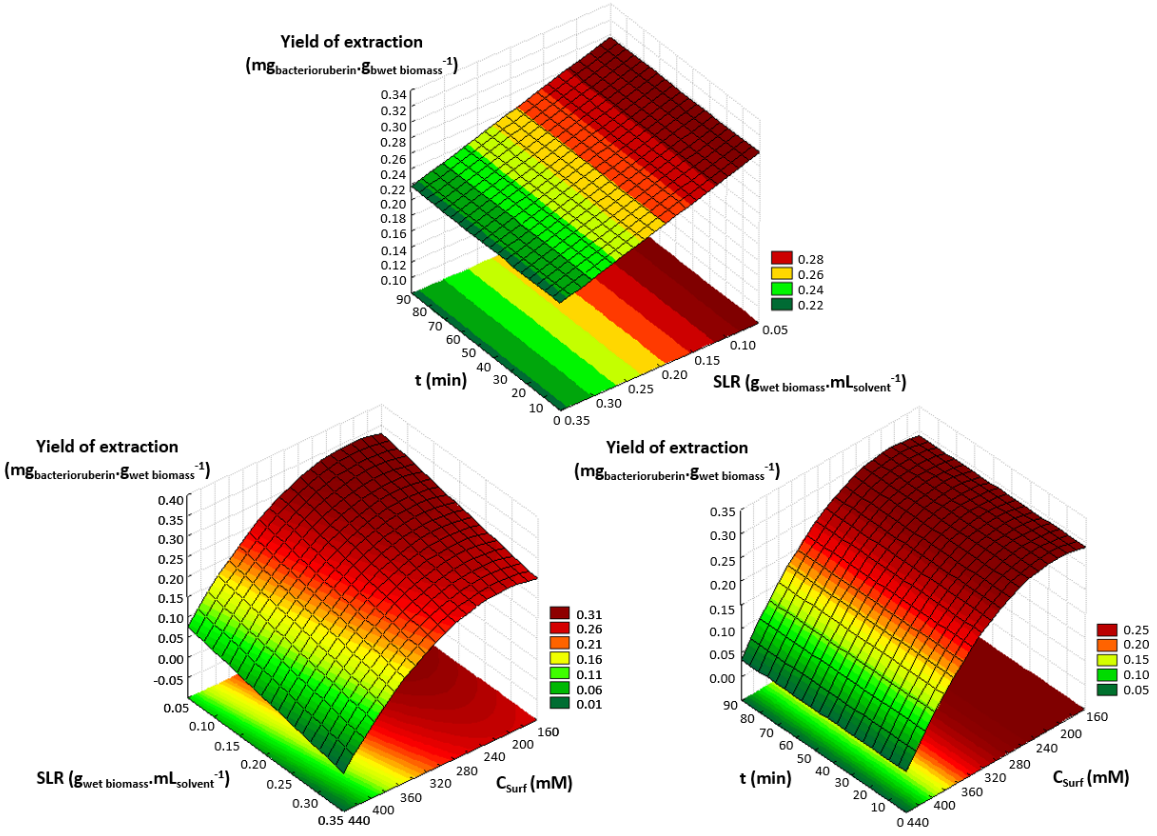


Figure 2.2.3. Response surface plots obtained for the CCRD (2^3) using an aqueous solution of Tween® 20 regarding SLR ($g_{wet\ biomass}.mL_{solvent}^{-1}$), concentration of surfactant in water (C_{Surf}, mM) and time of extraction (t, min) in terms of bacterioruberin yield of extraction (Yield of extraction, $mg_{bacterioruberin}.g_{wet\ biomass}^{-1}$).

Protein induced precipitation

Ethanol is a common solvent used as a precipitating agent since it possesses a lower dielectric constant compared to water,[132] which leads to an increase in the attraction forces between proteins, specifically at low temperatures.[133–135] To achieve a minimum of protein in the supernatant fraction (SF), an evaluation of the process conditions, such as temperature, volume of ethanol added to the initial extract, and time of the precipitation, was performed between -20 to 30 °C, 0.5 to 4 mL and 2 to 30 min, respectively (Figure 2.2.4).

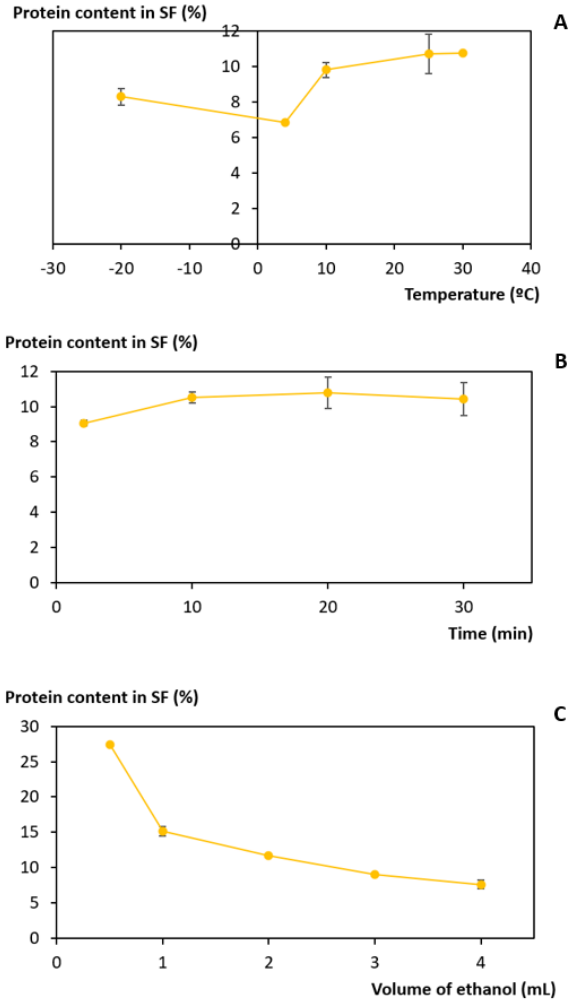


Figure 2.2.4. Protein content in the supernatant fraction (SF) after protein precipitation considering different temperatures (A), times of precipitation (B) and volumes of ethanol added to the initial extract (C).

The influence of temperature in the precipitation was carried out at a fixed time of 20 min and volume of ethanol of 3 mL. The results depicted in Figure 2.2.4A suggest that lower temperatures, particularly 4 ºC, reduce the protein losses of the process. However, as the difference in protein content between 4 °C and 25 °C corresponds only to 3.9 %, a temperature of 25 ºC was seen as preferable, as it lowers the energetic burden of the overall process. Taking this into account, the precipitation time was evaluated at a fixed 25 ºC and 3 mL of ethanol. As seen in Figure 2.2.4B, the data obtained show an almost constant protein content with a minimum at 2 min, being this time chosen to proceed. Finally, the volume of ethanol was considered (Figure 2.2.4C) under fixed conditions of 25 ºC and 2 min. Here, it is possible to observe that the volume of ethanol is the variable that mostly influences the precipitation process, reducing the protein content with an increase in the ethanol volume added to the initial extract. In this case, a volume of 3 mL was selected as the difference between 3 mL and 4 mL corresponds only to 1.5 % of protein content. Thus, with a set of optimum conditions of 25 ºC, 2 min and 3 mL of ethanol, an yield of 234.5 ± 0.9 $mg_{protein}.g_{wet\ biomass}^{-1}$ (quantified and calculated after protein redissolution in PBS) was obtained.

Moreover, further studies were conducted to understand the effect of consecutive precipitations (Figure B.7 in Appendix B). After the first precipitation, and consequent recovery of the pellet fraction, a second precipitation was carried out by adding another 3 mL to the supernatant fraction and repeating the procedure. Only a very small fraction with approximately 1.0 % of the total proteins present in the initial extract was obtained in the second precipitation corresponding to a yield of 2.7 ± 0.3 $mg_{protein}.g_{wet\ biomass}^{-1}$ (quantified and calculated after protein redissolution in PBS), substantially lower when compared to the first, and thus the application of a second step of precipitation was disregarded. This further confirms that over 90 % of the protein fraction was recovered in a single step of precipitation, as also proved by the abundance of proteins presented in the SDS-PAGE electrophoresis done to the precipitated proteins after dissolution in PBS (1X, pH 7.4), Figure B.8 in Appendix B. Lastly, bacterioruberin content was measured, after ethanol evaporation, to investigate bacterioruberin losses during induced precipitation, being noticed a loss of 12 %.

Bacterioruberin polishing

A bacterioruberin polishing step was accomplished to recycle Tween® 20 freeing the solvent for new cycles of extraction and to separate bacterioruberin from the solvent, thus facilitating its further application. Briefly, to perform this step, the water content was firstly removed from the bacterioruberin extract (extract obtained after induced precipitation and ethanol evaporation) by freeze-drying. Next, to induce the formation of two phases, ethanol was added to the sample [1:20 ($V_{bacterioruberin\ extract}$:$V_{ethanol}$)] and the solution obtained was stored in the freezer at -80 ºC for three days. This way, bacterioruberin successfully migrated to the ethanol-rich (top) phase, while Tween® 20 remained in the bottom phase. At the end, the ethanol-rich phase was analyzed by ^{1}H NMR spectrometry (Figure B.9 in Appendix B) to evaluate if Tween® 20 was fully separated, and no surfactant was detected. The presence of bacterioruberin in the top phase was confirmed through analysis of the collected sample by UV-Vis spectroscopy, Figure B.10 in Appendix B.

Integrated process design

A final diagram of the process developed in this work was proposed (Figure 2.2.5). Initially, a cell disruption/solid-liquid extraction of bacterioruberin and proteins from *Haloferax mediterranei* ATCC 33500 is carried out using an aqueous solution of Tween® 20 at 182.4 mM. To purify the pigment from the collected initial extract and to value proteins as a second product, ethanol was added in the proportion of 1:3 ($V_{bacterioruberin\ extract}$:$V_{ethanol}$), being the subproducts (in this case proteins) precipitated and then redissolved in PBS (1X, pH 7.4) to be ready for the final application. Lastly, bacterioruberin is recovered from the Tween® 20 (aq) by inducing a liquid-liquid extraction as previously discussed. The pigment can be provided in ethanolic solution or in dry form, depending on the desired end application. Drying should be performed using a vacuum dryer at low temperature and pressure to prevent pigment degradation, this way making the recycling of the solvents, Tween® 20 and ethanol, possible to be accomplished.

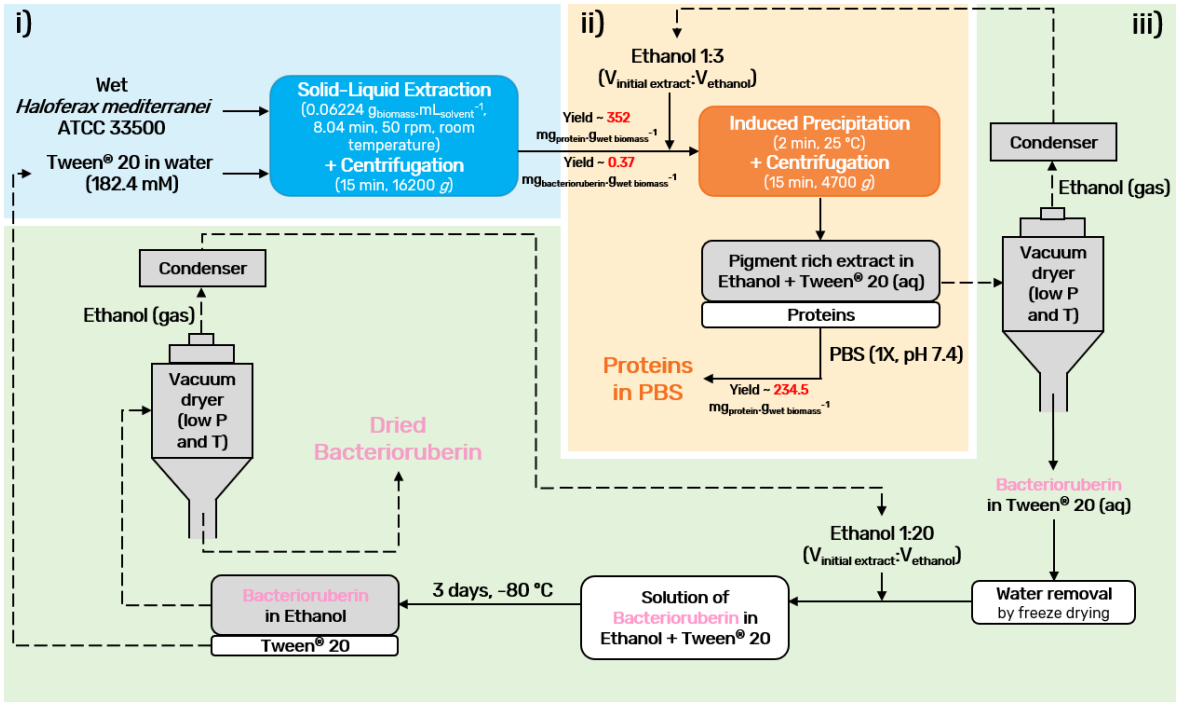


Figure 2.2.5. Schematic diagram of the downstream process developed in this work, consisting of (i) cell disruption/solid-liquid extraction of bacterioruberin and proteins using Tween® 20 (aq) as solvent, (ii) protein induced precipitation with ethanol and redissolution of proteins in PBS, and (iii) bacterioruberin polishing and recycling of the solvents. Dashed lines are only a suggestion of how the process could be industrially implemented, not being experimentally performed in this work.

Conclusions

In this work, the recovery of bacterioruberin and proteins from *Haloferax mediterranei* ATCC 33500 was successfully achieved using tensioactive solvents. Between the aqueous solutions of surfactants and ILs studied, Tween® 20 distinguished itself as the best, being achieved, after an optimization of the operational conditions, a maximum yield of bacterioruberin extraction of 0.37 ± 0.01 $mg_{bacterioruberin}.g_{wet\ biomass}^{-1}$ and proteins of 352 ± 9 $mg_{protein}.g_{wet\ biomass}^{-1}$. Furthermore, in a single protein precipitation step, over 90 % of the proteins were successfully separated from bacterioruberin. Lastly, it was possible to perform a polishing step of bacterioruberin and outline a proposal for the design of the final process to recycle the solvents and envisioning its industrial application.

CHAPTER 3. Final remarks and future perspectives

The establishment of the Sustainable Development Goals was, definitely, a wakeup call for everyone to join forces towards a sustainable future. However, achieving them is not an easy task. It requires promising disruptive conversion technologies for the sustainable transformation of natural resources into value-added products, materials, and fuels, considering the biorefinery and circular economy concepts.

In this thesis, two different procedures were developed to extract and purify xanthophylls, chlorophylls, and bacterioruberin from a microalga and a marine archaeon using alternative solvents. In the first work, it was found the remarkable performance of ammonium and phosphonium cations as a part of aqueous solutions of ILs to recover chlorophylls from AmberLite™ HPR900 OH, a step never achieved in literature before. This simple procedure allowed not only the fragmentation of chlorophylls and xanthophylls, but also the reuse and recycle of the resin in several cycles, which allowed its application in continuous mode. In addition, the addressed polishing step provided insights on how pigments could be isolated from the main solvent and the solvents recycled. In the second work, aqueous solutions of non-ionic surfactants revealed to efficiently extract bacterioruberin, a procedure usually performed using organic solvents. Furthermore, by a simple protein precipitation at room temperature bacterioruberin purification was attained allowing the recovery of proteins as a second valuable compound. In the end, the same polishing method of the previous work was successfully implemented showing its potential to be generalized for other pigments and solvents.

Although an economic and environmental analysis were not accomplished, the studied approaches took several factors into account when designed. First, the simplicity of the processes was a crucial aspect to consider them with potential industrial application. Second, the choice of the solvent was always based on its cost and sustainability. Aqueous solvents were used whenever possible, since water is the cheapest and most sustainable solvent that exists, and in some cases ethanol, which is one of the least hazardous organic solvents with reduced environmental impact. Lastly, to minimize energy and solvents consumption and to reach higher yields, the operational conditions of both procedures were optimized and the recycle of the solvents addressed. Moreover, none of the procedures required the use of pressure or additional equipment's, lowering their economic and environmental impacts.

As future perspective, and to improve the industrial applicability of the developed works, kinetic and adsorption studies should be developed to understand when the saturation of the solvents/adsorbent material or the maximum recovery of the target compound from the biomass is reached. In this way, being possible to overcome the limitations of the response surface methodology used in both processes regarding the variables of time and solid-liquid ratio. In general, more work should be developed considering the valorization of all biomass fractions without compromising their quality and applications. Moreover, there are several other issues that need to be further explored in this thematic, which may be divided in (i) the development of simple but efficient polishing steps to allow the solvents' reuse while allowing the biomolecules application in any field, (ii) the economic and environmental impacts of the process, and finally but also very important, the (iii) scale-up and continuous studies needed to prove (or not!) the processes' industrial applicability, an issue that is far from reality.

CHAPTER 4. Scientific contribution

Published articles

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, 9, 6599–6612.
2. **B.M.C. Vaz**, M. Martins, L.M. de Souza Mesquita, M.C. Neves, A.P.M. Fernandes, D.C.G.A. Pinto, M.G.P.M.S. Neves, J.A.P. Coutinho, S.P.M. Ventura, Using aqueous solutions of ionic liquids as chlorophyll eluents in solid-phase extraction processes, **Chemical Engineering Journal**, 2022, 428, 131073. (Part of this thesis, Chapter 2.1)

Oral communications

1. **B.M.C. Vaz**, M. Martins, L.M. de Souza Mesquita, M.C. Neves, A.P.M. Fernandes, D.C.G.A. Pinto, M.G.P.M.S. Neves, J.A.P. Coutinho, S.P.M. Ventura, A simple approach to recover chlorophylls from AmberLite™ HPR900 OH using tensioactive ionic liquids, **6th PATh Spring Workshop – A Path Towards Biomass Valorization Assisted by Green Chemistry Tools**, 30 September 2021, Aveiro, Portugal.
2. **B.M.C. Vaz**, M. Martins, L.M. de Souza Mesquita, M.C. Neves, A.P.M. Fernandes, J.A.P. Coutinho, S.P.M. Ventura, The challenge around elution of chlorophylls from AmberLite™ HPR900 OH with ionic liquids, **17th International Conference on Renewable Resources & Biorefineries**, 6-8 September 2021, Aveiro, Portugal.
3. **B.M.C. Vaz**, M. Martins, L.M. de Souza Mesquita, M.C. Neves, A.P.M. Fernandes, J.A.P. Coutinho, S.P.M. Ventura, Ionic liquids as a mean to elute chlorophyll from a commercial anion exchange resin, **PATh @ Internal Meeting on Ionic Liquids - "They talked so nice, they wanted it twice"**, 1 July 2021, online.
4. **B.M.C. Vaz**, M. Martins, L.M. de Souza Mesquita, M.C. Neves, A.P.M. Fernandes, J.A.P. Coutinho, S.P.M. Ventura, Aqueous solution of ionic liquids as eluents of pigments in solid phase extraction, **25th Annual Green Chemistry & Engineering Conference**, 14-18 June 2021, online.
5. M. Martins, L.M. de Souza Mesquita, **B.M.C. Vaz**, A.C.R.V. Dias, M.A. Torres-Acosta, J.A.P. Coutinho, S.P.M. Ventura, Liquid-liquid extractions based on tensioactive ionic liquids as a platform to purify pigments from algae, IMIL 2021 – **6th Iberoamerican meeting on ionic liquids**, 24-26 May 2021, online.
6. **B.M.C. Vaz**, M. Martins, L.M. de Souza Mesquita, M.C. Neves, A.P.M. Fernandes, J.A.P. Coutinho, S.P.M. Ventura, Ionic liquids as a mean to elute chlorophyll from a commercial anion exchange resin, **6th Iberoamerican meeting on ionic liquids**, 24-26 May 2021, online – flash presentation.

Panel communications

1. **B.M.C. Vaz**, M. Martins, L.M. de Souza Mesquita, M.C. Neves, A.P.M. Fernandes, D.C.G.A. Pinto, M.G.P.M.S. Neves, J.A.P. Coutinho, S.P.M. Ventura, Chlorophyll elution from an anion exchange resin using aqueous solutions of ionic liquids, Twitter Poster (GIF format) at **Jornadas do CICECO 2021 - Aveiro Institute of Materials**, 6-7 October 2021, Aveiro, Portugal.
2. M. Martins, L.M. de Souza Mesquita, **B.M.C. Vaz**, A.C.R.V. Dias, M.A. Torres-Acosta, J.A.P. Coutinho, S.P.M. Ventura, Fractionation of pigments using liquid-liquid systems based on aqueous solutions of ionic liquids, **17th International Conference on Renewable Resources & Biorefineries**, 6-8 September 2021, Aveiro, Portugal.
3. **B.M.C. Vaz**, M. Kholany, I.P.E. Macário, T. Veloso, T. Caetano, J.L. Pereira, J.A.P. Coutinho, S.P.M. Ventura, The potential behind bacterioruberin extraction and purification processes, **17th International Conference on Renewable Resources & Biorefineries**, 6-8 September 2021, Aveiro, Portugal.

Participation in events as part of the organization or exhibitor

1. **TechDays Aveiro**, National Forum of Technology, 14-16 October, 2021, Aveiro, Portugal.
2. **17th International Conference on Renewable Resources & Biorefineries – RRB 2021**, 6-8 September 2021, Aveiro, Portugal: Black T-shirt Team of the RRB Organizing Committee.

Experimental supervisions

1. Gonçalo Jorge Silva Coelho, Project student, BSc in Biotechnology, *Purification of bacterioruberin and its application on ocular gene therapy*, University of Aveiro, 2021.

Awards and distinctions

1. **Front cover of the journal** ACS Sustainable Chemistry & Engineering (https://pubs.acs.org/pb-assets/images/_journalCovers/ascecg/ascecg_v009i019-2.jpg?0.2613427284862495&fbclid=IwAR3ORP6e8DHEyLhz-ucfgJFY-aldSqaMz7jSmrU6e2Gernk4MTNaFx15oFk): Volume 9, Number 19, 17 May 2021, **2021**.

Bibliography

(1) Nations, U. Transforming our world: the 2030 Agenda for Sustainable Development https://sdgs.un.org/2030agenda (accessed Oct 11, 2021).

(2) Arora, N. K.; Mishra, I. United Nations Sustainable Development Goals 2030 and Environmental Sustainability: Race against Time. *Environ. Sustain.* **2019**, *2*, 339–342. https://doi.org/10.1007/s42398-019-00092-y.

(3) Visbeck, M.; Unger, S.; Kraemer, R. A.; Lubchenco, J.; Müller, A.; Neumann, B.; Rochette, J.; Schmidt, S.; Thiele, T. *The Ocean Dimension of the 2030 Agenda: Conservation and Sustainable Use of the Ocean, Seas, and Marine Resources for Sustainable Development*; 2017.

(4) Hoegh-Guldberg, O.; Beal, D.; Chaudhry, T.; Elhaj, H.; Abdullat, A.; Etessy, P.; Smits, M. *Reviving the Oceans Economy: The Case for Action - 2015*; World Wide Fund for Nature (WWF), 2015.

(5) Hamed, I.; Özogul, F.; Özogul, Y.; Regenstein, J. M. Marine Bioactive Compounds and Their Health Benefits: A Review. *Compr. Rev. Food Sci. Food Saf.* **2015**, *14*, 446–465. https://doi.org/10.1111/1541-4337.12136.

(6) Zhong, H. Exploitation and Utilization of Marine Resources and Protection of Marine Ecology. *IOP Conf. Ser. Earth Environ. Sci.* **2019**, *369*, 012009. https://doi.org/10.1088/1755-1315/369/1/012009.

(7) Filote, C.; Santos, S. C. R.; Popa, V. I.; Botelho, C. M. S.; Volf, I. *Biorefinery of Marine Macroalgae into High-Tech Bioproducts: A Review*; Springer International Publishing, 2021; Vol. 19. https://doi.org/10.1007/s10311-020-01124-4.

(8) Khan, M. I.; Shin, J. H.; Kim, J. D. The Promising Future of Microalgae: Current Status, Challenges, and Optimization of a Sustainable and Renewable Industry for Biofuels, Feed, and Other Products. *Microb. Cell Fact.* **2018**, *17*, 36. https://doi.org/10.1186/s12934-018-0879-x.

(9) Prabhu, M. S.; Israel, A.; Palatnik, R. R.; Zilberman, D.; Golberg, A. Integrated Biorefinery Process for Sustainable Fractionation of *Ulva Ohnoi* (Chlorophyta): Process Optimization and Revenue Analysis. *J. Appl. Phycol.* **2020**, *32*, 2271–2282. https://doi.org/10.1007/s10811-020-02044-0.

(10) Chandra, R.; Iqbal, H. M. N.; Vishal, G.; Lee, H.-S.; Nagra, S. Algal Biorefinery: A Sustainable Approach to Valorize Algal-Based Biomass towards Multiple Product Recovery. *Bioresour. Technol.* **2019**, *278*, 346–359. https://doi.org/10.1016/j.biortech.2019.01.104.

(11) Baghel, R. S.; Mantri, V. A.; Reddy, C. R. K. A New Wave of Research Interest in Marine Macroalgae for Chemicals and Fuels: Challenges and Potentials. In *Fuels, Chemicals and Materials from the Oceans and Aquatic Sources*; Kerton, F. M., Yan, N., Eds.; 2017; pp 43–63. https://doi.org/10.1002/9781119117193.ch3.

(12) di Visconte, G. S.; Spicer, A.; Chuck, C. J.; Allen, M. J. The Microalgae Biorefinery: A Perspective on the Current Status and Future Opportunities Using Genetic Modification. *Appl. Sci.* **2019**, *9* (22), 4793. https://doi.org/10.3390/app9224793.

(13) Suhag, M.; Sharma, H. R. Biorefinery Concept: An Overview of Producing Energy, Fuels and Materials from Biomass Feedstocks. *Iarjset* **2015**, *2* (12), 103–109. https://doi.org/10.17148/iarjset.2015.21219.

(14) Zollmann, M.; Robin, A.; Prabhu, M.; Polikovsky, M.; Gillis, A.; Greiserman, S.; Golberg, A. Green Technology in Green Macroalgal Biorefineries. *Phycologia* **2019**, *58* (5), 516–534.

https://doi.org/10.1080/00318884.2019.1640516.

(15) Balina, K.; Romagnoli, F.; Blumberga, D. Seaweed Biorefinery Concept for Sustainable Use of Marine Resources. *Energy Procedia* **2017**, *128*, 504–511. https://doi.org/10.1016/j.egypro.2017.09.067.

(16) Kümmerer, K.; Clark, J. H.; Zuin, V. G. Rethinking Chemistry for a Circular Economy. *Science (80-.).* **2020**, *367* (6476), 369–370. https://doi.org/10.1126/science.aba4979.

(17) Muscat, A.; Olde, E. M. de; Ripoll-Bosch, R.; Zanten, H. H. E. Van; Metze, T. A. P.; Termeer, C. J. A. M.; Ittersum, M. K. van; Boer, I. J. M. de. Principles, Drivers and Opportunities of a Circular Bioeconomy. *Nat. Food* **2021**, *2*, 561–566. https://doi.org/10.1038/s43016-021-00340-7.

(18) Stegmann, P.; Londo, M.; Junginger, M. The Circular Bioeconomy: Its Elements and Role in European Bioeconomy Clusters. *Resour. Conserv. Recycl. X* **2020**, *6*, 100029. https://doi.org/10.1016/j.rcrx.2019.100029.

(19) Levasseur, W.; Perré, P.; Pozzobon, V. A Review of High Value-Added Molecules Production by Microalgae in Light of the Classification. *Biotechnol. Adv.* **2020**, *41*, 107545. https://doi.org/10.1016/j.biotechadv.2020.107545.

(20) Bhattacharya, M.; Goswami, S. Microalgae – A Green Multi-Product Biorefinery for Future Industrial Prospects. *Biocatal. Agric. Biotechnol.* **2020**, *25*, 101580. https://doi.org/10.1016/j.bcab.2020.101580.

(21) Chew, K. W.; Yap, J. Y.; Show, P. L.; Suan, N. H.; Juan, J. C.; Ling, T. C.; Lee, D.-J.; Chang, J.-S. Microalgae Biorefinery: High Value Products Perspectives. *Bioresour. Technol.* **2017**, *229*, 53–62. https://doi.org/10.1016/j.biortech.2017.01.006.

(22) Bhalamurugan, G. L.; Valerie, O.; Mark, L. Valuable Bioproducts Obtained from Microalgal Biomass and Their Commercial Applications: A Review. *Environ. Eng. Res.* **2018**, *23* (3), 229–241. https://doi.org/10.4491/eer.2017.220.

(23) Koyande, A. K.; Show, P.-L.; Guo, R.; Tang, B.; Ogino, C.; Chang, J.-S. Bio-Processing of Algal Bio-Refinery: A Review on Current Advances and Future Perspectives. *Bioengineered* **2019**, *10* (1), 574–592. https://doi.org/10.1080/21655979.2019.1679697.

(24) Kirti, K.; Amita, S.; Priti, S.; Mukesh Kumar, A.; Jyoti, S. Colorful World of Microbes: Carotenoids and Their Applications. *Adv. Biol.* **2014**, 837891. https://doi.org/10.1155/2014/837891.

(25) Guedes, A. C.; Amaro, H. M.; Malcata, F. X. Microalgae as Sources of Carotenoids. *Mar. Drugs* **2011**, *9* (4), 625–644. https://doi.org/10.3390/md9040625.

(26) Nabi, F.; Arain, M. A.; Rajput, N.; Alagawany, M.; Soomro, J.; Umer, M.; Soomro, F.; Wang, Z.; Ye, R.; Liu, J. Health Benefits of Carotenoids and Potential Application in Poultry Industry: A Review. *J. Anim. Physiol. Anim. Nutr. (Berl).* **2020**, *104* (6), 1809–1818. https://doi.org/10.1111/jpn.13375.

(27) Darvin, M. E.; Sterry, W.; Lademann, J.; Vergou, T. The Role of Carotenoids in Human Skin. *Molecules* **2011**, *16*, 10491–10506. https://doi.org/10.3390/molecules161210491.

(28) Mendes-Silva, T. de C. D.; Andrade, R. F. da S.; Ootani, M. A.; Mendes, P. V. D.; Sá, R. A. de Q. C. de; Silva, M. R. F. da; Souza, K. S.; Correia, M. T. dos S.; Silva, M. V. da; Oliveira, M. B. M. de. Biotechnological Potential of Carotenoids Produced by Extremophilic Microorganisms and Application Prospects for the Cosmetics Industry. *Adv. Microbiol.* **2020**, *10* (08), 397–410. https://doi.org/10.4236/aim.2020.108029.

(29) Pareek, S.; Sagar, N. A.; Sharma, S.; Kumar, V.; Agarwal, T.; González-Aguilar, G. A.; Yahia, E. M. Chlorophylls: Chemistry and Biological Functions. In *Fruit and Vegetable Phytochemicals: Chemistry and Human Health*; Yahia, E. M., Ed.; Wiley-Blackwell, John Wiley & Sons Ltd., 2017; pp 269–284. https://doi.org/10.1002/9781119158042.ch14.

(30) Haznedaroglu, B. Z.; Rismani-Yazdi, H.; Allnutt, F. C. T.; Reeves, D.; Peccia, J. Algal Biorefinery for High-Value Platform Chemicals. In *Platform Chemical Biorefinery*; Elsevier Inc., 2016; pp 333–360. https://doi.org/10.1016/b978-0-12-802980-0.00018-3.

(31) Lanfer-Marquez, U. M.; Barros, R. M. C.; Sinnecker, P. Antioxidant Activity of Chlorophylls and Their Derivatives. *Food Res. Int.* **2005**, *38*, 885–891. https://doi.org/10.1016/j.foodres.2005.02.012.

(32) Abd El-Baky, H. H.; El Baz, F. K.; El Baroty, G. S. Evaluation of Marine Alga *Ulva Lactuca* L. as A Source of Natural Preservative Ingredient. *Electron. J. Environ. Agric. Food Chem.* **2008**, *3*, 434–444.

(33) Mishra, V. K.; Bacheti, R. K.; Husen, A. Medicinal Uses of Chlorophyll: A Critical Overview. In *Chlorophyll: Structure, Production and Medicinal Uses*; Le, H., Salcedo, E., Eds.; Nova Science Publishers, Inc.: Hauppauge, NY 11788, 2011; pp 177–196.

(34) Cai, J.-Q.; Liu, X.-M.; Gao, Z.-J.; Li, L.-L.; Wang, H. Chlorophylls Derivatives: Photophysical Properties, Assemblies, Nanostructures and Biomedical Applications. *Mater. Today* **2021**, *45*, 77–92. https://doi.org/10.1016/j.mattod.2020.11.001.

(35) Guedes, A. C.; Amaro, H. M.; Sousa-Pinto, I.; Malcata, F. X. Algal Spent Biomass—A Pool of Applications. In *Biofuels from Algae*; Elsevier B.V., 2019; pp 397–433. https://doi.org/10.1016/b978-0-444-64192-2.00016-0.

(36) Giani, M.; Garbayo, I.; Vílchez, C.; Martínez-Espinosa, R. M. Haloarchaeal Carotenoids: Healthy Novel Compounds from Extreme Environments. *Mar. Drugs* **2019**, *17* (9), 524. https://doi.org/10.3390/md17090524.

(37) Rodrigo-Baños, M.; Garbayo, I.; Vílchez, C.; Bonete, M. J.; Martínez-Espinosa, R. M. Carotenoids from Haloarchaea and Their Potential in Biotechnology. *Mar. Drugs* **2015**, *13*, 5508–5532. https://doi.org/10.3390/md13095508.

(38) Calegari-Santos, R.; Diogo, R. A.; Fontana, J. D.; Bonfim, T. M. B. Carotenoid Production by Halophilic Archaea Under Different Culture Conditions. *Curr. Microbiol.* **2016**, *72*, 641–651. https://doi.org/10.1007/s00284-015-0974-8.

(39) Giani, M.; Montero-Lobato, Z.; Garbayo, I.; Vílchez, C.; Vega, J. M.; Martínez-Espinosa, R. M. Haloferax Mediterranei Cells as C50 Carotenoid Factories. *Mar. Drugs* **2021**, *19* (2), 100. https://doi.org/10.3390/md19020100.

(40) Fariq, A.; Yasmin, A.; Jamil, M. Production, Characterization and Antimicrobial Activities of Bio-pigments by *Aquisalibacillus Elongatus* MB592, *Salinicoccus Sesuvii* MB597, and *Halomonas Aquamarina* MB598 Isolated from Khewra Salt Range, Pakistan. *Extremophiles* **2019**, *23* (4), 435–449. https://doi.org/10.1007/s00792-019-01095-7.

(41) Zalazar, L.; Pagola, P.; Miró, M. V.; Churio, M. S.; Cerletti, M.; Martínez, C.; Iniesta-Cuerda, M.; Soler, A. J.; Cesari, A.; De Castro, R. Bacterioruberin Extracts from a Genetically Modified Hyperpigmented *Haloferax Volcanii* Strain: Antioxidant Activity and Bioactive Properties on Sperm

Cells. *J. Appl. Microbiol.* **2019**, *126* (3), 796–810. https://doi.org/10.1111/jam.14160.

(42) Singh, O. V.; Gabani, P. Extremophiles: Radiation Resistance Microbial Reserves and Therapeutic Implications. *J. Appl. Microbiol.* **2011**, *110*, 851–861. https://doi.org/10.1111/j.1365-2672.2011.04971.x.

(43) Martins, M.; Ventura, S. P. M. Emerging Seaweed Extraction Techniques Using Ionic Liquids. In *Sustainable Seaweed Technologies*; Elsevier Inc., 2020; pp 287–311. https://doi.org/10.1016/b978-0-12-817943-7.00011-1.

(44) Khanra, S.; Mondal, M.; Halder, G.; Tiwari, O. N.; Gayen, K.; Bhowmick, T. K. Downstream Processing of Microalgae for Pigments, Protein and Carbohydrate in Industrial Application: A Review. *Food Bioprod. Process.* **2018**, *110*, 60–84. https://doi.org/10.1016/j.fbp.2018.02.002.

(45) Belwal, T.; Ezzat, S. M.; Rastrelli, L.; Bhatt, I. D.; Daglia, M.; Baldi, A.; Devkota, H. P.; Orhan, I. E.; Patra, J. K.; Das, G.; Anandharamakrishnan, C.; Gomez-Gomez, L.; Nabavi, S. F.; Nabavi, S. M.; Atanasov, A. G. A Critical Analysis of Extraction Techniques Used for Botanicals: Trends, Priorities, Industrial Uses and Optimization Strategies. *Trends Anal. Chem.* **2018**, *100*, 82–102. https://doi.org/10.1016/j.trac.2017.12.018.

(46) Ngamwonglumlert, L.; Devahastin, S.; Chiewchan, N. Natural Colorants: Pigment Stability and Extraction Yield Enhancement via Utilization of Appropriate Pretreatment and Extraction Methods. *Crit. Rev. Food Sci. Nutr.* **2017**, *57* (15), 3243–3259. https://doi.org/10.1080/10408398.2015.1109498.

(47) Mesquita, L. M. de S.; Martins, M.; Pisani, L. P.; Ventura, S. P. M.; Rosso, V. V. de. Insights on the Use of Alternative Solvents and Technologies to Recover Bio-Based Food Pigments. *Compr. Rev. Food Sci. Food Saf.* **2020**, *20* (1), 787–818. https://doi.org/10.1111/1541-4337.12685.

(48) Passos, H.; Freire, M. G.; Coutinho, J. A. P. Ionic Liquid Solutions as Extractive Solvents for Value-Added Compounds from Biomass. *Green Chem.* **2014**, *16*, 4786–4815. https://doi.org/10.1039/c4gc00236a.

(49) Kunz, W.; Häckl, K. The Hype with Ionic Liquids as Solvents. *Chem. Phys. Lett.* **2016**, *661*, 6–12. https://doi.org/10.1016/j.cplett.2016.07.044.

(50) Liu, B.; Jin, N. The Applications of Ionic Liquid as Functional Material: A Review. *Curr. Org. Chem.* **2016**, *20* (20). https://doi.org/10.2174/1385272820666160527101844.

(51) Rosen, M. J.; Kunjappu, J. T. Characteristic Features of Surfactants. In *Surfactants and Interfacial Phenomena*; 2012; pp 1–38. https://doi.org/10.1002/9781118228920.ch1.

(52) Sean Lai, Y.; Zhou, Y.; Eustance, E.; Straka, L.; Wang, Z.; Rittmann, B. E. Cell Disruption by Cationic Surfactants Affects Bioproduct Recovery from *Synechocystis* Sp. PCC 6803. *Algal Res.* **2018**, *34*, 250–255. https://doi.org/10.1016/j.algal.2018.08.010.

(53) Yazdi, A. S. Surfactant-Based Extraction Methods. *Trends Anal. Chem.* **2011**, *30* (6), 918–929. https://doi.org/10.1016/j.trac.2011.02.010.

(54) Chemat, F.; Abert-Vian, M.; Fabiano-Tixier, A. S.; Strube, J.; Uhlenbrock, L.; Gunjevic, V.; Cravotto, G. Green Extraction of Natural Products. Origins, Current Status, and Future Challenges. *TrAC - Trends Anal. Chem.* **2019**, *118*, 248–263. https://doi.org/10.1016/j.trac.2019.05.037.

(55) Haines, A.; Scheelbeek, P. European Green Deal: A Major Opportunity for Health Improvement.

Lancet **2020**, *395* (10233), 1327–1329. https://doi.org/10.1016/S0140-6736(20)30109-4.

(56) Jung, K. A.; Lim, S.-R.; Kim, Y.; Park, J. M. Potentials of Macroalgae as Feedstocks for Biorefinery. *Bioresour. Technol.* **2013**, *135*, 182–190. https://doi.org/10.1016/j.biortech.2012.10.025.

(57) Zimmerman, J. B.; Anastas, P. T.; Erythropel, H. C.; Leitner, W. Designing for a Green Chemistry Future. *Science (80-.).* **2020**, *367* (6476), 397–400. https://doi.org/10.1126/science.aay3060.

(58) Singh, S.; Kate, B. N.; Banerjee, U. C. Bioactive Compounds from Cyanobacteria and Microalgae: An Overview. *Crit. Rev. Biotechnol.* **2005**, *25* (3), 73–95. https://doi.org/10.1080/07388550500248498.

(59) Zhao, C.; Yang, C.; Liu, B.; Lin, L.; Sarker, S. D.; Nahar, L.; Yu, H.; Cao, H.; Xiao, J. Bioactive Compounds from Marine Macroalgae and Their Hypoglycemic Benefits. *Trends Food Sci. Technol.* **2018**, *72*, 1–12. https://doi.org/10.1016/j.tifs.2017.12.001.

(60) Manivasagan, P.; Bharathiraja, S.; Santha Moorthy, M.; Mondal, S.; Seo, H.; Dae Lee, K.; Oh, J. Marine Natural Pigments as Potential Sources for Therapeutic Applications. *Crit. Rev. Biotechnol.* **2018**, *38* (5), 745–761. https://doi.org/10.1080/07388551.2017.1398713.

(61) Pangestuti, R.; Kim, S.-K. Biological Activities and Health Benefit Effects of Natural Pigments Derived from Marine Algae. *J. Funct. Foods* **2011**, *3* (4), 255–266. https://doi.org/10.1016/j.jff.2011.07.001.

(62) De Souza Mesquita, L. M.; Ventura, S. P. M.; Braga, A. R. C.; Pisani, L. P.; Dias, A. C. R. V.; De Rosso, V. V. Ionic Liquid-High Performance Extractive Approach to Recover Carotenoids from *Bactris Gasipaes* Fruits. *Green Chem.* **2019**, *21* (9), 2380–2391. https://doi.org/10.1039/c8gc03283a.

(63) Martins, M.; Fernandes, A. P. M.; Torres-Acosta, M. A.; Collén, P. N.; Abreu, M. H.; Ventura, S. P. M. Extraction of Chlorophyll from Wild and Farmed *Ulva* Spp. Using Aqueous Solutions of Ionic Liquids. *Sep. Purif. Technol.* **2021**, *254*, 117589. https://doi.org/10.1016/j.seppur.2020.117589.

(64) Mesquita, L. M. de S.; Martins, M.; Maricato, É.; Nunes, C.; Quinteiro, P. S. G. N.; Dias, A. C. R. V.; Coutinho, J. A. P.; Pisani, L. P.; Rosso, V. V. de; Ventura, S. P. M. Ionic Liquid-Mediated Recovery of Carotenoids from the *Bactris Gasipaes* Fruit Waste and Their Application in Food-Packaging Chitosan Films. *ACS Sustain. Chem. Eng.* **2020**, *8* (10), 4085–4095. https://doi.org/10.1021/acssuschemeng.9b06424.

(65) Sun, C.; Liu, W.; Zhao, P. Micelle-Mediated Extraction and Cloud Point Preconcentration of Chlorophylls from Spinach. *Sep. Sci. Technol.* **2014**, *49* (18), 2921–2926. https://doi.org/10.1080/01496395.2014.948557.

(66) Rajabi, M. S.; Moniruzzaman, M.; Mahmood, H.; Sivapragasam, M.; Bustam, M. A. Extraction of β-Carotene from Organic Phase Using Ammonium Based Ionic Liquids Aqueous Solution. *J. Mol. Liq.* **2017**, *227*, 15–20. https://doi.org/10.1016/j.molliq.2016.12.008.

(67) Martins, M.; Albuquerque, C. M.; Pereira, C. F.; Coutinho, J. A. P.; Neves, M. G. P. M. S.; Pinto, D. C. G. A.; Faustino, M. A. F.; Ventura, S. P. M. Recovery of Chlorophyll *a* Derivative from *Spirulina Maxima*: Its Purification and Photosensitizing Potential. *ACS Sustain. Chem. Eng.* **2021**, *9* (4), 1772–1780. https://doi.org/10.1021/acssuschemeng.0c07880.

(68) Li, L.; Yuan, H. Chromoplast Biogenesis and Carotenoid Accumulation. *Arch. Biochem. Biophys.* **2013**, *539* (2), 102–109. https://doi.org/10.1016/j.abb.2013.07.002.

(69) Lodish, H.; Berk, A.; Zipursky, S.; Matsudaira, P.; Baltimore, D.; Darnell, J. Photosynthetic Stages and Light-Absorbing Pigments. In *Molecular Cell Biology*; 2000.

(70) Rosa, P. A. J.; Azevedo, A. M.; Sommerfeld, S.; Bäcker, W.; Aires-Barros, M. R. Aqueous Two-Phase Extraction as a Platform in the Biomanufacturing Industry: Economical and Environmental Sustainability. *Biotechnol. Adv.* **2011**, *29* (6), 559–567. https://doi.org/10.1016/j.biotechadv.2011.03.006.

(71) Jeyanthi Rebecca, L.; Sharmila, S.; Das, M. P.; Seshiah, C. Extraction and Purification of Carotenoids from Vegetables. *J. Chem. Pharm. Res.* **2014**, *6* (4), 594–598.

(72) Joseph, S.; Anandane, A. Process for Isolation and Purification of Carotenoids. Us 9,109,120 B2, 2015.

(73) Lennetech. AMBERSEP® 900 OH Product Data Sheet. *Rohm Haas* **1999**, 1–2.

(74) Bijttebier, S.; D'Hondt, E.; Noten, B.; Hermans, N.; Apers, S.; Voorspoels, S. Tackling the Challenge of Selective Analytical Clean-up of Complex Natural Extracts: The Curious Case of Chlorophyll Removal. *Food Chem.* **2014**, *163*, 147–153. https://doi.org/10.1016/j.foodchem.2014.04.098.

(75) Larsen, E.; Christensen, L. P. Simple Saponification Method for the Quantitative Determination of Carotenoids in Green Vegetables. *J. Agric. Food Chem.* **2005**, *53* (17), 6598–6602. https://doi.org/10.1021/jf050622+.

(76) Willauer, H. D.; Hardy, D. R.; Ndubizu, E. C.; Williams, F. W. Extraction of Carbon Dioxide from Seawater by Ion Exchange Resin Part II : Using Strong Base Anion Exchange Resin. *Navy Technol. Cent. Saf. Surviv.* **2009**, *NRL/MR/618*.

(77) Hrichi, S.; Chaabane-Banaoues, R.; Giuffrida, D.; Mangraviti, D.; Majdoub, Y. O. El; Rigano, F.; Mondello, L.; Babba, H.; Mighri, Z.; Cacciola, F. Effect of Seasonal Variation on the Chemical Composition and Antioxidant and Antifungal Activities of *Convolvulus Althaeoides* L . Leaf Extracts. *Arab. J. Chem.* **2020**, *13* (6), 5651–5668. https://doi.org/10.1016/j.arabjc.2020.04.006.

(78) Al-Alwani, M. A. M.; Mohamad, A. B.; Kadhum, A. A. H.; Ludin, N. A.; Safie, N. E.; Razali, M. Z.; Ismail, M.; Sopian, K. Natural Dye Extracted from *Pandannus Amaryllifolius* Leaves as Sensitizer in Fabrication of Dye-Sensitized Solar Cells. *Int. J. Electrochem. Sci.* **2017**, *12*, 747–761. https://doi.org/10.20964/2017.01.56.

(79) Adedokun, O.; Sanusi, Y. K.; Awodugba, A. O. Solvent Dependent Natural Dye Extraction and Its Sensitization Effect for Dye Sensitized Solar Cells. *Opt. - Int. J. Light Electron Opt.* **2018**, *174*, 497–507. https://doi.org/10.1016/j.ijleo.2018.06.064.

(80) Rajan, M.; Rajkumar, G.; Guedes, T. J. F. L.; Barros, R. G. C.; Narain, N. Performance of Different Solvents on Extraction of Bioactive Compounds, Antioxidant and Cytotoxic Activities in *Phoenix Loureiroi* Kunth Leaves. *J. Appl. Res. Med. Aromat. Plants* **2020**, *17*, 100247. https://doi.org/10.1016/j.jarmap.2020.100247.

(81) Faustino, M. V.; Faustino, M. A. F.; Silva, H.; Cunha, Â.; Silva, A. M. S.; Pinto, D. C. G. A. *Puccinellia Maritima*, *Spartina Maritime*, and *Spartina Patens* Halophytic Grasses: Characterization of Polyphenolic and Chlorophyll Profiles and Evaluation of Their Biological Activities. *molecules* **2019**, *24* (20), 3796. https://doi.org/10.3390/molecules24203796.

(82) Dean, A.; Voss, D. *Design and Analysis of Experiments*, 1st ed.; Springer-Verlag: New York, 1999.

https://doi.org/10.1007/b97673.

(83) Rodrigues, M. I.; Lemma, A. F. *Planejamento de Experimentos e Otimização de Processos*, 3rd ed.; Casa do Pão Editora, 2014.

(84) Jayaraman, S.; Knuth, M. L.; Cantwell, M.; Santos, A. High Performance Liquid Chromatographic Analysis of Phytoplankton Pigments Using a C16-Amide Column. *J. Chromatogr. A* **2011**, *1218* (22), 3432–3438. https://doi.org/10.1016/j.chroma.2011.03.058.

(85) Lena, G. Di; Casini, I.; Lucarini, M.; Lombardi-Boccia, G. Carotenoid Profiling of Five Microalgae Species from Large-Scale Production. *Food Res. Int.* **2019**, *120*, 810–818. https://doi.org/10.1016/j.foodres.2018.11.043.

(86) Wu, S.; Xie, X.; Huan, L.; Zheng, Z.; Zhao, P.; Kuang, J.; Liu, X.; Wang, G. Selection of Optimal Flocculant for Effective Harvesting of the Fucoxanthin-Rich Marine Microalga *Isochrysis Galbana*. *J. Appl. Phycol.* **2016**, *28*, 1579–1588. https://doi.org/10.1007/s10811-015-0716-0.

(87) Kim, S. M.; Kang, S.-W.; Kwon, O.-N.; Chung, D.; Pan, C.-H. Fucoxanthin as a Major Carotenoid in *Isochrysis* Aff. *Galbana*: Characterization of Extraction for Commercial Application. *J. Korean Soc. Appl. Biol. Chem.* **2012**, *55*, 477–483. https://doi.org/10.1007/s13765-012-2108-3.

(88) Obata, M.; Taguchi, S. The Xanthophyll-Cycling Pigment Dynamics of *Isochrysis Galbana* (Prymnesiophyceae) during Light–Dark Transition. *Plankt. Benthos Res.* **2012**, *7* (3), 101–110. https://doi.org/10.3800/pbr.7.101.

(89) Martins, M.; Oliveira, R.; Coutinho, joão A. P.; Faustino, M. A. F.; Neves, M. G. P. M. S.; Pinto, D. C. G. A.; Ventura, S. P. M. Recovery of Pigments from *Ulva Rigida*. *Sep. Purif. Technol.* **2021**, *255*, 117723. https://doi.org/10.1016/j.seppur.2020.117723.

(90) Wernet, G.; Bauer, C.; Steubing, B.; Reinhard, J.; Moreno-Ruiz, E.; Weidema, B. The Ecoinvent Database Version 3 (Part I): Overview and Methodology. *Int. J. Life Cycle Assess.* **2016**, *21*, 1218–1230. https://doi.org/10.1007/s11367-016-1087-8.

(91) Frade, R. F.; Afonso, C. A. Impact of Ionic Liquids in Environment and Humans: An Overview. *Hum. Exp. Toxicol.* **2010**, *29* (12), 1038–1054. https://doi.org/10.1177/0960327110371259.

(92) Kataki, R.; Chutia, R. S.; Mishra, M.; Bordoloi, N.; Saikia, R.; Bhaskar, T. Feedstock Suitability for Thermochemical Processes. In *Recent Advances in Thermo-Chemical Conversion of Biomass*; 2015; pp 31–74. https://doi.org/10.1016/B978-0-444-63289-0.00002-8.

(93) ’t Lam, G. P.; Vermuë, M. H.; Eppink, M. H. M.; Wijffels, R. H.; van den Berg, C. Multi-Product Microalgae Biorefineries: From Concept Towards Reality. *Trends Biotechnol.* **2018**, *36* (2), 216–227. https://doi.org/10.1016/j.tibtech.2017.10.011.

(94) Sherwood, J. The Significance of Biomass in a Circular Economy. *Bioresour. Technol.* **2020**, *300*, 122755. https://doi.org/10.1016/j.biortech.2020.122755.

(95) Gupta, M. N. Purification of Naturally Occurring Biomaterials. In *Natural-Based Polymers for Biomedical Applications*; 2008; pp 54–84. https://doi.org/10.1533/9781845694814.1.54.

(96) Nunes, J. C. F.; Almeida, M. R.; Faria, J. L.; Silva, C. G.; Neves, M. C.; Freire, M. G.; Tavares, A. P. M. *Overview on Protein Extraction and Purification Using Ionic-Liquid-Based Processes*; Springer US, 2021. https://doi.org/10.1007/s10953-021-01062-x.

(97) Sharma, N.; Farooqi, M. S.; Chaturvedi, K. K.; Lal, S. B.; Grover, M.; Rai, A.; Pandey, P. The Halophile Protein Database. *Database* **2014**, 1–9. https://doi.org/10.1093/database/bau114.

(98) Vílchez, C.; Forján, E.; Cuaresma, M.; Bédmar, F.; Garbayo, I.; Vega, J. M. Marine Carotenoids: Biological Functions and Commercial Applications. *Mar. Drugs* **2011**, *9* (3), 319–333. https://doi.org/10.3390/md9030319.

(99) Gong, M.; Bassi, A. Carotenoids from Microalgae: A Review of Recent Developments. *Biotechnol. Adv.* **2016**, *34* (8), 1396–1412. https://doi.org/10.1016/j.biotechadv.2016.10.005.

(100) Mezzomo, N.; Ferreira, S. R. S. Carotenoids Functionality, Sources, and Processing by Supercritical Technology: A Review. *J. Chem.* **2016**, *2016*, 16. https://doi.org/10.1155/2016/3164312.

(101) Vega, M. de la; Sayago, A.; Ariza, J.; Barneto, A. G.; León, R. Characterization of a Bacterioruberin-Producing Haloarchaea Isolated from the Marshlands of the Odiel River in the Southwest of Spain. *Biotechnol. Prog.* **2016**, *32* (3). https://doi.org/10.1002/btpr.2248.

(102) Britton, K. L.; Baker, P. J.; Fisher, M.; Ruzheinikov, S.; Gilmour, D. J.; Bonete, M.-J.; Ferrer, J.; Pire, C.; Esclapez, J.; Rice, D. W. Analysis of Protein Solvent Interactions in Glucose Dehydrogenase from the Extreme Halophile *Haloferax Mediterranei*. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103* (13), 4846–4851. https://doi.org/10.1073/pnas.0508854103.

(103) Mevarech, M.; Frolow, F.; Gloss, L. M. Halophilic Enzymes: Proteins with a Grain of Salt. *Biophys. Chem.* **2000**, *86*, 155–164. https://doi.org/10.1016/S0301-4622(00)00126-5.

(104) Payá, G.; Bautista, V.; Camacho, M.; Bonete, M.-J.; Esclapez, J. Functional Analysis of Lsm Protein under Multiple Stress Conditions in the Extreme Haloarchaeon *Haloferax Mediterranei*. *Biochimie* **2021**, *187*, 33–47. https://doi.org/10.1016/j.biochi.2021.05.002.

(105) C., E.; D., M.; G., Z. Molecular Adaptation of Halophilic Proteins. In *Extremophiles*; 2009; Vol. 2.

(106) Kumar, A.; Alam, A.; Tripathi, D.; Rani, M.; Khatoon, H.; Pandey, S.; Ehtesham, N. Z.; Hasnain, S. E. Protein Adaptations in Extremophiles: An Insight into Extremophilic Connection of Mycobacterial Proteome. *Semin. Cell Dev. Biol.* **2018**, *84*, 147–157. https://doi.org/10.1016/j.semcdb.2018.01.003.

(107) Siglioccolo, A.; Paiardini, A.; Piscitelli, M.; Pascarella, S. Structural Adaptation of Extreme Halophilic Proteins through Decrease of Conserved Hydrophobic Contact Surface. *BMC Struct. Biol.* **2011**, *11* (50). https://doi.org/10.1186/1472-6807-11-50.

(108) Abbes, M.; Baati, H.; Guermazi, S.; Messina, C.; Santulli, A.; Gharsallah, N.; Ammar, E. Biological Properties of Carotenoids Extracted from *Halobacterium Halobium* Isolated from a Tunisian Solar Saltern. *BMC Complement. Altern. Med.* **2013**, *13*, 255. https://doi.org/10.1186/1472-6882-13-255.

(109) Müller, W. J.; Smit, M. S.; Heerden, E. van; Capes, M. D.; DasSarma, S. Complex Effects of Cytochrome P450 Monooxygenase on Purple Membrane and Bacterioruberin Production in an Extremely Halophilic Archaeon: Genetic, Phenotypic, and Transcriptomic Analyses. *Front. Microbiol.* **2018**, *9*, 2563. https://doi.org/10.3389/fmicb.2018.02563.

(110) Alvares, J. J.; Furtado, I. J. Kinetics of - DPPH• Scavenging by Bacterioruberin from *Haloferax Alexandrinus* GUSF-1 (KF796625). *J. Anal. Sci. Technol.* **2021**, *12* (44). https://doi.org/10.1186/s40543-021-00293-3.

(111) Manikandan, M.; Hasan, N.; Wu, H.-F. Rapid Detection of Haloarchaeal Carotenoids via Liquid–

Liquid Microextraction Enabled Direct TLC MALDI-MS. *Talanta* **2013**, *107*, 167–175. https://doi.org/10.1016/j.talanta.2013.01.005.

(112) Mishra, S.; Chanotiya, C. S.; Shanker, K.; Tripathi, A. K. Characterization of Carotenoids and Genes Encoding Their Biosynthetic Pathways in *Azospirillum Brasilense*. *FEMS Microbiol. Lett.* **2021**, *368* (5). https://doi.org/10.1093/femsle/fnab025.

(113) Higa Herminia, L.; Schilrreff, P.; Martín Briski, A.; Emanuel Jerez, H.; Alexandre de Farias, M.; Villares Portugal, R.; Lilia Romero, E.; Jose Morilla, M. Bacterioruberin from Haloarchaea plus Dexamethasone in Ultra-Small Macrophage-Targeted Nanoparticles as Potential Intestinal Repairing Agent. *Colloids Surfaces B Biointerfaces* **2020**, *191*, 110961. https://doi.org/10.1016/j.colsurfb.2020.110961.

(114) Kesbiç, F. I.; Gültepe, N. C50 Carotenoids Extracted from *Haloterrigena Thermotolerans* Strain K15: Antioxidant Potential and Identification. *Folia Microbiol. (Praha).* **2021**, No. 0123456789. https://doi.org/10.1007/s12223-021-00905-w.

(115) Biswas, J.; Haque, F. N.; Paul, A. K. Carotenogenesis in *Haloferax* Sp . Strain BKW301 , a Halophilic Archaeon from Indian Solar Saltarns. *J. Adv. Microbiol.* **2016**, *1* (3), 1–11. https://doi.org/10.9734/JAMB/2016/31559.

(116) Ghosh, S.; Ray, A.; Pramanik, N. Self-Assembly of Surfactants: An Overview on General Aspects of Amphiphiles. *Biophys. Chem.* **2020**, *265*, 106429. https://doi.org/10.1016/j.bpc.2020.106429.

(117) Aguirre-Ramírez, M.; Silva-Jiménez, H.; Banat, I. M.; Díaz De Rienzo, M. A. Surfactants: Physicochemical Interactions with Biological Macromolecules. *Biotechnol. Lett.* **2021**, *43*, 523–535. https://doi.org/10.1007/s10529-020-03054-1.

(118) Smirnova, N. A.; Safonova, E. A. Ionic Liquids as Surfactants. *Russ. J. Phys. Chem. A* **2010**, *84* (10), 1695–1704. https://doi.org/10.1134/S0036024410100067.

(119) Li, Y.; Fang, F.; Sun, M.; Zhao, Q.; Hu, Y.; Sui, Z.; Liang, Z.; Zhang, L.; Zhang, Y. Ionic Liquid-Assisted Protein Extraction Method for Plant Phosphoproteome Analysis. *Talanta* **2020**, *213*, 120848. https://doi.org/10.1016/j.talanta.2020.120848.

(120) Danwanichakul, D.; Rattanaphan, O.; Srisatjang, J.; Danwanichakul, P. Extraction of Protein from Skim Natural Rubber Latex Using PEG as a Surfactant via Low Speed Centrifugation and Continuous Flow. *J. Appl. Polym. Sci.* **2014**, *131* (4). https://doi.org/10.1002/app.39900.

(121) Lin, X.; Wang, Y.; Zeng, Q.; Ding, X.; Chen, J. Extraction and Separation of Proteins by Ionic Liquid Aqueous Two-Phase System. *Analyst* **2013**, *138* (21), 6445–6453. https://doi.org/10.1039/c3an01301d.

(122) Martins, M.; Ooi, W. C.; Neves, M. C.; Pereira, J. F.; Coutinho, J. A. Extraction of Recombinant Proteins from *Escherichia Coli* by Cell Disruption with Aqueous Solutions of Surface-Active Compounds. *J. Chem. Technol. Biotechnol.* **2018**, *93*, 1864–1870. https://doi.org/10.1002/jctb.5596.

(123) Kholany, M.; Trébulle, P.; Martins, M.; Ventura, S. P. M.; Nicaud, J.-M.; Coutinho, J. A. P. Extraction and Purification of Violacein from *Yarrowia Lipolytica* Cells Using Aqueous Solutions of Surfactants. *J. Chem. Technol. Biotechnol.* **2020**, *95* (4), 1126–1134. https://doi.org/10.1002/jctb.6297.

(124) Martins, M.; Mesquita, L. M. de S.; Vaz, B. M. C.; Dias, A. C. R. V.; Torres-Acosta, M. A.;

Quéguineur, B.; Coutinho, J. A. P.; Ventura, S. P. M. Extraction and Fractionation of Pigments from *Saccharina Latissima* (Linnaeus, 2006) Using an Ionic Liquid + Oil + Water System. *ACS Sustain. Chem. Eng.* **2021**, *9* (19), 6599–6612. https://doi.org/10.1021/acssuschemeng.0c09110.

(125) Burgess, R. R. Protein Precipitation Techniques. In *Methods in Enzymology*; Burgess, R. R., Deutscher, M. P., Eds.; Elsevier Inc., 2009; Vol. 463, pp 331–342. https://doi.org/10.1016/S0076-6879(09)63020-2.

(126) Bell, D. J.; Hoare, M.; Dunnill, P. The Formation of Protein Precipitates and Their Centrifugal Recovery. In *Downstream Processing. Advances in Biochemical Engineering/Biotechnology*; Springer, Berlin, Heidelberg, 1983; pp 1–72. https://doi.org/10.1007/978-3-662-39694-0_1.

(127) Martins, M.; Soares, B. P.; Santos, J. H. P. M.; Bharmoria, P.; Torres Acosta, M. A.; Dias, A. C. R. V.; Coutinho, J. A. P.; Ventura, S. P. M. Sustainable Strategy Based on Induced Precipitation for the Purification of Phycobiliproteins. *ACS Sustain. Chem. Eng.* **2021**, *9* (10), 3942–3954. https://doi.org/10.1021/acssuschemeng.0c09218.

(128) Castro-Alonso, M. J.; Montañez-Hernandez, L. E.; Sanchez-Muñoz, M. A.; Franco, M. R. M.; Narayanasamy, R.; Balagurusamy, N. Microbially Induced Calcium Carbonate Precipitation (MICP) and Its Potential in Bioconcrete: Microbiological and Molecular Concepts. *Front. Mater.* **2019**, *6*, 126. https://doi.org/10.3389/fmats.2019.00126.

(129) Laemmli, U. K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*, 680–685. https://doi.org/10.1038/227680a0.

(130) Vieira, F. A.; Guilherme, R. J. R.; Neves, M. C.; Abreu, H.; Rodrigues, E. R. O.; Maraschin, M.; Coutinho, J. A. P.; Ventura, S. P. M. Single-Step Extraction of Carotenoids from Brown Macroalgae Using Non-Ionic Surfactants. *Sep. Purif. Technol.* **2017**, *172*, 268–276. https://doi.org/10.1016/j.seppur.2016.07.052.

(131) Martins, M.; Vieira, F. A.; Correia, I.; Ferreira, R. A. S.; Abreu, H.; Coutinho, J. A. P.; Ventura, S. P. M. Recovery of Phycobiliproteins from the Red Macroalga *Gracilaria* Sp. Using Ionic Liquid Aqueous Solutions. *Green Chem.* **2016**, *18*, 4287–4296. https://doi.org/10.1039/C6GC01059H.

(132) Mohsen-Nia, M.; Amiri, H.; Jazi, B. Dielectric Constants of Water, Methanol, Ethanol, Butanol and Acetone: Measurement and Computational Study. *J. Solution Chem.* **2010**, *39*, 701–708. https://doi.org/10.1007/s10953-010-9538-5.

(133) Oss, C. J. van. On the Mechanism of the Cold Ethanol Precipitation Method of Plasma Protein Fractionation. *J. Protein Chem.* **1989**, *8* (5), 661–668. https://doi.org/10.1007/BF01025606.

(134) Taskila, S.; Ahokas, M.; Järvinen, J.; Toivanen, J.; Tanskanen, J. P. Concentration and Separation of Active Proteins from Potato Industry Waste Based on Low-Temperature Evaporation and Ethanol Precipitation. *Sci.* **2017**, *2017*, 5120947. https://doi.org/10.1155/2017/5120947.

(135) Schilcher, G.; Schlagenhauf, A.; Schneditz, D.; Scharnagl, H.; Ribitsch, W.; Krause, R.; Rosenkranz, A. R.; Stojakovic, T.; Horina, J. H. Ethanol Causes Protein Precipitation — New Safety Issues for Catheter Locking Techniques. *PLoS One* **2013**, *8* (12), e84869. https://doi.org/10.1371/journal.pone.0084869.

(136) Vicente, F. A.; Cardoso, I. S.; Sintra, T. E.; Lemus, J.; Marques, E. F.; Ventura, S. P. M.; Coutinho, J.

A. P. Impact of Surface Active Ionic Liquids on the Cloud Points of Nonionic Surfactants and the Formation of Aqueous Micellar Two-Phase Systems. *J. Phys. Chem. B* **2017**, *121* (37), 8742–8755. https://doi.org/10.1021/acs.jpcb.7b02972.

(137) García-Mateos, I.; Velázquez, M. M.; Rodríguez, L. J. Critical Micelle Concentration Determination in Binary Mixtures of Ionic Surfactants by Deconvolution of Conductivity/Concentration Curves. *Langmuir* **1990**, *6* (6), 1078–1083. https://doi.org/10.1021/la00096a009.

(138) Wu, S.; Liang, F.; Hu, D.; Li, H.; Yang, W.; Zhu, Q. Determining the Critical Micelle Concentration of Surfactants by a Simple and Fast Titration Method. *Anal. Chem.* **2020**, *92* (6), 4259–4265. https://doi.org/10.1021/acs.analchem.9b04638.

(139) Shi, Y.; Luo, H. Q.; Li, N. B. Determination of the Critical Premicelle Concentration, First Critical Micelle Concentration and Second Critical Micelle Concentration of Surfactants by Resonance Rayleigh Scattering Method without Any Probe. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **2011**, *78* (5), 1403–1407. https://doi.org/10.1016/j.saa.2011.01.018.

(140) Merk. Genapol® C-100 https://www.sigmaaldrich.com/PT/en/product/sigma/61028 (accessed Oct 24, 2021).

Appendix

Appendix A

Using aqueous solutions of ionic liquids as chlorophyll eluents in solid-phase extraction processes

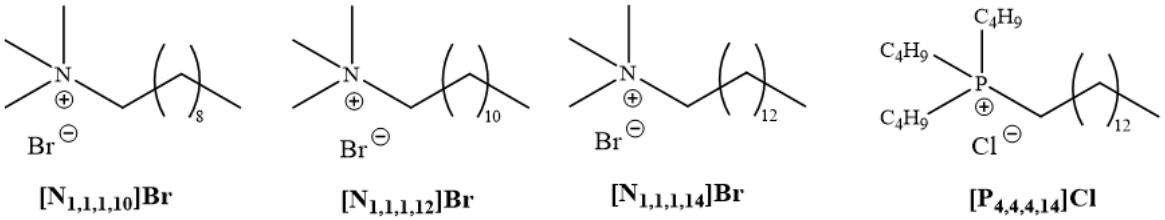


Figure A.1. Molecular structure and respective abbreviation of the ILs screened in this work.

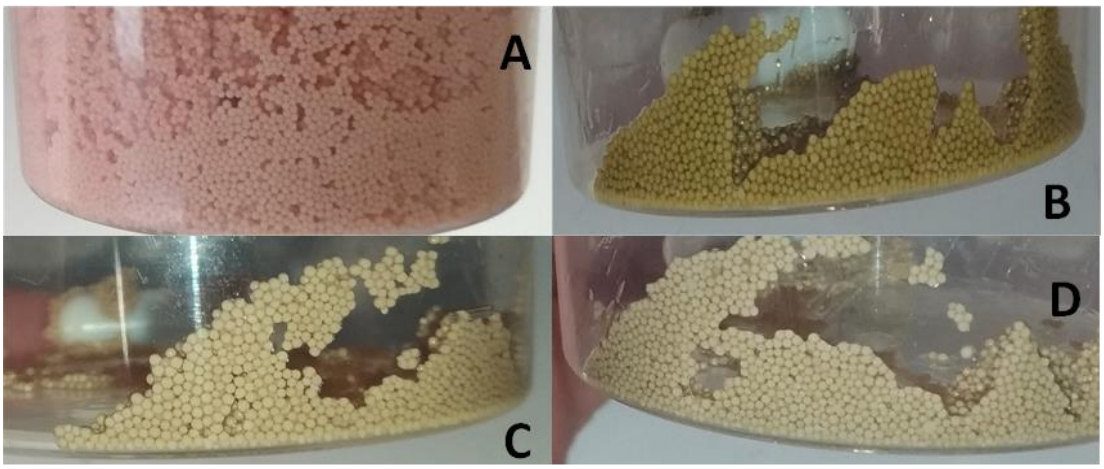
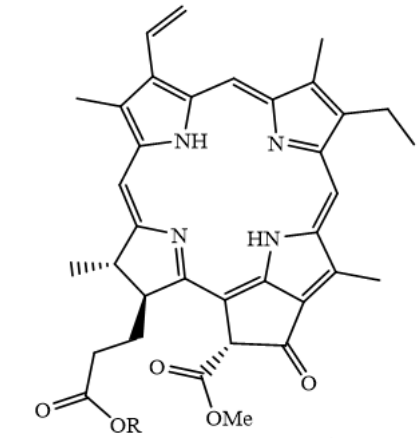
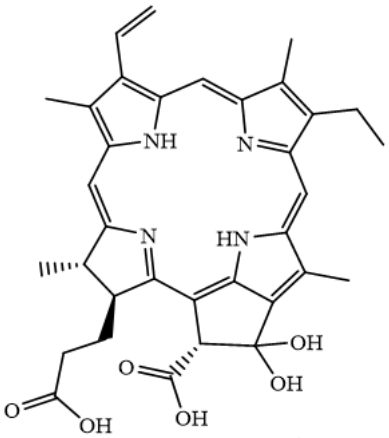


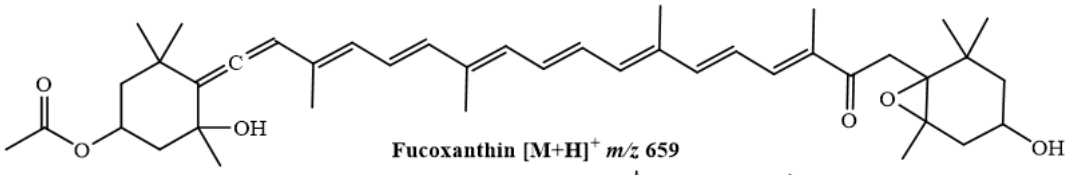
Figure A.2. Photographs of the resin AmberLite™ HPR900 OH: (A) resin before usage; (B) resin after chlorophyll adsorption and collection of the carotenoid extract; (C) resin after elution with aqueous solution of $[N_{1,1,1,12}]Br$ using the optimized conditions of elution by CCRD; and (D) resin after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



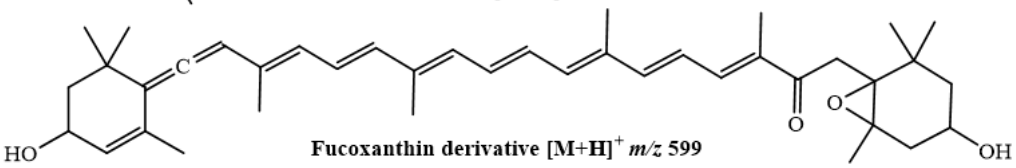
1) Pheophorbide *a* (R=H) $[M+H]^+$ *m/z* 593
2) Pheophytin *a* (R=Phytyl) $[M+H]^+$ *m/z* 871



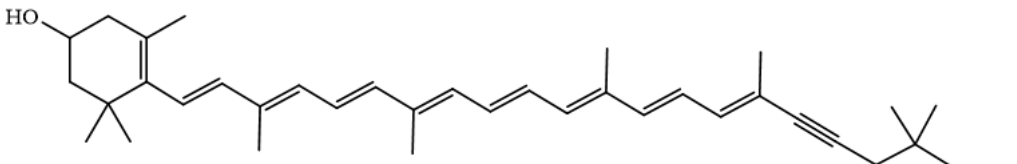
Pheophorbide derivative $[M+H]^+$ *m/z* 597



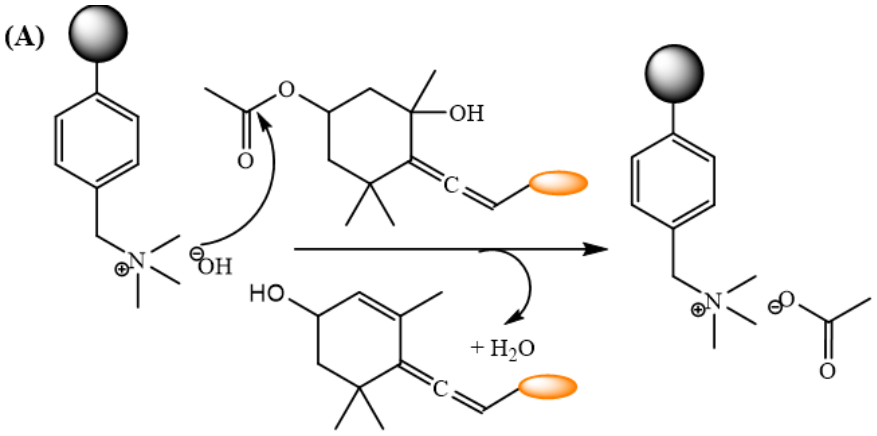
Fucoxanthin $[M+H]^+$ *m/z* 659



Fucoxanthin derivative $[M+H]^+$ *m/z* 599



Diatoxanthin $[M+H]^+$ *m/z* 567



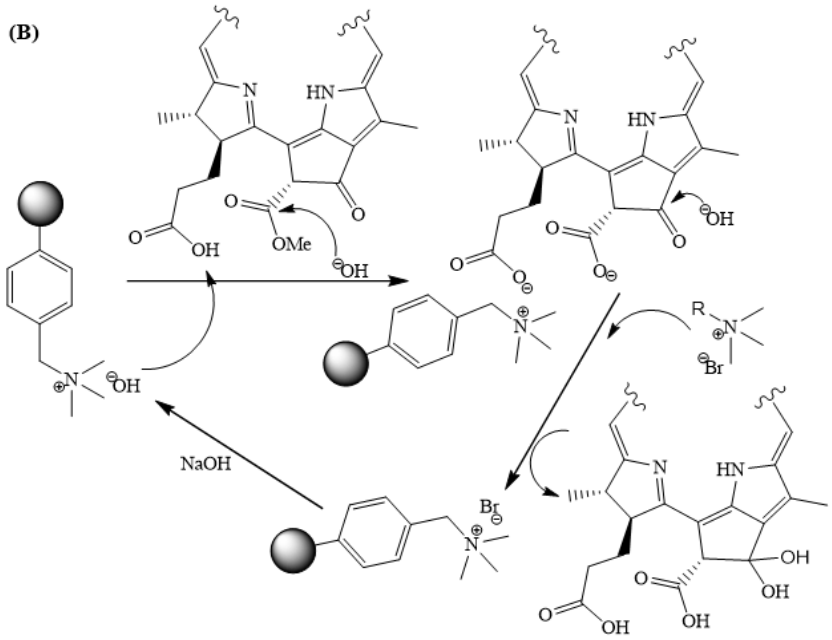


Figure A.3. Compounds, chemical structures, and proposed reactions between fucoxanthin (A) and pheophorbide (B) with the strong basic resin.

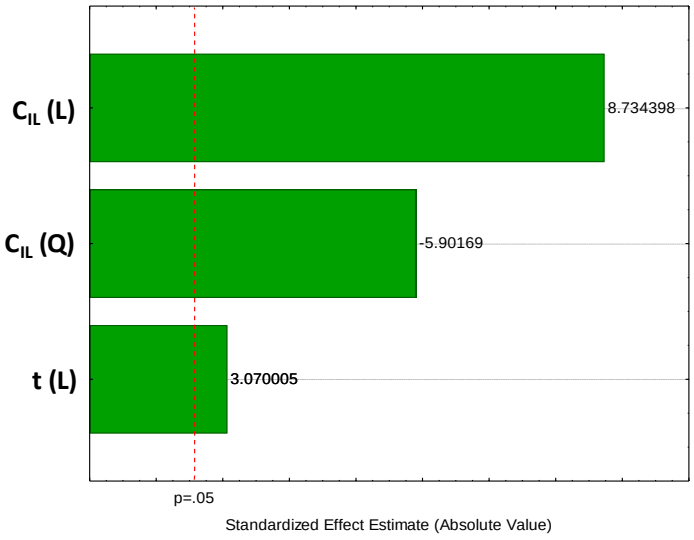


Figure A.4. Pareto Chart of the CCRD (2^3) regarding the chlorophyll recovery from the resin (%) using aqueous solutions of $[P_{4,4,4,14}]Cl$.

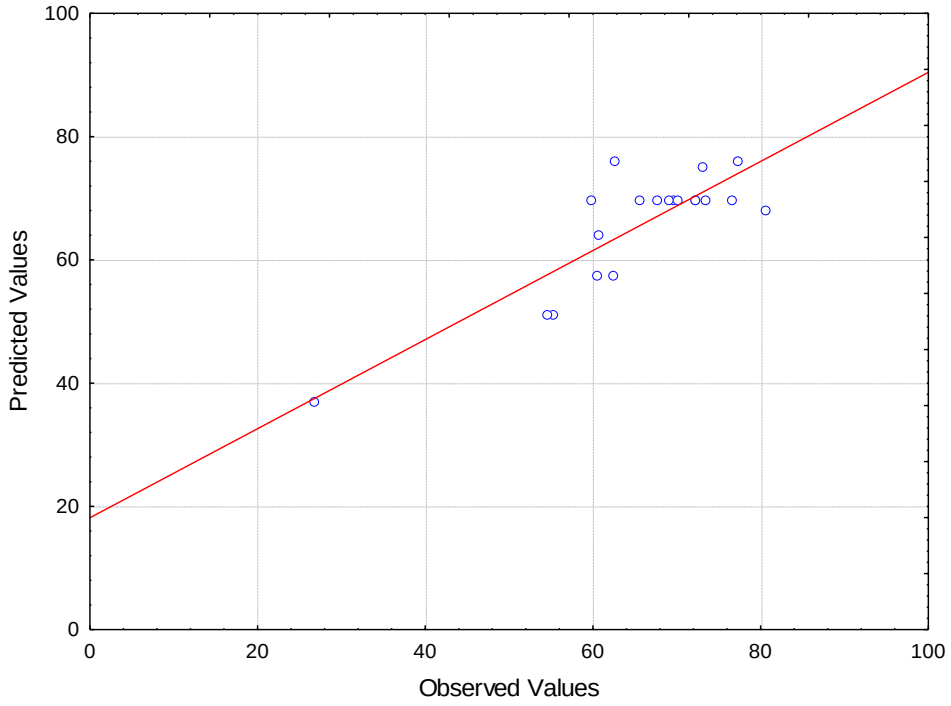


Figure A.5. Predicted *vs.* experimental values of the CCRD (2^3) regarding the chlorophyll elution from the resin (%) using aqueous solutions of $[P_{4,4,4,14}]Cl$.

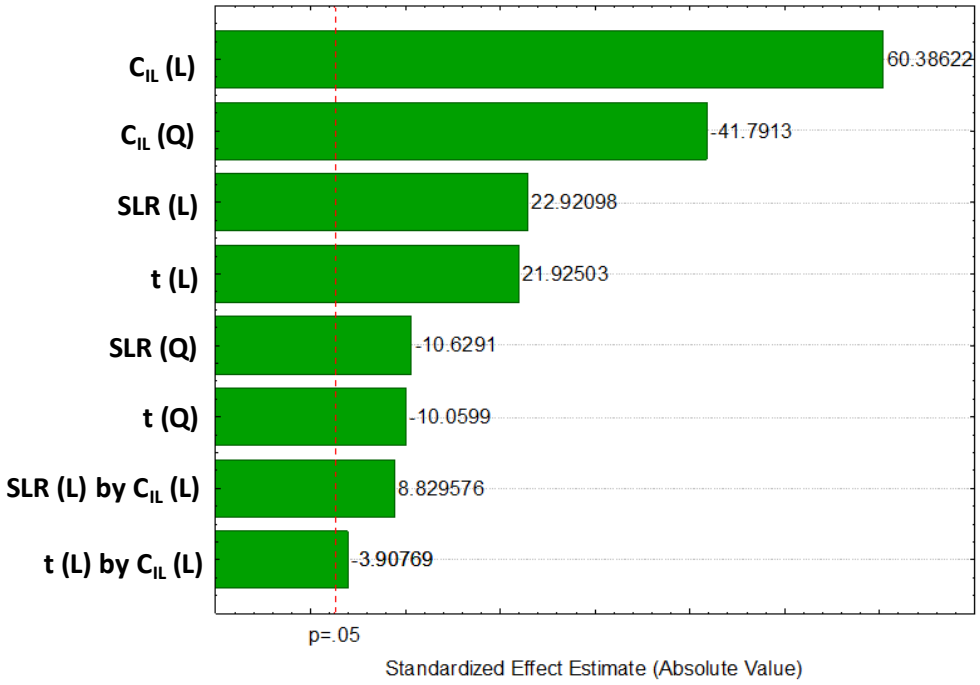


Figure A.6. Pareto Chart of the CCRD (2^3) regarding the chlorophyll recovery from the resin (%) using aqueous solutions of $[N_{1,1,1,12}]Br$.

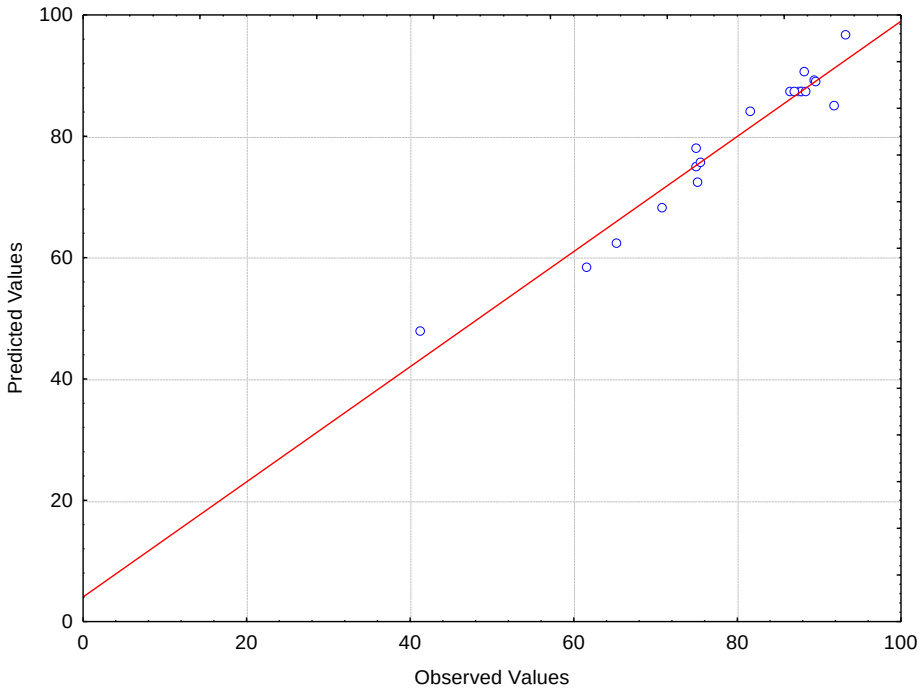


Figure A.7. Predicted *vs.* experimental values of the CCRD regarding the chlorophyll recovery from the resin (%) using aqueous solutions of $[N_{1,1,1,12}]Br$.

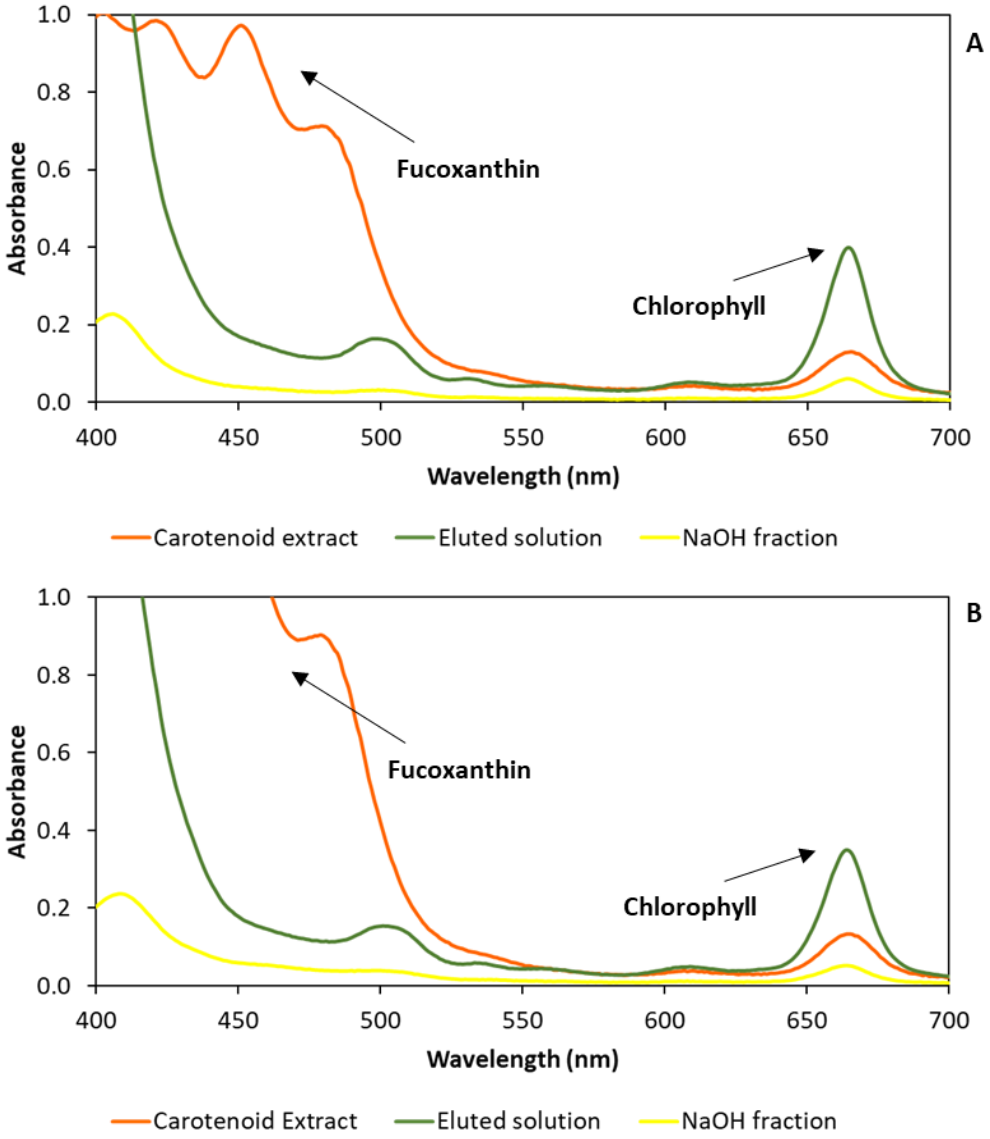


Figure A.8. UV-visible spectra of the collected fractions for batch studies in the first cycle, namely carotenoid extract, eluted solution and NaOH fraction using (A) an aqueous solution of $[N_{1,1,1,12}]Br$; and (B) an aqueous solution of $[P_{4,4,4,14}]Cl$.

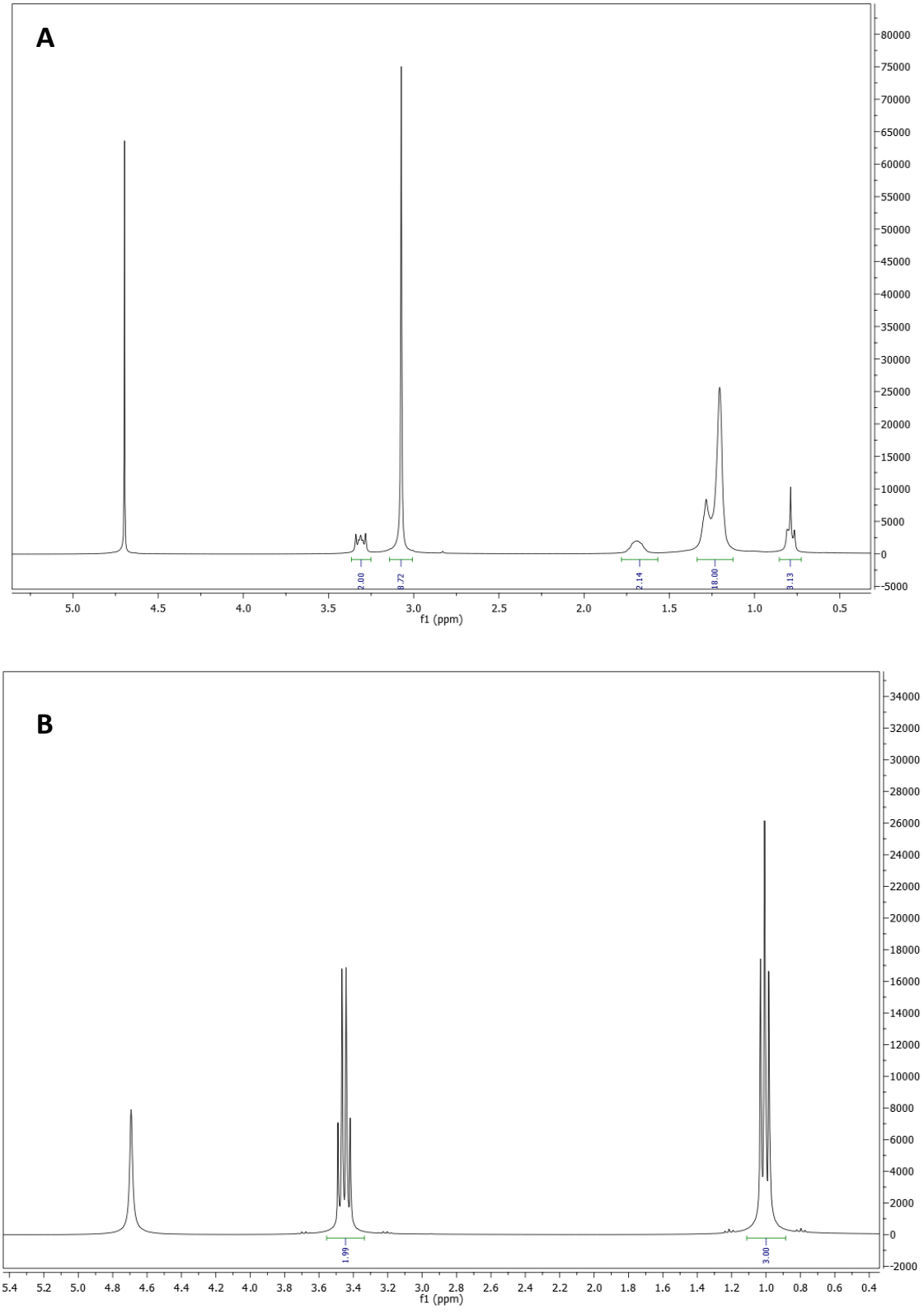


Figure A.9. ^{1}H NMR spectroscopy of (A) pure [$N_{1,1,1,12}$]Br and (B) ethanolic fraction rich in chlorophylls (after the polishing step) dissolved in D_2O.

Table A.1. Critical Micellar Concentration (CMC) of surface-active ILs used to elute the chlorophylls.

Surface-active compound	CMC (mM)	Reference
$[P_{4,4,4,14}]Cl$	4.69	[136]
$[N_{1,1,1,10}]Br$	25.20	[136]
$[N_{1,1,1,12}]Br$	15.6	[137]
$[N_{1,1,1,14}]Br$	3.8	[137]

Table A.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 A.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 A.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 A.5. Effect of the estimates for chlorophyll recovery optimized by the CCRD 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 A.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.

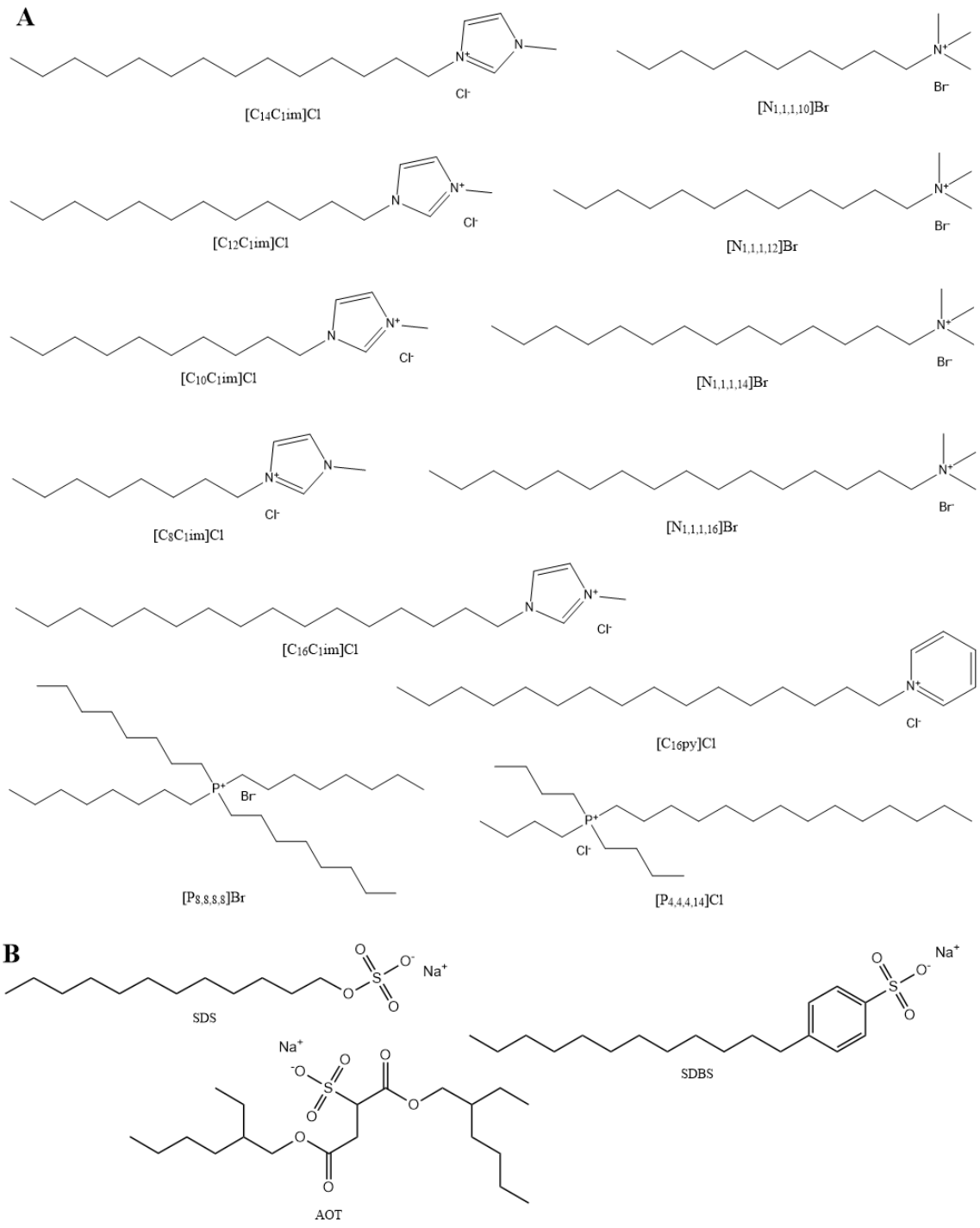
Assay	SLR (g_{resin}. mL_{eluent}^{-1})	t (min)	C_{IL} (mM)	Chlorophyll recovery (%) using $[N_{1,1,1,12}]Br$		Relative deviation (%)
				Experimental values	Predicted values	
	X1	X2	X3	Y	Predicted Y	
V1	0.070	48.5	370	96.7	100	-3.3
V2				98.1		-1.9
V3				96.3		-3.7
					Mean of deviation	-3.0

Table A.7. 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

Appendix B

Recovery of bacterioruberin using aqueous solutions of surface-active compounds



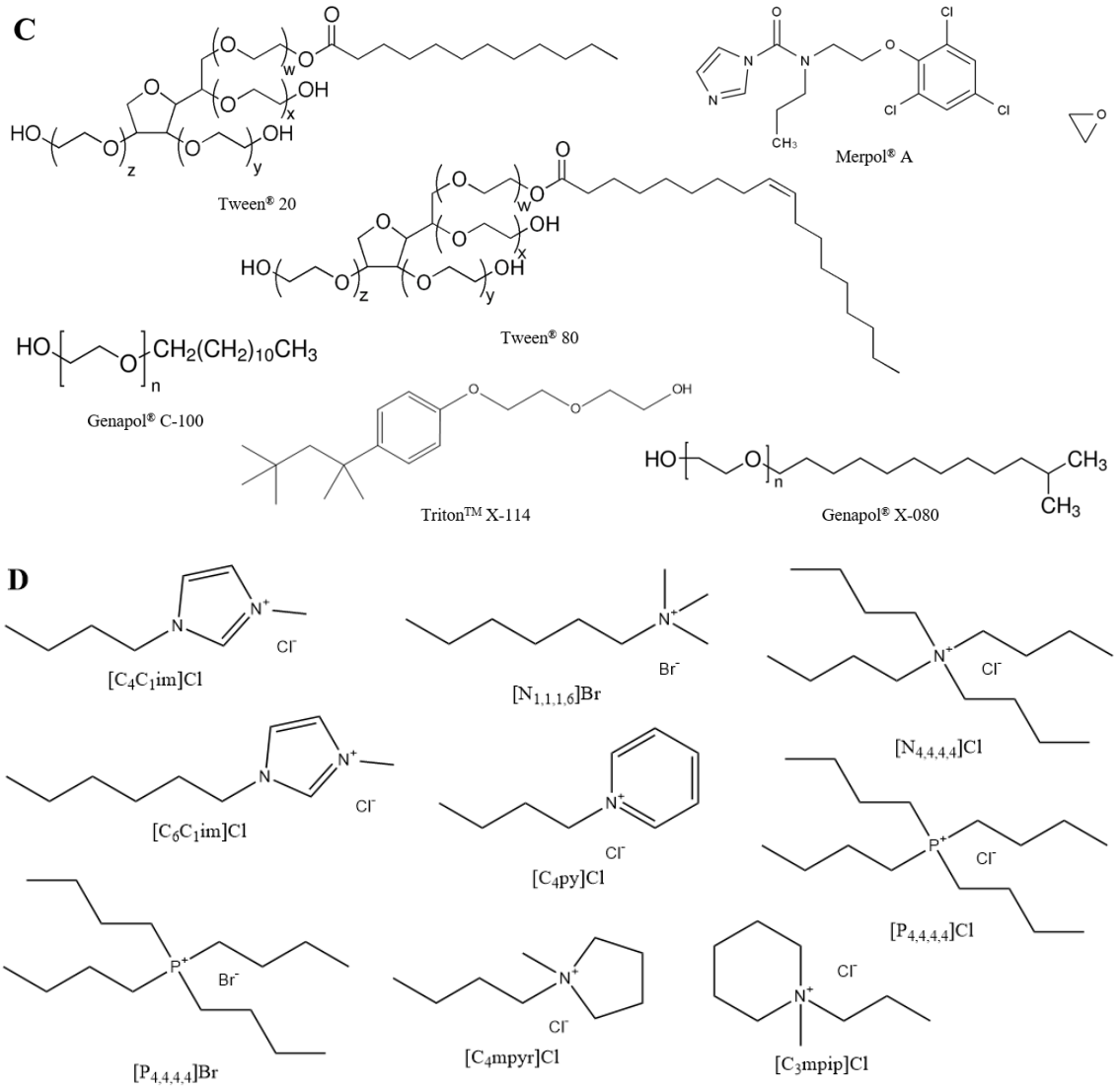
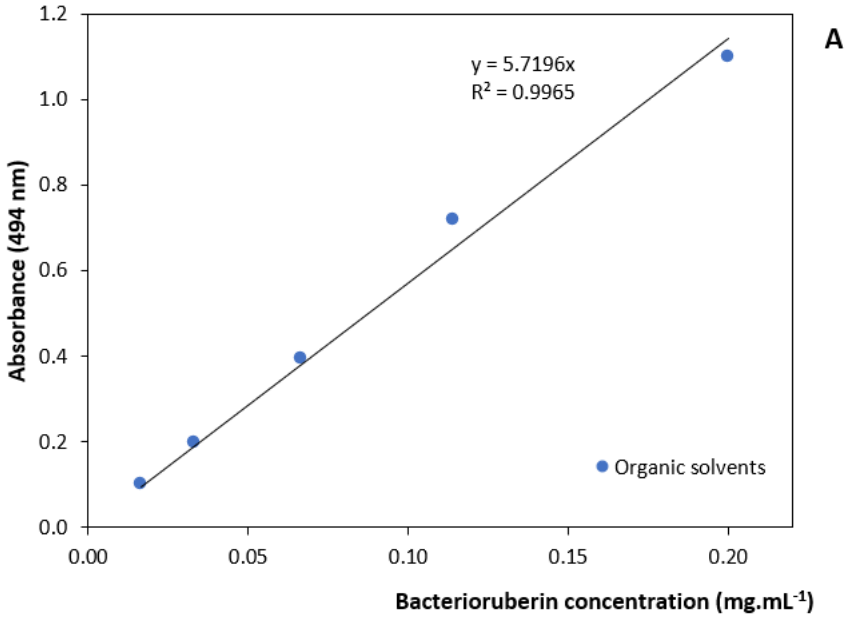
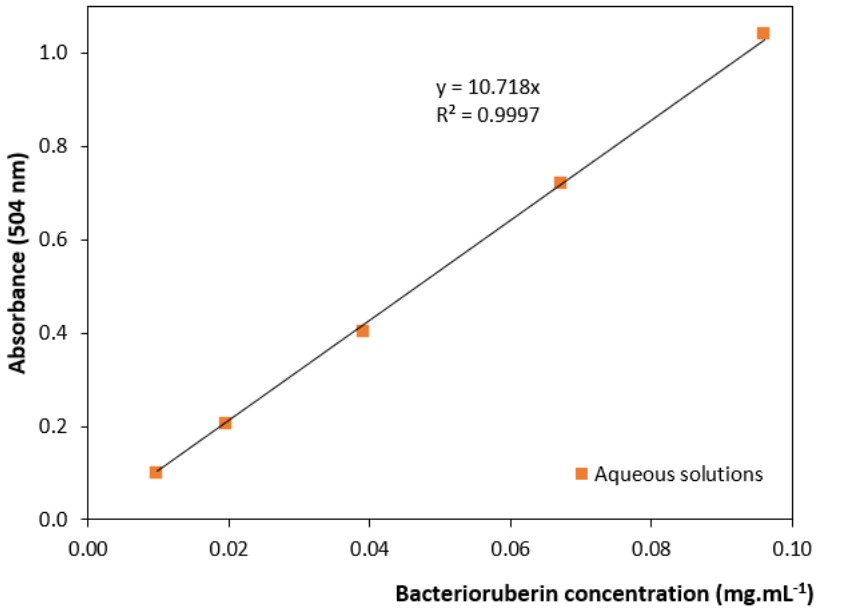


Figure B.1. Molecular structures and abbreviation names of the cationic (A), anionic (B), non-ionic (C), and non-tensioactive (D) compounds screened in this work.


A
y = 5.7196x
R² = 0.9965
Organic solvents
Absorbance (494 nm)
Bacterioruberin concentration (mg.mL-1)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0.00
0.05
0.10
0.15
0.20


y = 10.718x
R² = 0.9997
Aqueous solutions
Absorbance (504 nm)
Bacterioruberin concentration (mg.mL-1)
0.0
0.2
0.4
0.6
0.8
1.0
0.00
0.02
0.04
0.06
0.08
0.10

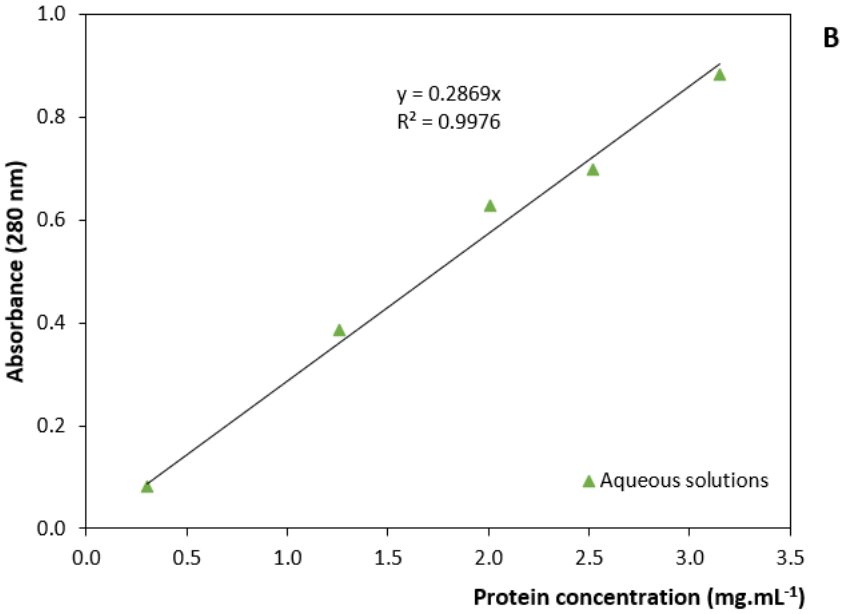


Figure B.2. Calibration curves experimentally determined and used to quantify bacterioruberin in organic solvents and aqueous solutions (A) and total proteins in aqueous solvents (B) using the Synergy HT microplate reader – BioTek.

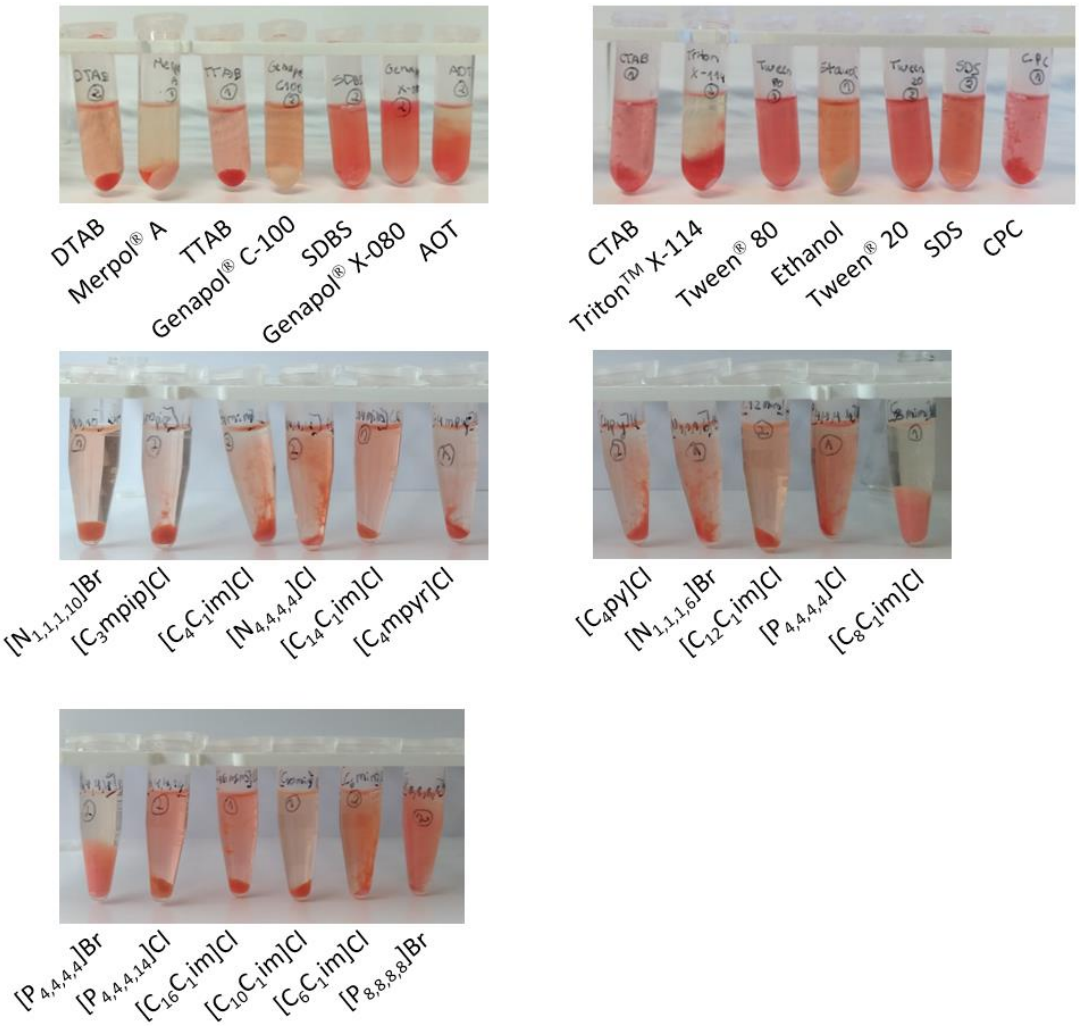


Figure B.3. Photographs of the extracts obtained in the screening of solvents at 100 mM.

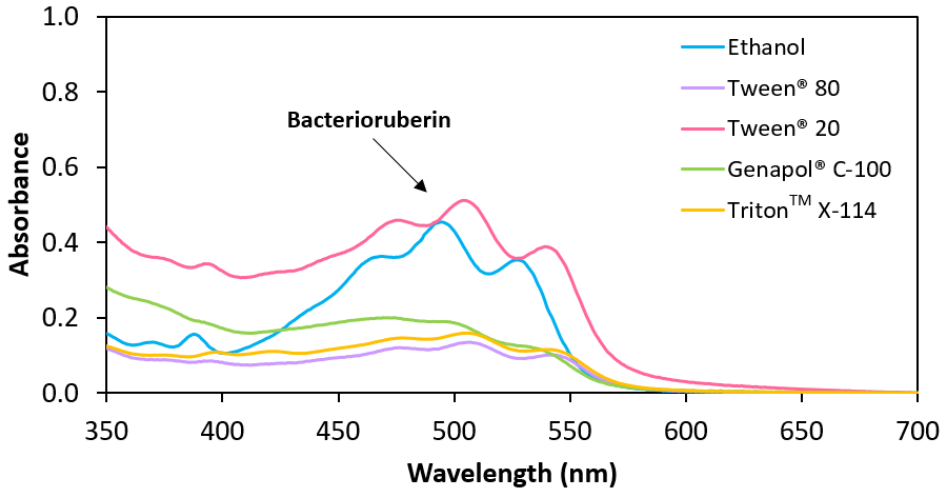


Figure B.4. UV-Vis spectroscopy of the extracts obtained in the screening of the non-ionic compounds at 250 mM.

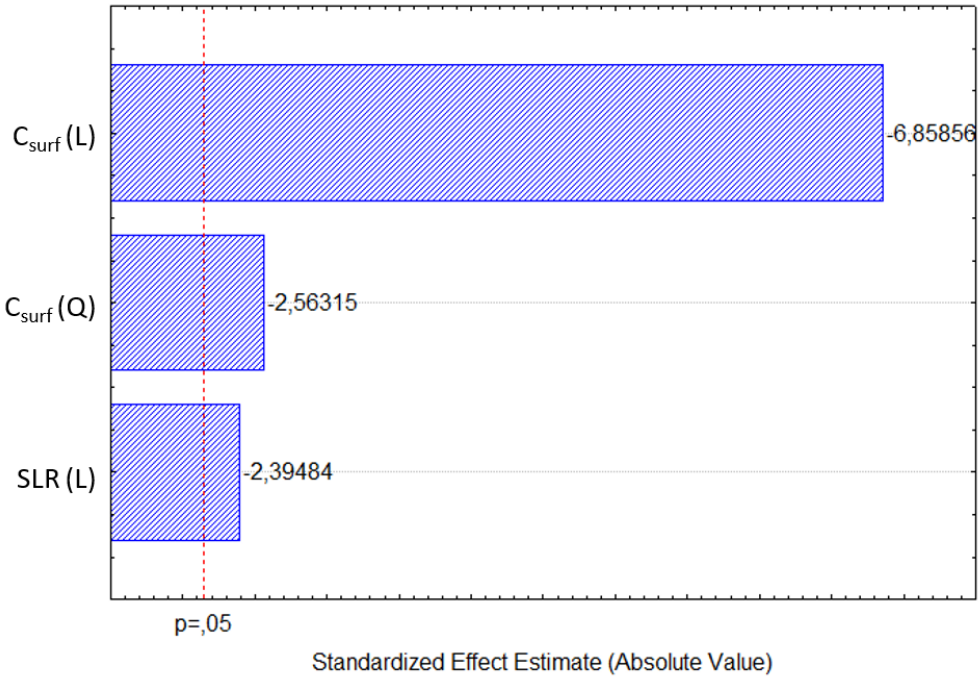


Figure B.5. Pareto Chart of the CCRD (2^3) regarding bacterioruberin yield of extraction ($mg_{bacterioruberin}.g_{wet\ biomass}^{-1}$) using aqueous solutions of Tween® 20.

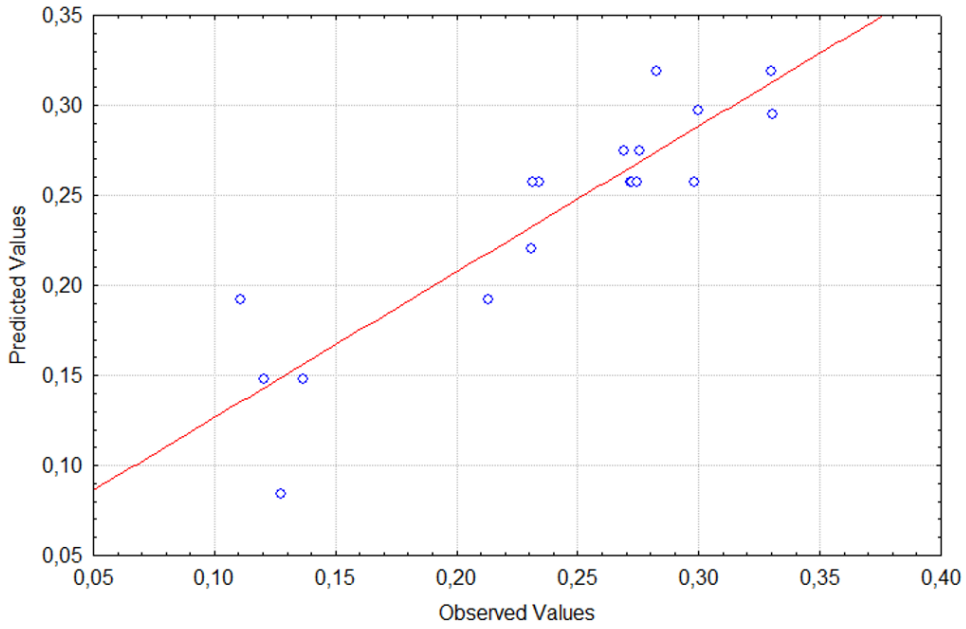


Figure B.6. Predicted *vs.* experimental values of the CCRD (2^3) regarding bacterioruberin yield of extraction ($mg_{bacterioruberin}.g_{wet\ biomass}^{-1}$) using aqueous solutions of Tween® 20.

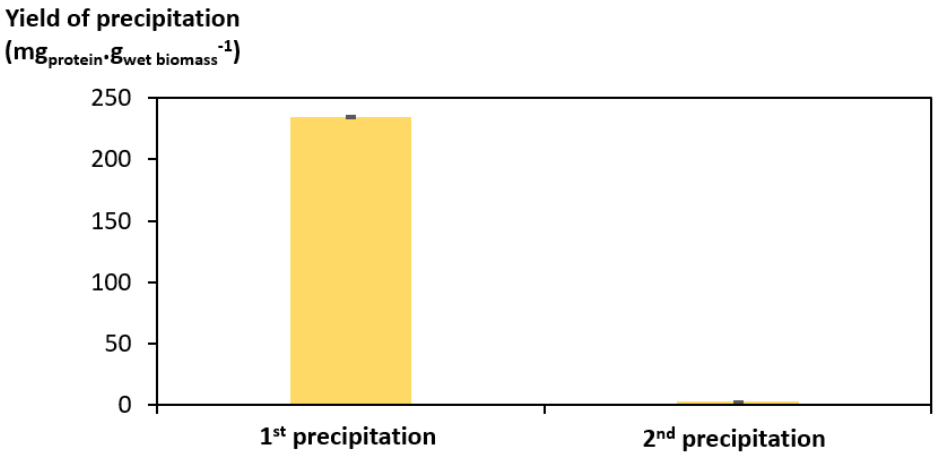


Figure B.7. Yield of precipitation ($mg_{protein}.g_{wet\ biomass}^{-1}$) for two consecutive protein precipitations using the same operational conditions, measured after proteins redissolution in PBS.

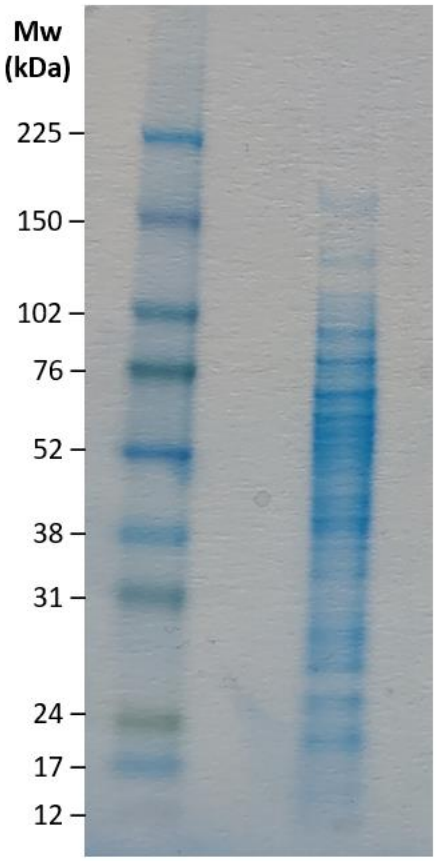


Figure B.8. SDS-PAGE of the recovered proteins redissolved in PBS after protein induced precipitation.

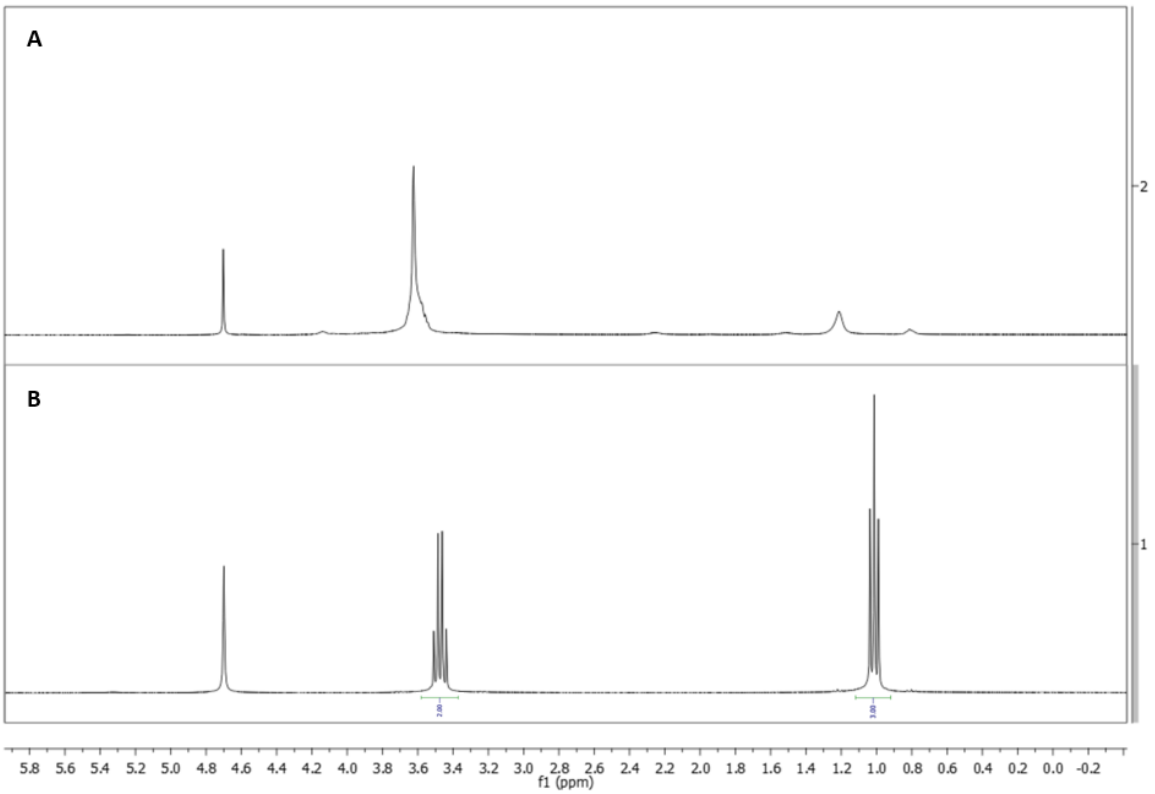


Figure B.9. ^{1}H NMR spectroscopy of (A) pure Tween® 20 and (B) ethanolic fraction rich in bacterioruberin (after the polishing step) dissolved in D_2O.

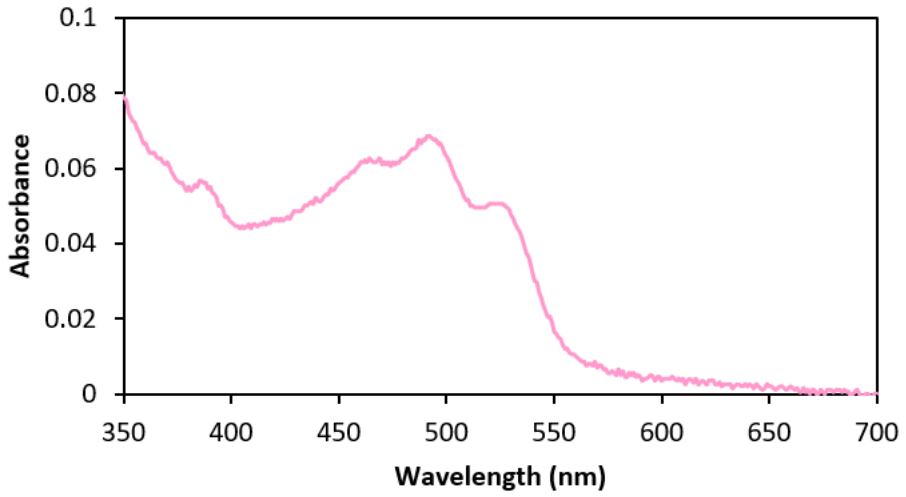


Figure B.10. UV-Vis spectroscopy of the collected sample from ethanol-rich phase after the polishing step.

Table B.1. Critical Micellar Concentration (CMC) of surface-active ILs used to elute the chlorophylls.

Surface-active compound	CMC (mM)	Reference
Triton™ X-114	0.29	[138]
Tween® 20	0.078	[139]

Tween® 80	0.014	[139]
Genapol® C-100	0.075	[140]*

***** Manufacturer data

Table B.2. Real values used in the optimization process by CCRD (2^3) expressed by yield of extraction of bacterioruberin ($mg_{bacterioruberin}.g_{wet\ biomass}^{-1}$) using aqueous solutions of Tween® 20.

Run	SLR ($g_{wet\ biomass}.mL_{solvent}^{-1}$)	t (min)	C_{Surf} (mM)	Yield of extraction ($mg_{bacterioruberin}.g_{wet\ biomass}^{-1}$)
1	0.118	23.0	230.0	0.330
2	0.282	23.0	230.0	0.276
3	0.118	67.0	230.0	0.283
4	0.282	67.0	230.0	0.269
5	0.118	23.0	370.0	0.111
6	0.282	23.0	370.0	0.120
7	0.118	67.0	370.0	0.213
8	0.282	67.0	370.0	0.137
9	0.0622	45.0	300.0	0.331
10	0.338	45.0	300.0	0.231
11	0.200	8.04	300.0	0.234
12	0.200	82.0	300.0	0.272
13	0.200	45.0	182.4	0.300
14	0.200	45.0	417.6	0.128
15	0.200	45.0	300.0	0.232
16	0.200	45.0	300.0	0.298
17	0.200	45.0	300.0	0.273
18	0.200	45.0	300.0	0.275

Table B.3. List of the surface-active compounds tested to recover bacterioruberin and their success in the extraction.

	Surface-active compound	Success	Observations
Cationic	$[C_8C_1im]Cl$	x	Formed 2 phases, inviable to read UV-Vis spectra
	$[C_{10}C_1im]Cl$	✓	White cloudy initial solution but viable to read UV-Vis spectra

	$[C_{12}C_1im]Cl$	✓	-
	$[C_{14}C_1im]Cl$	✓	-
	$[C_{16}C_1im]Cl$	✓	White cloudy initial solution but viable to read UV-Vis spectra
	$[N_{1,1,1,10}]Br$	✓	-
	$[N_{1,1,1,12}]Br$	✓	-
	$[N_{1,1,1,14}]Br$	✓	-
	$[N_{1,1,1,16}]Br$	✓	-
	$[C_{16}py]Cl$	✓	-
	$[P_{8,8,8,8}]Br$	x	Biomass did not settle in centrifuge; white cloudy initial solution
	$[P_{4,4,4,14}]Cl$	✓	-
Anionic	SDBS	x	Biomass did not settle in centrifuge
	SDS	✓	Very slimy
	AOT	x	Formed 3 phases; white cloudy initial solution; very slimy inviable to separate mixture
Non-ionic	Tween® 20	✓	Very slimy
	Tween® 80	✓	Very slimy
	Triton™ X-114	✓	Formed 2 phases; slimy solution but possible to separate from sample
	Merpol® A	x	Formed 2 phases; very slimy inviable to separate mixture from sample; white cloudy initial solution
	Genapol® X-080	x	Biomass did not settle in centrifuge; white cloudy initial solution
	Genapol® C-100	✓	-
Non-tensioactive	$[C_4C_1im]Cl$	x	Biomass did not settle in centrifuge
	$[C_6C_1im]Cl$	x	Biomass did not settle in centrifuge
	$[P_{4,4,4,4}]Cl$	x	Biomass did not settle in centrifuge
	$[P_{4,4,4,4}]Br$	x	Formed 2 phases, inviable to read UV-Vis spectra
	$[N_{4,4,4,4}]Cl$	x	Biomass did not settle in centrifuge
	$[N_{1,1,1,6}]Br$	x	Biomass did not settle in centrifuge

	[C_3mpip]Cl	x	Biomass did not settle in centrifuge
	[C_4mpyr]Cl	x	Biomass did not settle in centrifuge
	[C_4py]Cl	x	Biomass did not settle in centrifuge

Table B.4. Effect of the estimates for bacterioruberin yield of extraction ($mg_{bacterioruberin}.g_{wet\ biomass}^{-1}$) using aqueous solutions of Tween® 20, optimized by the CCRD (2^3) with significant factors at 95 % confidence level.

Factor	Effect	Standard error	Calculated t^*	p-value
Mean/Interaction	0.257	0.0107	24.1	0.000
SLR ($g_{wet\ biomass}.mL_{solvent}^{-1}$) – (X1)	-0.0443	0.0185	-2.39	0.0312
C_{Surf} (mM) – (X3)	-0.127	0.0185	-6.86	0.000
C_{Surf} (mM) – ($X3^2$)	-0.0475	0.0185	-2.56	0.0225

*Degrees of freedom.

Table B.5. Predicted *vs.* experimental values (real) obtained by the fitted model and the respective relative deviation (%) from the independent variables fixed at the optimum conditions for bacterioruberin yield of extraction ($mg_{bacterioruberin}.g_{wet\ biomass}^{-1}$) using aqueous solutions of Tween® 20. V1, V2, and V3 represent the validation assays.

Assay	SLR ($g_{wet\ biomass}.mL_{solvent}^{-1}$)	C_{Surf} (mM)	Yield of extraction ($mg_{bacterioruberin}.g_{wet\ biomass}^{-1}$)		Relative deviation (%)
			Experimental values	Predicted values	
	X1	X3	Y	Predicted Y	
V1	0.06224	182.4	0.373	0.329	11.7
V2			0.361		8.84
V3			0.384		14.3
Mean of deviation					11.6